“BIO-REMEDIATION OF HEAVY METALS DERIVED FROM DELTAMETHRIN PESTICIDES USING POTENTIAL MICROBES”

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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Certificate by Supervisor

The work described in this project report entitled "Bio-remediation of Heavy Metals derived from Deltamethrin pesticides using Potential Microbes" has been carried out by Avinash Jyoti under my supervision. I certify that this is her bonafide work. The work described is original and has not been submitted for any degree to this or any other university.

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DECLARATION

This is to submit that this written submission in my project report entitled "Bio-remediation of Heavy Metals derived from Deltamethrin pesticides using Potential Microbes" represents original ideas in my own words and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have stuck to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be cause for disciplinary action by the School and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when required.

I assure and hold full responsibility for its genuineness.

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ABBREVIATION

HM	Heavy Metals
Cu	Copper
Zn	Zinc
Hg	Mercury
ug/L	Microgram per litre
mg/L	Miligram per litre
ppm	Parts Per Million
rpm	Rotation Per Minute

hrs.	Hours
Conc.	Concentration
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
EPA	Environmental Protection Agency
DNA	Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
HNO_3	Nitric acid
$HCLO_4$	Perchloric acid
spp.	Species
B.subtilis	*Bacillus subtilis*
A.flavus	*Aspergillus flavus*
P.citrinum	*Penicillium citrinum*
q_e	Biosorption capacity

CHAPTER 1

INTRODUCTION

Heavy metal contamination is a major global concern in the environment as a result of these hazardous contaminants, which tend to accumulate in the living organisms includes microorganisms, plants, animals and humans & are responsible for various physiological and metabolic disorders [Rajendran *et al*., 2003; Banerjee *et al*., 2015].

Heavy metals are non- biodegradable pollutants & can be defined as the elements with metallic properties (such as conductivity, ductility, stability as cations, ligand specificity etc.), with atomic masses ranging from 54.63 to 200.59 & with a density higher than 5g cm-3. Unlike most of the organic matter that can be metabolized with natural microbiota, these heavy metals being non-biodegradable, persist in the environment for a long period beyond their permissible limits. Heavy metals are extremely hazardous & accumulate in the biological systems & concentrate in the food chain at each trophicallevel [Siddique *et al*., 2015].

The anthropogenic activities may results into accumulation of heavy metals in the soil other than by natural source. Natural sources of heavy metals (HM) such as emissions of volcanoes, transport of continental particulates & weathering of metal enriched rocks as a result of prolonged exposure to air, greatly inserts higher quantity of HM to soils. Instead, human activities such as utilization of metal-based pesticides, exploitation of mines & smelters, metal-enriched waste sludge in the agriculture [Khan *et al*., 2016; Smith *et al*., 2016], metallurgical industries, fossil fuel combustion, military training & weapons etc., contribute greatly to contaminate the soil with heavy metals. The anthropogenic activities have been categorized into 5 groups such as (i) metalliferous mining & smelting that give rise to accumulation of various HM such as arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) etc. (ii) industries that give rise to accumulation of various HM like arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), mercury (Hg), nickle (Ni) & zinc (Zn) etc., (iii) atmospheric deposition of various HM such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), uranium (U) & lead (Pb), (iv) agricultural deposition of varied heavy metals such as, lead (Pb), cadmium (Cd), copper (Cu), selenium (Se), arsenic (As), uranium (U) & zinc (Zn) & (v) waste disposal, that contribute to accumulation of various HM such as arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), zinc (Zn) & lead (Pb). On the whole, chief heavy metals that are associated with environmental pollution & offer potential threat to the ecosystem, includes copper (Cu), zinc (Zn), silver (Ag), lead (Pb), mercury (Hg), arsenic (As), cadmium (Cd), chromium (Cr), strontium (Sr), cesium (Cs), cobalt (Co), nickel (Ni), tin (Sn) & vanadium (V) [Wang *et al*., 2009]. Copper, zinc & nickle are essential trace metals in the human body, but its excessive intake from outside adversely affect the human health [Chen, 2011].

Agricultural practices such as excessive usage of phosphatic fertilizers for optimal production of crops, extensive application of toxic pesticides, & usage of sewage sludge cause soil pollution [Oves *et al*., 2015] & results into depredation of humans and other life forms under the precept "if little is good, a lot more will be better" [Aktar *et al.,* 2009].

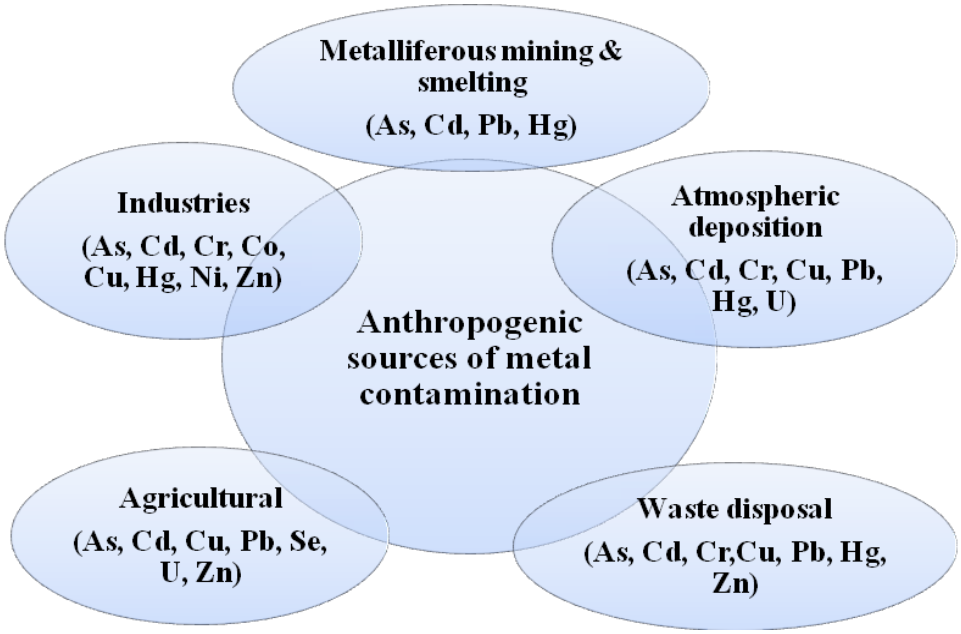

Figure 1.1 : Anthropogenic activities leading to the contamination of soils with heavy metals [Gupta *et al*., 2016].

Deltamethrin-pyrethroid insecticides includes copper (Cu), zinc (Zn) & mercury (Hg) heavy metals. Pyrethroids are synthetic analogs of naturally occuring pyrethrum, isolated from *Chrysanthemum* flower [Ali *et al.,* 2013 ; Rehman *et al.,* 2014]. After the world war II, the pesticide production arose precipitously from approximately 5 lakh t/a to over 3 million t/a globally, in the time period from 1950s to twenty first century [Mondal *et al.,* 2015]. Pyrethroid insecticides has been introduced in the market in 1980, contributed greatly to pest control & agricultural output [Aktar *et al.,* 2009] and account for appx. 25% of the global insecticide market. At present, 16 pyrethroid insectides are certified for use in the USA in varied formulations of agricultural or consumer products [Shafer *et al.,* 2005]. More than thousands of pyrethroids have been made & are reported to constitute majority of the commercialized household insecticides [Ali *et al.*] & used to control pest in

agriculture horticulture, forestry, public health & for indoor home use for more than 20 yrs. Because pyrethroids were considered to be a safer than organophosphate pesticides (OP's), their applications significantly increased when the use of alternative OP's was banned or limited [Cycon and Seget., 2016]. Among pyrethroids, deltamethrin is frequently used as an insecticide for agriculture, public health & livestock applications in various countries. Mode of action of Deltamethrin includes a quick knockdown effect, thus rapidly paralyze the nervous system of insects. Deltamethrin was first described in 1974 & marketed in year around 1978 for the first time [Ali *et al.*, 2013].

Pyrethroids are categorized into two subclasses: type I (first generation pyrethroids) & type II (second generation pyrethroids) on the basis of induction of either T syndrome or CS syndrome. T syndrome includes whole body tremors & a coarse whole body tremors advancing to choreoathetosis with a condition of salivation is CS syndrome [Schleier III and Peterson, 2011].

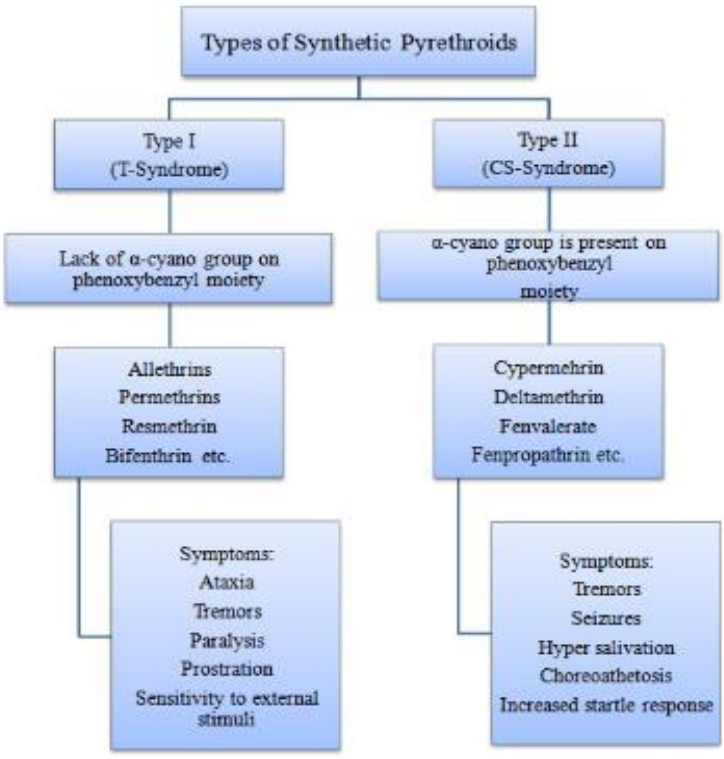

Figure 1.2: Classification of Synthetic Pyrethroid insecticides [Rehman *et al.*, 2014].

Although, pyrethroids have agricultural benefits, its widespread use and continual application has led to their parmanent occurance in some environment, especially in soils where they can be toxic to target & non-target organisms. Due to their highly hydrophobic properties, pyrethroid strongly bind to soil particles and organic matter which allow them to leach into the groundwater and to form residues of these compounds thereby, adversely affecting the ecosystem.Since they are not degraded immediately after application & because their residues are detected in soils, there is an urgent need to remediate pyrethroids-polluted environments [Cycon and Seget., 2016].

Prolonged & widespread use of pyrethroids results into depredation of humans and other life forms [Aktar *et al.*, 2009]. According to a report, residues of 360 metric tones of synthetic pyrethroid insecticides were prevalent in agriculture that affected the aquatic system, in California (USA). Furthermore, the presence of synthetic pyrethroid insecticides ranging from 0.100 µg/l to 1.000 µg/l has been revealed by rain water residue testing in Hisar, (Haryana, India). Among the synthetic pyrethroids that were detected, cypermethrin was found to be present in ample quantity (1µg/lt). Presence of mentioned insecticide's residues in rain water was confirmed thoroughly, as the vapours of these compounds come along with water. Above mentioned significant reports interprets the existance of toxicants as a result of excessive use of synthetic pyrethroids, and the necessity to reduce them [Thatheyus and Selvam, 2013].

To reduce the environmental & public health risks associated with pyrethroid use, it is necessary to develop rapid and effective methods to remove or minimize the concentration of heavy metals derived from insecticides in the environment [Cycon and Seget., 2016]. Various techniques are available for the remediation of contaminated environment includes precipitation, electrolysis, membrane filtration, ion exchange, reverse osmosis, sorptive floatation, & adsorption with the activated carbon. However the appliance of such processes is not feasible due to various reasons such as high capital and operating cost & high energy requirements particularly for small & medium scale industries, partial elimination of the pollutants

& production of the secondary pollutants and less efficient at low concentrations [Ghosh *et al*., 2015].

Among various methods that are applied for remediation of contaminated environments, the biological approach that is based on catabolic activity of the pyrethroids-degrading bacteria, seems to be the most effective approach. [Cycon and Seget., 2016]. Thus, the ideal way out of the abatement of pollution for these non-biodegradable metals is "bioremediation" which is found to be an efficient & promising strategy in order to deal with the contaminants from the environment [Banerjee *et al*., 2015]. Outcome of various studies have proven that pyrethroids & heavy metals derived from them can be successfully removed from media & soil by potential microorganisms, belongs to different taxonomic groups. [Cycon and Seget., 2016].

Bioremediation is defined as an efficient, innovative & promising technology intended for the elimination and recovery of HM in polluted water & lands. The process mediated by microbes for immobilization or clearance of pollutants such as agrochemicals, hydrocarbons, & other organic toxicants. Active metabolizing capabilities of microorganisms forms the basis for heavy metal bioremediation [Dixit *et al*., 2015]. Bioremediation techniques are more advantageous as compared to conventional methods as it can be implemented on site, therefore, minimizing personnel risks. It minimizes the sludge disposal problems due to generation of less effluent volume when compared to traditional approaches [Yadav *et al*., 2017].

Numerous microscopic organisms (bacteria, fungi, yeast and algae etc.), either naturally or genetically modified, possess the potential to degrade, alter/ transform, or chelate different toxic chemicals & hence forms the basis of better strategies to eradicate environmental pollution [Mosa *et al*., 2016]. Several strategies have been developed & adopted by these microbial communities in order to overcome repressive effects of toxic heavy metals [Oves *et al*., 2015]. These mechanisms includes biosorption, bioaccumulation, biomineralization & biotransformation. These detoxifying mechanisms can be employed either *insitu* or *excitu.* In an elaborate way, the strategies includes metal exclusion via permeable barriers, metal's active transport far-off from the cell, sequestration of metals by protein binding intracellularly, extracellularly sequestration of metals, Transformation of toxic metals to less toxic

form by detoxification enzymatically and, by reducing the sensitivity of cellular targets to the metal ions. It has been surveyed that 51% of the respondents favored environment-friendly approaches, which includes 16% of phytoremediation & 35% of microbial remediation [Dixit *et al*., 2015]. Phytoremediation is defined as the process that involves the usage of plants & concerned microbial organisms to partially or completely eliminate selected pollutants from wastewater, ground water, sludge, soil & also for remediation of organic pollutants, radionuclides & heavy metals. It is an efficient, eco-friendly & cost-effective *in situ* remediation technology impelled by solar energy. This technique comprises various processes such as phytoextraction, phytostabilization, phytofiltration, phytovolatilization & phytodegradation. It employs various plant processes & plant's physical properties in order to help in remediation of polluted sites. Various plant species has been reported for phytoremediation of heavy metals such as *Salix spp.* (*Salix viminalis, Salix fragilis*) for remediation of Cd, Cu, Pb, Zn; *Ricinus communis for* remediation of Cd; *Zea mays* for remediation of Zn, Cd, Pb; *Populus* spp. (*Populus nigra, Populus deltoides* & *Populus trichocarpa*) for remediation of Cd, Cu, Pb, Zn and *Populus canescens* for remediation of Zn heavy metal [Dixit *et al*., 2015]. Factors affecting the detoxification mechanisms can includes the type of the microorganism [Oves *et al*., 2015].

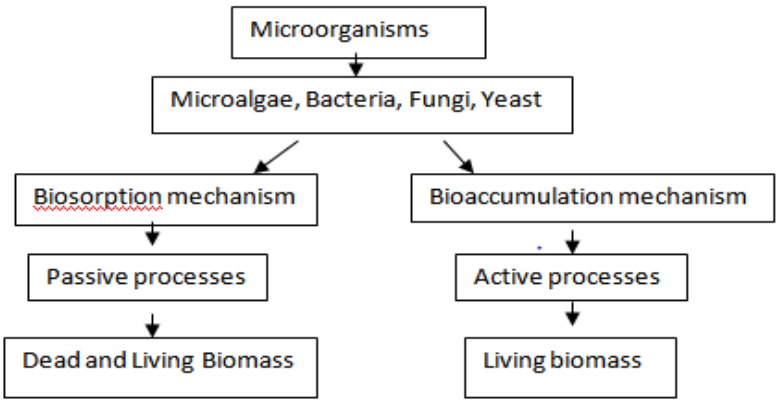

Figure 1.3: Microorganisms commonly used for Bioremediation of metals.

[Coelho et al., 2015; Rajendran *et al*., 2003]

Active uptake and/or passive uptake are two ways via which microbial organisms uptake the heavy metals. Active uptake includes the bioaccumulation & passive

uptake includes the adsorption. The cell wall of microorganisms, primarily incorporates polysaccharides, lipids & proteins, presents various functional groups which can adhere heavy metal ions. These functional groups includes the carboxylate, hydroxy, phosphate & amino groups. Out of various processes mediated by microbes, the biosorption, is suggested as more feasible for industrial applications at large scale as in comparison to the bioaccumulation process, as a result of microbes would require addition of nutrients for heavy metal's uptake actively that increases the biological oxygen demand (BOD) & chemical oxygen demand (COD) in the waste matter. Furthermore, maintaining a healthy population is microbes is challenging as a result of toxicity by HMs & else environmental factors [Dixit *et al*., 2015].

Microscopic organisms are ubiquitous that prevails in heavy metal contaminated soil & acquire the ability to transform toxic HMs into non-toxic forms. In the bioremediation processes, mineralization of the organic toxicants by microbial agents into end pdts like CO_2 & H_2O, or into metabolic intermediates is being done that are used for cell growth as primary substrates. Also, microbial organisms are competent of 2 way defence by producing degradative enzymes for the contaminants as well as tolerance to associated HMs [Dixit *et al*., 2015]. Microbial organisms can sustain in all environments as a result of their innate ability to adopt the toxicants as nutrients like heavy metals due to accumulative and/or absorptive potentiality. Soil microflora are best-known to play a key role in the mobilization & immobilization of metal cations, thereby altering their availability to the plants [Abd et al., 2015].

Bacteria, as an efficient biosorbents, have been reported for heavy metal biosorption as a result of their small size & being ubiquitous in all envirnments. Recently, differnt types of bacteria have been reported to remediate varied heavy metals in their different growth phases.Another biosorbent, fungi, constitutes a versatile group that comprises the ability to adapt & proliferate under varied harsh circumstances [Anand *et al*., 2006] that depends on a range of their tolerance mechanism. Major benefit in use of fungi for bioremediation of heavy metals comprises its significant uptake potential at low anticipated price. [Shivakumar *et al*., 2014]. Species such as *Penicillium, Aspergillus, Pseudomonas, Sporophyticus, Bacillus & Phanerochaete* are found to be potential for removal of heavy metals such as chromium (Cr) & nickel (Ni) [Abd *et al*., 2015]. *Aspergillus niger* and *Aspergillus flavus* play a vital role in

removal of the heavy metals from aqueous metal solution & metal contaminated effluent via mechanism of bioaccumulation [Shivakumar *et al*., 2014]. Many microscopic organisms such as *Pseudomonas* sp., *Rhizopus* sp., *Bacillus* sp., *Aspergillus* sp., *Penicillium* sp., are reported as potential microbes that have ability to accumulate the metal nutrients in higher quantities [Heinfling *et al*.,1997]. *Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus aureus* have the ability to eliminate heavy metals. Heterotrophic fungi such as *Aspergillus* sp., *Mucor* sp., *Penicillium* sp.,& *Yarrowta* sp. can remediate both insoluble & soluble metal species from solution & are capable to eliminate cations of metals from solid waste [Heinfling *et al*.,1997; Khare *et al*., 2010].

Despite of lethality to targeted pest, prolonged application of insecticides and their affiliated heavy metals poses serious intimidation to human health as well as to the ecosystem [Gupta, 2008]. Cadmium, copper, arsenic, chromium, lead, mercury, nickel & zinc are considered the most hazardous heavy metals and are included on the EPA's (Environmental protection agency) list of priority pollutants. Thus, bioremediation presents an ideal way out for the removal of pollution for these non-biodegradable pollutants [Cycon and Seget., 2016].

CHAPTER 2
REVIEW OF LITERATURE

In this chapter, the literature pertaining to the identification & screening of potential pyrethroid insecticide resistant microbes and bioremediation of heavy metals derived from pyrethroids, have been reviewed.

2.1 Background

Ali *et al*., (2013), made a study on quantitative determination of deltamethrin with high performance liquid chromatography (HPLC) & highlighted the consequences, of excessive usage of deltamethrin insecticide by farmers without considering their recommended limits, to non-target species. It presents the scenario of an agricultural area located in Pakistan (Hydyara, Lahore), that grass of sprayed fields, being provided as a food to the animals such as buffalos. Diseases such as paresthesia, salivation,

nausea and vomiting & reddening of skin has been observed in the individuals working in the fields. As we all know that human's metabolism may have similarity to the metabolism of animals, animal samples such as milk, blood & urine were then analyzed for the presence of residual quantities of deltamethrin and comparative studies were performed with Maximum Residue Limit (MRL) values primed by Environmental Protection Agency (EPA). Residual concentrations of deltamethrin in blood and urine samples of animals were reported to be 0.1418 & 0.0708 mg/kg resp., which were higher in values when compared with MRL values of deltamethrin conc. in blood & urine having 0.05 and 0.02 mg/kg respectively. Also, presence of deltamethrin was observed in the breast milk of females through systemic passage & found to be absorbed in the vegetables about 0.2 mg/ kg of the vegetables [Ali *et al*., 2013].

Diverse patterns of biological materials, particularly bacteria, algae, yeasts & fungi have received accelerative attention for heavy metal removal and recovery due to their good performance, low cost & large available quantities. Biosorbents offer cheaper & more effective alternatives for the elimination of metallic elements, especially heavy metals from aqueous solution [Wang and Chen., 2009].

Dixit *et al*., (2015) has discussed various approaches for bioremediations such as bioremediation by Adsorption, by Physio-Bio-Chemical Mechanism, exploration of the involvement of Molecular Mechanisms in bioremediation Process, Phytoremediation, "Designer" Microbes Approach along with application of Nano-Biotechnology. Designer Microbes Approach involves the

"Microbial biosensors" have been developed by Genetic engineering, in order to measure the level of pollution in polluted locales, rapidly with quality. In order to measure HM concentrations such as cadmium (Cd), mercury (Hg), nickel (Ni), copper (Cu) &arsenic (As), a variety of biosensors have been designed. Practically, the molecular approaches have been restrictedly applied to strains such as *Pseudomonas putida, Bacillus subtilis, Escherichia coli etc.* This entails the need for exploration of other microbes for their applicability in HM bioremediation through with molecular intervention [Dixit *et al*., 2015].

Qayyum *et al*., (2016) explored the potential of fungi Rhizomucor pusillus (F6) & Aspergillus flavus (F4) as biosorbent for removal of high concentrated heavy metals such as Pb, Cr & Cd from soil and industrial effluents, using Sabouraud dextrose

broth (SDB) medium. 39.58 mg/g, 68.02 mg/g & 68.87 mg/g of maximum uptake was observed by *A. flavus* (F4) &*R. pusillus* (F6) [Qayyum *et al.*, 2016].

Abd *et al*., (2015) made a study on 26 fungal cultures (isolated from different phosphatic source) and tested them for biosorption of heavy metal on the solid medium containing different metal ions at 50ppm conc. using two diiferent media such as potato dextrose agar (PDA) and czapek dox agar medium. Selected isolates were screened for their potential of heavy metal removal with different conc. ranging from 0 to 150 ppm in the liquid medium. 5 isolates were selected for that were able to withstand higher concentrations (150 ppm) of heavy metals such as U, Zn, Cu, Co and Pb. The isolates were identified based on their morphological properties & reported as *Aspergillus niger* and *Aspergillus flavus* [Abd *et al*., 2015].

Khare *et al*., (2010) reported four isolates named as *Bacilus subtilis* (NIA2,NIP2) *Staphylococcus aureus* (AIN3) and *Pseudomonas aeruginosa* (NIA4) that showed greater tolerance against detrimental heavy metals such as Zn, Hg and Pb, were mentioned on the basis of their morphology, physiological & biochemical characteristics. Further, these isolates were compared with their standard MTCC strains. About $15x10^9$ cfu/ml tolerance in 100 μg/ml of mercury chloride was showed by Bacilus subtilis NIA2, $15x10^9$ cfu/ml tolerance in 400 μg/ml of zinc acetate was showed by *Bacilus subtilis* NIP2, $11x10^9$ cfu/ml tolerance in 150 μg/ml of lead acetate by *Pseudomonas aeruginosa* (NIA4), and *Staphylococcus aureus* (AIN3) showed tolerance upto 450 μg/ml of zinc sulphate [Khare *et al*., 2010].

Zhang *et al*., (2016) reported the catabolic potential of *Bacillus cereus* Y1 to degrade Deltamethrin pyrethroid insecticide, isolated from deltamethrin contaminated soil (china). The dissipation or profligacy rates of were reported as 99.4 and 22.8% at initial concentrations of 10 and 100 mg/L respectively at optimal conditions of pH 7.5 and 30 ℃ temperature [Zhang *et al*., 2016].

Tiwari and dubey., (2015) reported a cypermethrin-pyrethroid degrading bacteria *Bacillus sp.* AKD1, isolated from sludge (India), having catabolic mode of action at optimum conditions of pH 8 and 37.8 ℃ temperature. The degrading capacity of the bacteria was reported as 86, 73, 67, 51 and 47% of cypermethrin insecticide at 100, 150, 200, 400 and 500 mg/L respectively, in 7 days [Tiwari and dubey., 2015].

Chen *et al*., (2012a) explored *Bacillus sp.*DG-02 for its capability to degrade various pyrethroid insecticides, isolated from source of pyrethroid-manufacturing wastewater treatment system (China), holding catabolic mode of action at optimum conditions of pH 7.5 and 30 ℃ temperature. 94.1% of Deltamethrin (50 mg/L) was degraded with *Bacillus sp.* DG-02 within incubation period of 72 hours. Fanpropathrin insecticide was reported to degrade in 72 hours by *Bacillus sp.*DG-02, by 100, 93.3, 90.4, 87.6, 84.7, 80.5, 75.8, 67.2 and 61 % of fanpropathrin at various concentrations of 25, 50, 100, 200, 400, 600, 800, 1000 and 1200mg/L respectively. 89.2% of cypermethrin insecticide (50mg/L) was removed within 72 hrs. 65.1% and 63.6% of bifenthrin and permethrin resp. was degraded within time period of 72 hours [Chen *et al*., 2012a].

Xiao *et al*., (2015) made study on *Bacillus subtilis* BSF01 and explored its catabolic potential to degrade various pyrethroids, isolated from activated sludge from pesticide manufacturing waste-water treatment system (China), at optimum conditions of pH 6.7 and 34.5 ℃ temperature. 86.9% of Deltamethrin (50 mg/L) was removed within 7 days. 93.9, 89.4 and 84.7% of Cypermethrin (50 mg/L) was removed within 7 days at conc. of 25, 50 and 100 ppm respectively [Xiao *et al*., 2015].

Chen *et al*., (2015) made study on *Bacillus thuringiensis* ZS-19 and explored its catabolic potency to degrade various pyrethroids isolated from activated sludge from pesticide manufacturing waste-water treatment system (China), at optimum conditions of pH 7.5 and 30 ℃ temperature. 92.4% of Deltamethrin (100mg/L), 81% of Cypermethrin (100mg/L), 86% of Cyfluthrin (100mg/L), 50.9% of Bifenthrin (100mg/L), 98% Fanpropathrin & 100, 95.5, 87.4, 84, and 82.1 % of Cyhalothrin at 100, 200, 400, 600, 800 mg/L concentrations respectively were degraded in 72 hrs of incubation [Chen *et al*., 2015].

Bacillus sp. SG2, was reported to aggrade 82% of Cypermethrin pyrethroid in 15 days of incubation at optimal conditions of pH 7 and 32 ℃ temperature, isolated from contaminated soil (India) [Bhat *et al*., 2016]. *Bacillus amyloliquefaciens* APO1, isolated from contaminated soil (Korea), was reported having degradation capacity of about 45% of Cypermethrin (50 mg/L) within 5 days of incubation at pH 7 and 30 ℃temperature, harboring catabolic and co-metabolic mode of action [Lee *et al.*, 2016].

Akbar *et al*., (2015b) explored the pyrethroid degradation capacity of *Bacillus megaterium* JCm2, isolated from contaminated soil (Pakistan), at optimal conditions of pH 7 and 30 °Ctemperature. About 83% of Deltamethrin (100 mg/L), 10% of Cyhalothrin (100 mg/L) and 75% of Bifenthrin (100 mg/L) was remediated in 7 days of incubation period, And 89% of Cypermethrin (100 mg/L) was degraded in 10 days [Akbar *et al*., 2015b].

Pseudomonas aeruginosa JQ-41, isolated from pyrethroid-treated soil (China), was reported to hold degradation capacity of various pyrethroid insecticides at optimal pH 7 and 30 °Ctemperature. 90.4% of Deltamethrin (50 mg/L), 87.2% of Cypermethrin (50 mg/L), 70.1% of Bifenthrin (50 mg/L), 74.1 % of Cyhalothrin (50 mg/L) and 91.7% of Fenpropathrin (50 mg/L) was degraded by *Pseudomonas aeruginosa* JQ-41 within 7 days of incubation period [Song *et al.*, 2015].

Bacterial strain *Streptomyces aureus* HP-S-01, isolated from activated sludge from wastewater treatment system (China), was reported to degrade 99% of Deltamethrin (50 mg/L) within 4 days of incubation period & complete degradation of Cyfluthrin, Bifenthrin and Fenvelrate (50 mg/L) within 5 days of incubation period. 90% of Fanpropathrin(50 mg/L) and 87.4 % degradation of Permethrin (50 mg/L) was achieved in 5 days of incubation period [Chen et al., 2011d].

Fungal strain *Aspergillus niger,* isolated from Type Culture Lab, IARI (India), was reported to degrade 10 % of Cyfluthrin within 30 days of incubation period at 6.5 pH and 28℃temperature [Saikia and Gopal., 2004].

Aktar *et al.*, (2009) reported a pesticide (fenitrothion) resistant bacteria identified as *Bacillus sp.* in order to facilitate bioremediation through microbes. Research were carried out to isolate strains of microbes from the fenitrothion-contaminated soil by using enrichment culture technique comprising fenitrothion as exclusive carbon source, isolated in pure culture & chosen for fenitrothion degrading ability. Identification of isolate was resolute through biochemical & morphological characteristics; and confirmation/verification was done by 16s rRNA gene sequencing. The strain was found to harbor endogenous plasmid [Aktar *et al.*, 2009].

Kumar, S., (2011) reported six isolates of bacteria, known as *Pseudomonas spp., 2 Staphylococcus spp., Flavobacterium spp., Azomonas spp.* & *Streptococcus spp.* that were capable of degrading chloropyrifos (an organophosphorus pesticide). Basis of

identification was physical & morphological colony characteristics, staining behaviour and different biochemical tests. MIC of chloropyrifos resistant bacteria were noted. Isolates were capable to grow in the presence of chloropyrifos in 60-100 mg/L, varied between temperature range 27°C-37°C and 6-8 pH range. Optimum growth of majority of the isolates were reported at temperature 37°C and at 7.5 pH. It was concluded that optimization of factors such as temperature & pH is absolutely essential before culture at large scale [Kumar, S., 2011].

Ragib, O.S.A., (2010) reported sixteen bacterial strains isolated from soil collected from a pepper plantation with history of deltamethrin application, putatively identified to five different genera include *Acetobacter spp., Pseudomonas spp., Acidiphilium spp, Aminobacter spp., and Aeromonas spp.,* designated as the prolific deltamethrin-tolerant bacteria that were able to tolerate 9600 ppm concentration of deltamethrin. Further, heavy metal tolerace of isolates was assessed. *Aminobacter spp.* showed resistance towards mercury, copper and zinc, and proved to be a potential candidate for biodegrader in co-contaminated soil environment. It was concluded that bacterial resistivity towards deltamethrin and some heavy metals were chromosomal encoded enzymatic reaction [Ragib, O.S.A., 2010].

Cycon and Seget, (2016) review article presents an overview of the microorganisms isolated from pyrethroid-polluted sites, characterized and applied for the pyrethroid degradation. Bacterial strains from the genera *Achromobacter, Acidomonas, Bacillus, Brevibacterium, Catellibacterium, Clostridium, Lysinibacillus, Micrococcus, Ochrobacterum, Pseudomonas, Serratia, Sphingobium, Streptomyces,* and the fungal strains from the genera *Aspergillus, Candida, Cladosporium and Trichoderma* were characterized by their ability to degrade various pyrethroids such as deltamethrin, permethrin, allethrin, bifenthrin, fenpropathrin, cyfluthrin, cyhalothrin and cypermethrin. This article also discussed about the pyrethroid degradation pathways, the enzymes which are responsible for cleavage of pesticide molecules and the factors or conditions that influence the survival of strains introduced into soil and removal rate of pyrethroids [Cycon and Seget., 2016].

Chen *et al,* (2012d)., explored the fungal strain *Candida pelliculosa* ZS-02 for its pyrethroid degrading capability at 6.5 pH and 28 ℃ temperature. Degradation rates of

Bifenthrin (100 mg/L) were reported as 97.1, 95.8 and 93.8 at 200, 300, and 400 mg/L concentration during 5 days of incubation period. 93.4% of degradation of Deltamethrin (50 mg/L) and 87.7% of cypermethrin (50 mg/L) within 5 days of incubation period was reported [Chen etal, 2012d].

Banerjee *et al*., (2015) explored one of the mechanism of heavy metal tolerance by microorganisms, named as Bioaccumulation, that rely on catabolic & anabolic energy of microorganisms. Four types of bacteria was isolated from ash dyke samples collected from different places located in Chattisgarh. Three screened isolates were tested for their tolerance against different concentrations of Pb, Co, Ni, Hg,Cu and Mn heavy metals that showed maximum tolerance existed upto 0.6 mM/ml. Through 16S rRNA sequencing, the isolates were identified as *Bacillus cereus* and *Bacillus subtillus* [Banerjee *et al*., 2015]**.**

Shivakumar *et al*., (2014) explored the bioaccumulation mechanism of heavy metal resistance by two strains of fungal genus *Aspergillus*, named as *Aspergillus niger* and *Aspergillus flavus* in aqueous medium of 6 major heavy metals named as Copper (Cu), Zinc (Zn), Lead (Pb), Chromium (Cr), Cadmium (Cd), and Nickel (Ni) in industrial treated effluent at optimal conditions of pH 5.0, 25°C temperature & 120 hrs of incubation, for maximum metal accumulation. *Aspergillus niger* showed 75% tolerance towards Lead (Pb), 49% of Zinc (Zn), 45% of Copper (Cu), 41 % of Chromium (Cr), & 25% of Nickel (Ni). Similarly, *Aspergillus flavus s*howed high percentage of accumulation of Lead (Pb) upto 82%, 40% of Zinc (Zn), 34% of Copper (Cu), & 20% of Nickel (Ni) [Shivakumar *et al*., 2014].

2.2 Pyrethroid insecticide

Since historic times, Pyrethrum, a broad spectrum natural insecticide, has been used which is isolated from the flowerhead of the *Chrysanthemum cinerariaefolium* & *Chrysanthemum cineum* plants. Pyrethrum's higher concentration is existing within flower structures known as achenes, located in the flowerhead of chrysanthemum. The lipophilic six esters collectively known as pyrethrins, are responsible for the insecticidal activity of pyrethrum to kill insects and are found within pyrethrum extract [Schleier III and Peterson, 2011., Thatheyus and Selvam, 2013].

Figure 2.1: Source of Pyrethrum: *Chrysanthemum* flower

Despite of the fact that natural pyrethrins possess insecticidal activity, they were found to exhibit higher rates of photo-degradation and a little "knockdown" effect (rapid paralysis). Therefore, after the disclosure of pyrethrin's constituents, search for their derivatives, attaining high resistivity towards photo-degradation, began, which led directly to the discovery of synthetic pyrethroids. Assets of pyrethroids includes their highly lipophilic nature, short shelf-life in the surroundings, reduced levels of toxicity towards terrestrial vertebrates & absence of capability of biomagnification like older chemical classes such as organochlorines [Schleier III and Peterson, 2011]. All pyrethroids encompass some common features such as: an ester bond located centrally, an acid moiety & an alcohol moiety. The acid moiety consist of two chiral carbons; responsible for existance of pyrethroids as stereoisometric compounds. Also, in some compounds, a chiral carbon is present on the alcohol moiety, which allow for three chiral carbons and a total of eight different stereoenantiomers [Shafer *et al.*, 2005].

A mixture of various isomers may be present in their commercial products. Varrying isomeric ratios may be responsible for variation in toxicity level of same compound. Efficiency of the pyrethroid insecticides is enhanced with compounds like piperonyl sulfoxide, piperonyl butoxide & sesamex, which function as synergists, in the formulation. Furthermore, formulations contain a high percentage of some different inert ingredients, that are extremely toxicant. Pyrethroid's efficacy and their selectivity b/w insect species depends upon considerations such as: form, physical attributes (e.g. volatile compounds holds good against flying insects), chemical attributes (e.g. polar compounds better for knockdown & high lipophilicity), key structural attributes such as ester or non ester, specific chirality & cis or trans stereochemistry over cyclopropane ring. Pyrethrins and pyrethroids along with DDT

& its analogs, belongs to a neurotoxic chemical grouping and share an allied mode of action. Several ways are there through which pyrethrins & pyrethroids enters in the organism's body, in order to employ their effect. 1st mode includes the entrance through epidermis, which is non-stereospecific, following uptake by hemolymph carriers protein or blood & subsequent circulation throughout the body. The primary route of distribution to CNS (central nervous system) is pyrethroid diffusion through epidermis cells followed by penetration. Also, it can enter in the central nervous system directly by contact with sense organs of the peripheral nervous system. The sensory organs of both vertebrates and invertebrates are highly sensitive to the pyrethroids insecticides. Second mode includes the enterance through airways in vapour phase. And, third mode is by ingestion & penetration into blood-hemolymph through the alimentary canal that can play an important role in perniciousness [Schleier III and Peterson, 2011].

Pyrethroids includes a variety of 1000 insecticides [Thatheyus and Selvam, 2013]. It has been toxicologically classified, into 2 types, type I & type II, on the basis of induction of either T syndrome or CS syndrome. T syndrome includes whole body tremors and a coarse whole body tremors advancing to choreoathetosis with a condition of salivation is CS syndrome. Type I pyrethroids includes allethrin, bifenthrin, permethrin, phenothrin, resmethrin tefuthrin, teramethrin & type 2 pyrethroids includes cyfluthrin cyhalothrin, cypermethrin deltamethrin fenvalerate fenpropathrin tralomethrin [Schleier III and Peterson, 2011]. The cyano group is present in second generation pyrethroids and is absent in first generation pyrethroid insecticides [Thatheyus and Selvam, 2013].

Second generation pyrethroids are more toxic to mammals as compared to First generation pyrethroids. Primerely, 2nd generation pyrethroids are responsible for paresthesia, indicated by transient burning/itching sensation of exposed skin. Also, it has been reported that some pyrethroids led to developmental neurotoxicity [Rehman *et al.*, 2014].

ACUTE TOXINDROMES OF PYRETHROIDS			
Pesticide	**Acute symptoms**	**Diagosis**	**Treatment**
Pyrethroids			

Ist Type E.g.Allethrin Permethrin Tetramethrin	Tremor Ataxia irritability	Urinary 3-phenoxybenzoic acid (in research studies)	Supportive care Symptomatic decontamination
IInd Type E.g. Deltamethrin Cypermethrin Fenvalerate	Salvation Temporary paraesthesias seizures	Urinary 3-phenoxybenzoic acid (in research studies)	Supportive care Symptomatic decontamination
Organo-chlorines E.g. Lindane Endosulfan	GABA blockage Tremors Dizziness seizures	Detectable in blood	Supportive care Symptomatic decontamination

Table 2.1: Acute toxindromes of Pyrethroids

Deltamethrin, an alpha-cyano pyrethroid insecticide, kills insects through dermal contact & digestion [Shrivastva *et al.*, 2011] used as crop pesticide & in public or domestic hygiene as a biocide [Junquera, 2007-2016].

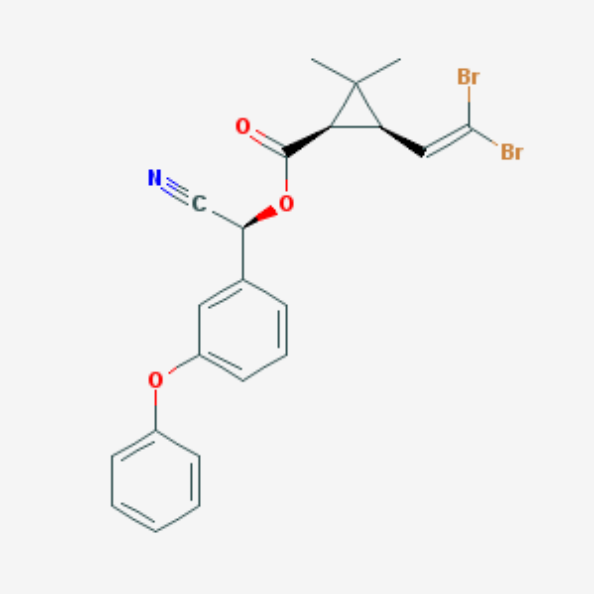

Figure 2.2: Chemical structure of Deltamethrin

Physical and Chemical Properties of Deltamethrin	
Chemical formula	$C_{22}H_{19}Br_2NO_3$
Boiling point	300 ℃
Molar mass	505.21 g/mole
Density	1.5 g/cm^2
Melting point	98 ℃
Vapor pressure	1.5×10^{-8} mm Hg at 25 ℃
Octanol-Water Partition coefficient (log K_{ow})[3]	6.1
Solubility	0.002 to 0.0002 mg/L
Soil Sorption Coefficient (K_{0c})	7.05×10^5 to 3.14×10^6

Table 2.2: Physical and Chemical Properties of Deltamethrin

Deltamethrin, in its most pure form, is colorless /white to light beige crystals that are odourless. As a lipophillic compound, deltamethrin is not soluble in H2O and therefore, is highly stable in the physical surrounding or environment [Shrivastva *et al.*, 2011]. It was first described in 1974 & enter in the market in the year 1978. Deltamethrin insecticide products can be formulated as dusts, sprays, aerosols, wettable powders & granules. The illegitimate formulations such as "Chinese Chalk" or "Miraculous Chalk" often contains deltamethrin as the active ingredient.

APPLICATIONS OF DELTAMETHRIN	
Pyrethroid	Deltamethrin
Insects	Aphids, Beetles, Bollworm, Bud-Worm, Caterpillars Cicadas, Codingmoths, Totrix Moths, Weevils, Whitefly, Winter Moths

Crops	Alfalfa, Beet, Cerels, Coffee, Cotton, Figs, Fruits, Hops, Maize, Oilseed Rape, Olives, Oil Palms, Potatoes, Rice, Soybeans, Sunflower, Tea, Tobacco, Vegetables
Other locations and applications	Households, forests, animal houses, stored products.

Table 2.3: Various applications of Deltamethrin insecticide

Deltamethrin's mode of action is primerely, central in action or originate in brain's higher nerve centers. Death of insects may be due to irreversible damage to the nervous system when poisoning occurs lasts more than a few hours [Rehman *et al.*, 2014]. Deltamethrin acts on the nerve cell's membrane, (blocking the closure of sodium channel ion gates during re-polarization) followed by disruption of the transmission of nerve impulses, causing repetitive discharges. Insects & other arthropods suffer from hyperactivity when there is low concentration of deltamethrin but, increased concentrations led to paralysis & death. Besides neurotoxic effects, deltarmethrin exhibits hepatotoxic effects & induces microsomal enzymes in the liver [Junquera, 2007-2016].

Acute Effects Of Deltamethrin Pyrethroid Formulation On Non- Target Organisms	
Birds (mg pyrethroid /kg body wt.)	>4640 mg/kg
Fish	Toxic
Bees	Highly toxic
Rats	>2940 mg/kg
Dogs	300 mg/kg
Rabbits	>2000 mg/kg

Table 2.4: Acute effects of Deltamethrin formulation on non-target organisms

Primarily, the nervous & muscular systems are affected by prime symptoms of intoxication through deltamethrin as well as other synthetic pyrethroids. Most

frequent symptoms includes uncoordinated trembling/ shaking movements (tremor), uncoordinated movements (ataxia), skin sensation of tingling, tickling, prickling (paresthesia), exhaustion (lethargy, fatigue), hypersalivation, hyperreactivity (exaggerated reaction to stimuli) & urinary incontinence.

Deltamethrin insecticide is water insoluble, tends to adhere to particles of soil and doesn't bioaccumulate. Therefore, groundwater pollution is unlikely to occur.

Persistency of water rely on the factors such as pH & temperature. Soil particles are having moderate persistency that breaks down within 6-14 days. Soil bacteria greatly contribute to the biological degradation of deltamethrin insecticide. deltarmethrin exhibits hepatotoxic effects & induces microsomal enzymes in the liver [Junquera, 2007-2016].

2.3 Impact of heavy metal on human health and plants

The ingestion of zinc (Zn) at higher level than recommended level can have adverse effects on health. If doses are 10 –15 times higher than the recommended & are taken by mouth, even for a short time, the stomach cramps, nausea & vomiting may occur.

Anaemia, damage to the pancreas, & decreased levels of high-density lipoprotein (HDL) cholesterol may be caused by consumption of large amount of Zn for several months [Khare et al., 2010]. Cu consumption is responsible for wilson's disease, kidney failures, liver failures, brain damage, & even death. Arsenic (Ar) causes an endemic blackfoot disease. Cadmium (Cd) is reported to cause lung insufficiency, lung insufficiency, renal disturbances, cancer, bone injury, & hypertension in humans [Ghosh *et al*., 2015]. Mercury (Hg) is one of the major global pollutants in the environment which is highly toxic [Khare et al., 2010]. Genotoxicity & varied neurological and physiological disorders are caused by mercury as it adheres to the sulfur & nitrogen atoms of proteins and nucleic acids [Ghosh *et al*., 2015].

Heavy metal	EPA Regulatory limit (ppm)	Toxic effects of heavy metals on human health	References

Hg	2.0	Insomnia, autoimmune diseases, depression, drowsiness, fatigue, hair loss, loss of memory, restlessness, disturbance of vision, tremors, temper outbursts, brain damage, lung & kidney failure.	[Dixit *et al*., 2015., Yadav *et al*., 2017]
Cu	1.3	Brain & kidney damage, elevated levels results in liver cirrhosis and chronic anemia, stomach and intestine irritation.	[Dixit *et al*., 2015., Yadav *et al*., 2017]
Zn	0.5	Tiredness, dizziness etc.	[Dixit *et al*., 2015., Yadav *et al*., 2017]

Table 2.5: Toxic effect of heavy metal on human health.

Some of the metals, like Zn, Cu, Mn, Ni & Co are micronutrients, essential for plant growth while others, like cadmium (Cd), lead (Pb) and mercury (Hg) have no known biological function and very toxic even at lower concentration of 0.001-0.1 mg/L [Yadav *et al*., 2017].

Heavy metal	Toxic effect on plants	References

Hg	Interferes the mitochondrial activity and induces oxidative stress by triggering the generation of reactive oxidative species (ROS) resulting disruption of biomembrane lipids & cellular metabolism	[Yadav *et al*., 2017]
Cu	It interferes with cellular process, such as photosynthesis and respiration, and Inhibit plant growth.	[Yadav *et al*., 2017]
Zn	Inhibition of metabolic functions by Zn results in retarded growth & senescence, limits the root and shoot growth, chlorosis.	[Yadav *et al*., 2017]

Table 2.6: Toxic effect of heavy metal on plants.

2.4 Bioremediation: Biosorption and Bioaccumulation

Transformation/alteration of toxic metals & radionuclides by aid of microbial organisms may change their solubility, mobility & bioavailability. Processes assocoiated with microbial agents may affect mobilization or immobilization of toxic pollutants by one or more of the following executive mechanisms: Chelation of

elements by metabolites, Oxidation-Reduction of metals which affect the solubility or valence state, Change in pH which affect the ionic state, Biosorption by functional groups on the cell surface, bioaccumulation by an energy-dependent transport system, Immobilization due to formation of stable materials, Biomethylation, Biodegradation of organic complex of metals and radionuclides [Gazso, 2001].

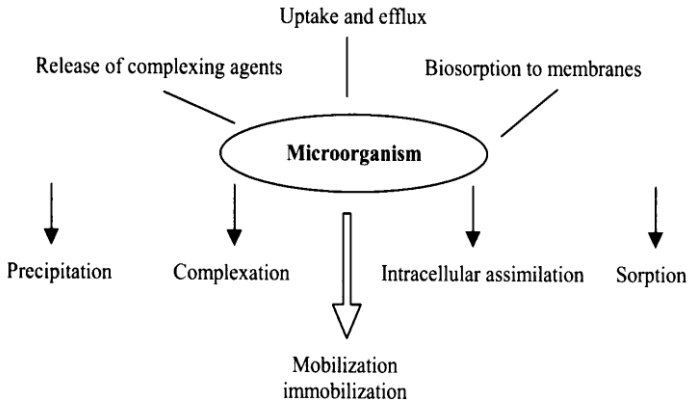

Figure 2.3: Metal processing mechanisms of Microorganisms [Gazso, 2001].

Biosorption & bioaccumulation are two efficient processes that includes the binding & accumulation of HMs & contaminants from the environment with aid of microbial agents or associated biomass. However, both processes of biosorption & bioaccumulation execute their work in distinct ways. During process of biosorption, pollutants are adsorbed onto the cellular surface of sorbents in quantities which depends on the constitution & kinetic equilibrium of the cellular surface. Thus, biosorption, being a passive metabolic process, does not require energy or respiration.

On the other hand, bioaccumulation, being an active metabolic process, requires respiration & energy. Toxicants (such as HMs) adhere to the cellular surface of microorganisms during biosorption, which is a reversible process, whereas bioaccumulation is only partially reversible process. Biosorption was also shown to be rapid & to produce a greater number of concentrations. Biosorption is an emerging technique that came into practice about two decades ago. It holds an eminent potential as a cost-efficient process for environmental cleanup & reducing pollution by heavy metals resulting from industrial & agricultural sources. This technique rely on the sequestration of hazardous heavy metals by the moieties of biosorbent cell surfaces such as those present in bacteria, fungi/yeast, and algae. Its applicability comprises

heavy metal elimination from soil, landfill leachates & water as well as several other roles.

Bioaccumulation process is applicable, especially when the absorption rate of the contaminant is higher than the rate of losing it. Thus, the contaminant remains contained & accumulated in the organism. It is a toxico-kinetic process that affects the sensitivity of living organisms to chemicals. Organisms can usually resist concentrations of chemicals up to a certain limit, beyond which these chemicals become toxic & endanger the organism. The organism's sensitivity to various chemicals is highly variable that depends upon the organism's type & chemicals involved. Candidate organisms for bioaccumulation should have a tolerance ranging between one or more contaminants to higher levels. Furthermore, they may exhibit superior biotransformational potentiality, transforming the toxic chemical to a non-toxic form that enables the organism to lessen the toxicity of the contaminant while keeping it contained [Mosa *et al*., 2016]. Simply, it can be defined as the uptake of toxicants by living cells. The toxicant can transport into the cell across the cell membrane & accumulate intracellularly [Shivakumar *et al*., 2014].

2.5 Bergey's manual

Bergey's manual of systematic bacteriology defines its purpose in classification, cultural characteristics & diseases caused by particular genus or species of bacteria. Volume 4 states genus description & separate listing for each species in alphabetical order. Some bacteria might not appear in any of the volumes as that was not known to scientic community until the publication of that particular volume. On the basis pf molecular classification the species are classified into following groups: Archaea (highly branched phototropic bacteria), Proteobacteria (contain most of gram negative bacteria, having subclasses of (α, β, δ and ε), Firmicutes (gram positive bacteria having low percentage of GC content) and Actinobacteria (gram positive bacteria having high %age of GC content and some species of this class are good antibiotic producers). The recognition of unknown cultures is a practical application of classification system, in order to have a new isolate that can be identified as a member of pre-existing species. Bergey's manual can be used to find an unknown bacteria by following steps:

1. Identify the group no. of the unknown microorganism

2. The genus of the unknown should be determined

3. Check the different genuses with your genus to see if there is any similarity.

4. Identification of the unknown microbe at species level.

5. Troubeshooting the results.

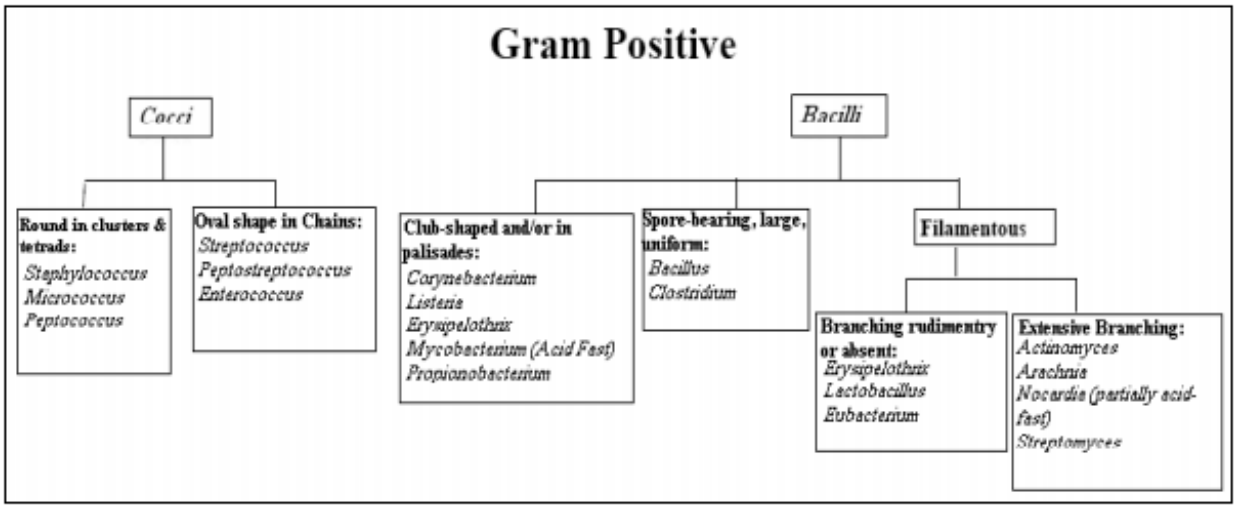

Figure 2.4: Differentiation via Gram Stains & Cell Morphological properties (Gram +ve)

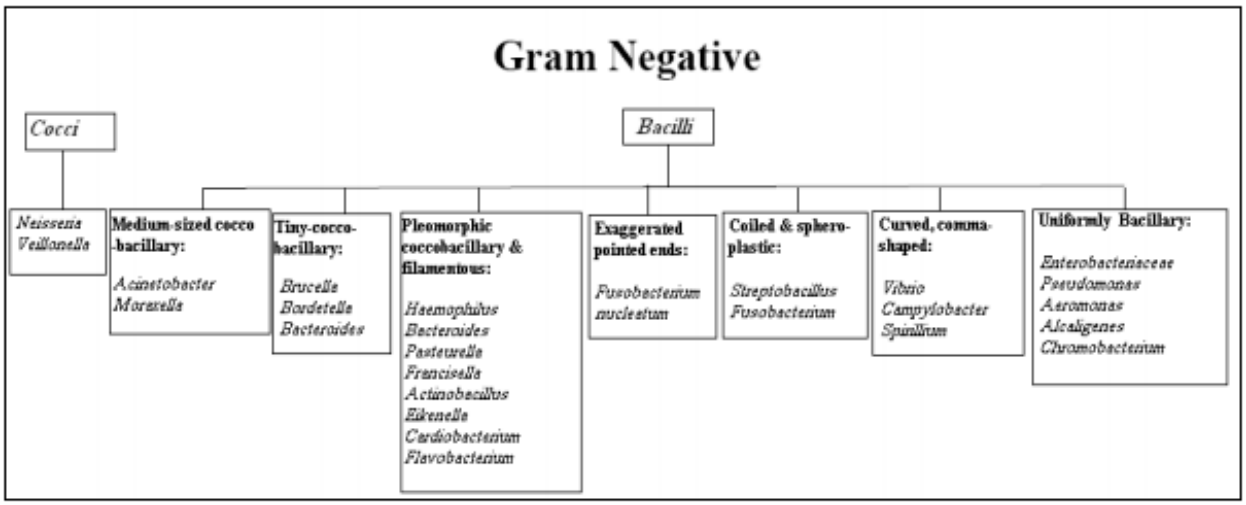

Figure 2.5: Differentiation via Gram Stains & Cell Morphological properties (Gram -ve)

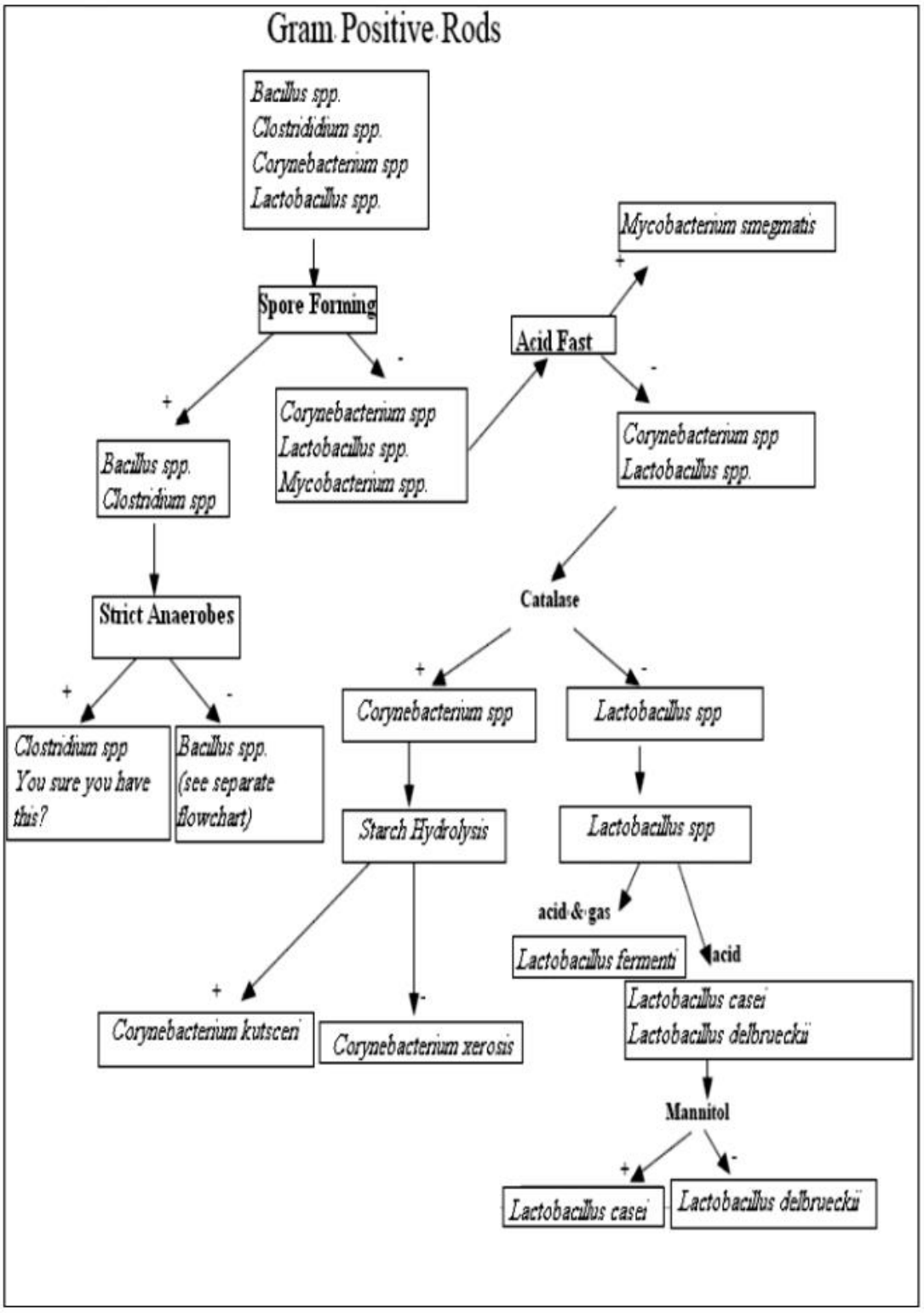

Figure 2.6: Gram Positive Rods ID Flow Chart

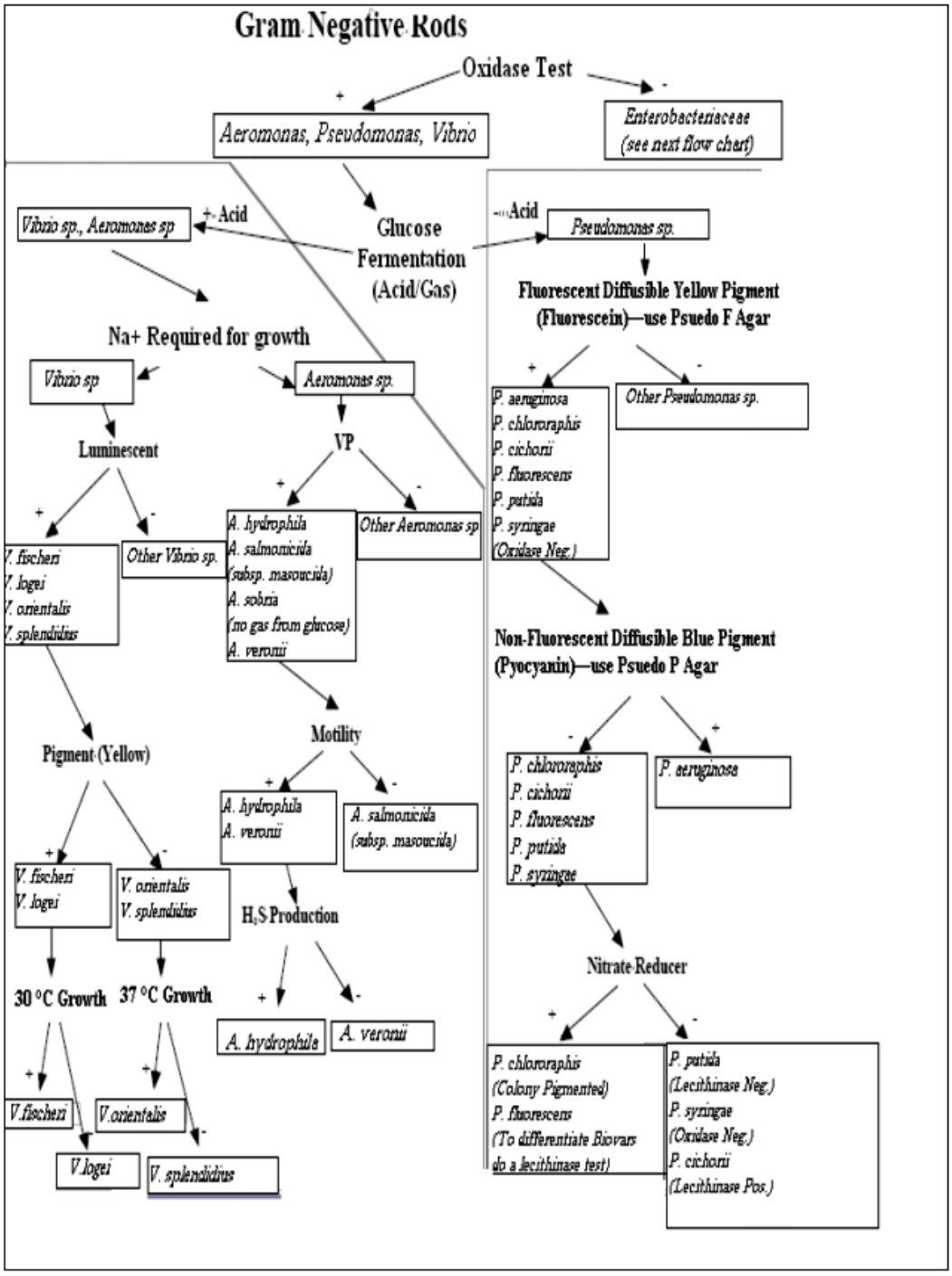

Figure 2.7: Gram Negative Rods ID Flow Chart

Bergey's manual is used to classify bacteria on the basis of their structural and functional properties by arranging them into specified familial orders. Group number determination is of prime importance. To find the group number, look through the table of contents of the manual as follows :

- Gram-Negative, Aerobic or Micro aerophilic Rods and Cocci --**Group 4**

- Facultatively Anaerobic Gram-Negative Rods --**Group 5**
- Gram-Positive Cocci --**Group 17**
- Endospore-Forming Gram-Positive Rods and Cocci --**Group 18**
- Regular, Nonsporing Gram-Positive Rods --**Group 19**
- Irregular, Nonsporing Gram-Positive Rods --**Group 20**
- Aerobic, Nonmotile, Nonsporing, Acid-fast, Weakly Gram-Positive Rods --**Group 21.**

2.6 PCR amplification & 16S & 18S rRNA gene sequencing

Primarily, 16S rRNA, the ribosomal operons, has evidenced to be a stable & specific molecular marker for bacterial identification. The 16S rDNA exist in disordered form in the whole genome of any bacteria. For phylogenetic analysis & molecular taxonomy of bacteria, these ribosomal sequences are usable. The 16S rDNA's mosaic arrangement of phylogenetically conserved & inconsistent/variable region within the gene, is actually responsible, for being a common target for taxonomical purposes & identification at molecular level. The upstream region of 16S rRNA can also be used for the confirmation of the thermodynamic stability based on conserved secondary RNA structures [Singh *et.al.*, 2012]. The gene sequences of 16S rRNA are the most frequently used constitutive genetic marker because of some genuine reasons such as: 16S rRNA is present in about all bacteria, oftentimes existing as operons; 16S rRNA gene function with time remains unchanged; and is large enough (consisting 1500 bp) for Informatics utility [Janda and Abbott., 2007]. Genotype identification of fungal isolates can be carried out by 18S rRNA sequencing.

CHAPTER 3

MATERIAL AND METHODS

3.1 Sample collection

Soil samples were taken from a vegetable cultivation field having history of insecticide application at Ranipur village, situated in Phagwara (Pb). To increase the variety of microflora in the soil, approximately a total of 500 g of fresh soil was taken from three different spots in the farm. To obtain the soil sample, surface of the area to be sampled were made clear of any vegetation in order to get accurate measurement of soil content. A sterilized hand trowel were used to dug approximately 6 inch deep, mixed up the soil in the hole and collected together from different spots in the field in a sterile polyethylene bag. Aseptic techniques were applied to prevent contamination during sample collection. Moist soil sample was then, air dried at room temperature and kept in 4°C prior to begin the experiment.

Figure 3.1: Collection of soil sample

3.2 Preparation of Growth medium

Four most frequently used growth media are enlisted in table 3.1, which were used for cultivation & enumeration of bacteria and fungi. Appropriate amount of media powder was suspended in respective amount of distilled water. To dissolve the medium completely, it was heated till boiling. The prepared media was sterilized prior to use, at 121ºC at 15 psi for 15 minutes. The mixture was mixed thoroughly prior to pouring into the petri dish.

S.No.	MEDIA	COMPOSITION		COMPANY
1.	Nutrient Agar (NA)	Ingredients	Gms/lt	HiMedia
		Peptic digest of animal tissue Sodium chloride Beef extract Yeast extract Agar	5.00 5.00 1.50 1.50 15.00	
2.	Nutrient Broth (NB)	Peptic digest of animal tissue Sodium chloride Beef extract Yeast extract	5.00 5.00 1.50 1.50	HiMedia
3.	Soyabean Casein Digest Agar	Pancreatic digest of casein Papaic digest of soyabean meal Sodium chloride Agar	15.00 5.00 5.00 15.00	HiMedia
4.	Potato Dextrose Agar (PDA)	Potatoes infusion from Dextrose Agar	200.00 20.00 15.00	HiMedia

5.	Potato Dextrose Broth (PDB)	Potatoes infusion from Dextrose	200.00 20.00	HiMedia
6.	Sabouraud Dextrose Chloramphenicol Agar	Peptones Dextrose monohydrate Tetracycline Agar	10.000 40.000 0.050 15.000	HiMedia
7.	Rose Bengal Agar (with Chloramphenicol)	Dextrose Papaic Digest of Soybean Meal Monopotassium Phosphate Magnesium Sulfate Chloramphenicol Rose Bengal Agar	15.0 5.0 1.0 0.5 0.1 0.05 15.0	HiMedia

Table 3.1: Manufacturer and composition of media used

3.3 Physiochemical characterization of the sample

Soil sample was characterized for its physiochemical properties from soil testing laboratory in Punjab Agricultural University (PAU), ludhiana.

3.4 Isolation of soil microflora by serial dilution & plating method

Enumeration of soil bacteria and fungi was done by dilution and plating method. For preparation of solution sample 10 g soil was mixed in 90 ml of distilled water & mixed thoroughly, representing 10^{-1}dilution & labelled as solution A. By using a sterilized measuring pipette, 1 ml of solution sample was transfered to 9 ml distilled water labelled as solution B representing10^{-2} dilution. Tube was thoroughly mixed and this step was repeated upto solution E, representing 10^{-6} dilution. Approximately 0.1 ml of each diluted culture was then spread over the surface of Soyabean Casein Digest Agar for growth of bacteria & Rose Bengal and Saubraud Dextrose Chloramphenicol Agar for growth of Fungi, using sterile glass spreader. Plates were

then incubated until colonies appeared, at 35-37ºC & 25-27 ºC for baceria and fungi respectively.

STEPS	SOLUTIONS	DILUTIONS
10 g soil (w/v)	90 mL distilled water (solution A)	10^{-1}
1 mL solution A (v/v)	9 mL distilled water (solution B)	10^{-2}
1 mL solution B (v/v)	9 mLdistilled water (solution C)	10^{-3}
1 mL solution C (v/v)	9 mL distilled water (solution D)	10^{-4}
1 mL solution D (v/v)	9 mL distilled water (solution E)	10^{-5}
1 mL solution E (v/v)	9 mL distilled water (solution F)	10^{-6}

Table 3.2: Serial dilution method

3.5 Physical characterization of bacterial & fungal isolates

Colony morphology refers to the visible characteristics of a colony. A bacterial colony consists of numerious bacterial cells, derived from one parent . Description of a colony's morphology includes its shape, edges, color & surface features. Some colonies are round & smooth, others can have wavy edges and a wrinkled appearance. Basically, a bacterial colony seems like a dot that is growing on the medium & is composed of millions of bacteria that originate through binary fission from one initial bacterium i.e., the parent. Differentiation based on the appearance of the bacterial colonies is a crude step, but is fundamental step in isolating the different types of bacteria as well as fungi in the sample that often found in thin mycelial monolayers. Being able to visibly differentiate bacteria based on the appearance of their colonies is a crude, but essential first step in isolating the different types of bacteria as well as fungi in the sample that often exist in thin mycelial monolayers. Thus, bacterial colonies were examined on the basis of colony shape, colony height, colony margin, surface refraction, opacity and color. And, fungal colonies were examined on the

basis of their texture, color, shape, surface appearance & form of conidia, phialides and conidiophores.

3.6 Biochemical Characterization of Bacterial isolates

In order to identify bacteria, various biochemical reagents were used. Availability of large number of biochemical procedures for bacterial identification is there. They are being done depending upon the type of bacteria isolated & are described as follows.

3.6.1 Gram Staining

Differentiation between Gram positive and Gram negative bacteria is done by gram staining. Gram positive bacteria hold on to primary dye iodine complex and appear violet in color while Gram negative bacteria get de-colorized thus, taking up the counter stain (pink/red) appear red/pink in color.

Procedure

Bacterial culture was taken on a clean glass slide & heat fixed. Flooded the smear with Crystal violet for 1 minute with the help of dropper & washed with water. (Primary stain). Followed by addition of gram's Iodine to the smear for 1 minute & washed with water. Ethanol was added then which acted as a decolorizer for about 30 seconds & was quickly washed with water. The smear was then flooded with safranin (Counterstain) for about 1 minute & washed with water. Smear was cleaned with blotting paper, air dried & examined under microscope.

3.6.2 Lactophenol Cotton Blue Staining

This method is used for staining and examining fungi. Consist of Phenol, Lactic acid & cotton blue (LPCB). Phenol : Live organism get killed, Lactic acid : Fungal structures preservation, Cotton blue : The chitin and cellulose of the fungal cell wall is stained intensely blue.

Procedure

A drop of 70% ethanol was placed on a clean glass slide & submerged the specimen in the drop of alcohol. Before drying of alcohol, one drop of the LPCB was added & covered with a cover slip for avoiding formation of air bubbles. Examined under microscope, initially with low power objective & then with high power objective lens (40X).

3.6.3 Catalase Test

Confirmation of the presence of catalase enzyme in the organism is determined by catalase test. Catalase directs the breakdown of hydrogen peroxide (H2O2) into oxygen and water. When a small inoculum is introduced into hydrogen peroxide (30% for slide test), the immediate effervescence of O2 bubbles occurs which confirms the presence of the enzyme in a bacterial isolate. If their is no bubble production then it means their is scarcity of catalase enzyme.

Procedure

One drop of 3% of hydrogen peroxide (H_2O_2) was placed on the bacterial slide but didn't allow it to get mixed. Observed it for either immediate bubbling or no bubbling. *Bacillus subtilis* was taken as positive control & *Streptococcus pyregenes* was taken as negative control.

3.6.4 Indole Test

Monitorization of the ability of an organism to degrade the amino acid tryptophan & produce indole. The transformation of pink to red color ("cherryred ring") confirms indole production reagent by development of a layer on top of the medium within few seconds of adding. The reagent layer remains yellow or slightly cloudy if indole is not produced.

Procedure

Tryptone broth tube was inoculated with a loopful of pure culture, incubated at 37°C for a period of 24-48 hours. 5 drops of Kovác's reagent were then added directly to the tube & observed for the color transformation in the tube. *E.coli* was taken as positive control & *Bacillus subtilis* was taken as negative control.

3.6.5 Citrate test

Screening of a bacterial isolate is done for the ability to use citrate as a carbon & energy source. Positive diagnosis depends on the generation of alkaline by-products of cit-rate metabolism. Color change of a pH indicator determines the increase in pH of the medium. If the color of Simmon's citrate agar media changes to prussian blue

means it is citrate positive whereas if no color change is observed means it is citrate negative.

Procedure

Simmon's citrate agar slant was prepared. A single bacterial colony from a freshly prepared culture (18-24 hours old) was taken & streaked it on the slant followed by incubation at 37℃ for about 24 hours to 7 days, reason being their limited growth rate. Slants were then observed for color change. *Bacillus subtilis* was taken as positive control & *E.coli* was taken as negative control.

3.6.6 Urease Test

By performing urease test, an organism's ability to split urea by forming 2 molecules of NH_3 & CO_2, through the action of urease enzyme with resulting alkalinity is determined. If the color of tubes of rapid urease test broth changes to pink the test is positive & is negative if there is no color change in the urease broth.

Procedure

Tubes of rapid urease test broth were prepared & inoculated with freshly prepared (18-24 hours) bacterial culture followed by Incubation at 37℃ for up-to 24 hours. Tubes were then observed for change in color. *Staphylococcus aureus* was taken as positive control & *Bacillus subtilis* was taken as negative control.

3.6.7 Methyl Red (MR) Test

Certain bacteria have the ability to utilize glucose and convert it to form a stable acid. The bacteria initially metabolize glucose to pyruvic acid and later undergo mixed acid pathway to form a stable acid. The color of MR-VP broth will changes from yellow to red if bacteria uses glucose.

Procedure

Test tubes were prepared containing MR-VP broth & inoculated with freshly prepared (18-24 hours) bacterial culture. Followed by incubation at 37℃ for 48 hours. 2-3 drops of methyl red were added to the culture tubes and observed for change in color. *E.coli* was taken as positive control & *Bacillus subtilis* was taken as negative control.

3.6.8 Voges-Proskauer (VP) test

This test is used to determine whether an organism produces acetylmethyl carbinol from glucose fermentation as if it does then it is converted to diacetyl in presence of a strong alkali (α-naphthol) and atmospheric oxygen. In MR-VP broth if development of red color is there then test is positive indicating presence of diacetyl else it is negative if color does not change.

Procedure

Test tubes containing MR-VP broth were prepared, inoculated with freshly prepared (18-24 hours) bacterial culture followed by incubation at 37℃for 48 hours. Few drops of Barrit's reagent were added & exposed the tubes to atmospheric oxygen by keeping them undisturbed for 15 minutes. Broth was then observed for color change. *Bacillus subtilis* was taken as positive control & *E.coli* was taken as negative control.

3.6.9 Triple Sugar Iron Agar Test

TSIA is a differential media that contains lactose, sucrose, small amount of glucose, ferrous sulfate and pH indicator phenol red. Organisms are tested on the basis of their ability to reduce sulfur and ferment carbohydrates. In TSIA if organism can reduce sulfur the media will produce a black precipitate else if no precipitate is formed then the test is negative.

Procedure

TSIA slants were prepared & bacterial culture was streaked on them. Incubated at 30℃-37℃ for 24-48 hours & Observed for change in color of media. *E.coli* was taken as positive control & *bacillus subtilis* was taken as negative control.

3.6.10 MacConkey Agar Test

MacConkey Agar is a selective and differential media used for non-fastidious gram negative rods. It ferments lactose fermenting bacteria from lactose non-fermenting bacteria. If bacterial growth is observed in the media then result is positive else if no growth is seen test is negative.

Procedure

MacConkey Agar plated were prepared & streak with bacterial culture followed by incubation for 24 hours at 37℃. Results were then observed. *Pseudomonas aeruginosa* was taken as positive control & *bacillus subtilis* was taken as negative control.

3.6.11 Starch Hydrolysis

The ability of bacteria to hydrolyze starch is studied by this test, as they can produce sacchrolytic enzyme. Transparent clear zones are observed around bacterial colonies if test is positive else no clear zones are formed in case of negative test.

Procedure

Plates containing starch agar media were prepared and incubated at 30 ℃-37 ℃ for 24 to 48 hours. The plates are flooded with iodine solution when bacterial growth was visible on the plates. Observed for results. *Bacillus subtilis* was taken as positive control & *Staphylococcus aureus* was taken as negative control.

3.6.12 Motility Test

This test is done to observe the motility or movement potential of bacteria of interest. If the bacteria are motile then the motility medium becomes turbid and opaque but if bacteria is non-motile the growth is confined to the stab line.

Procedure

Tubes of semi-solid motility agar medium were prepared & inoculation was done by single stab to about half the medium depth at the center of the tube by straight wire. Incubated at 30℃-37℃ for 24-48 hours. *Bacillus subtilis* was taken as positive control & *Staphylococcus aureus* was taken as negative control.

3.7 Molecular Characterization of Bacterial and Fungal isolates

Conformation of biochemical test results of bacteria and fungi via 16S r RNA Sequencing & 18S rRNA Sequencing respectively, was being done by Yaazh Xenomics, Tamilnadu (India). The procols for sequencing submitted by them are enlisted as follows.

3.7.1 Bacterial Identification- 16S r RNA Sequencing

3.7.1.1 DNA Extraction

1) Lysis/homogenization: Cells grown in monolayer should be lysed by suspend 1-3 colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and cell lysis through repeated pipetting.
2) Add 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer.
3) Vortex the content and incubate the tubes for 30 minutes at 65˚C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.
4) Centrifuge the tubes for 15 minutes at 14,000 rpm at 10 ˚C.
5) Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet.
6) Add 600 µl of "B Cube" binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes.
7) Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.
8) Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
9) Reassemble the spin column and the collection tube then transfer the remaining 600 µl of the lysate.
10) Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
11) Add 500 µL "B Cube" washing buffer I to the spin column. Centrifuge at 14,000 rpm for 2 mins and discard flow-through
12) Reassemble the spin column and add 500 µl "B Cube" washing buffer II and Centrifuge at 14,000 rpm for 2 mins and discard flow-through
13) Transfer the spin column to a sterile 1.5-ml microcentrifuge tube
14) Add 100 µl of "B Cube" Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filter.
15) Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000 rpm for 1 min.
16) Repeat the above mentioned step 14 and 15 for complete elution. The buffer in the microcentrifuge tube contains the DNA.
17) DNA concentrations were measured by running aliquots on 1% agarose gel.

18) The DNA samples were stored at -20°C until further use.

3.7.1.2 PCR Protocol

Polymerase Chain Reaction (PCR) is an *invitro* DNA replication process that uses primers in order to amplify specific cloned or, genomic DNA sequences with the help of a very unique enzyme known as DNA polymerase enzyme which directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA (ss DNA) template. The DNA polymerase enzyme adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer DNA template. Hence, DNA polymerase enzyme can use the oligonucleotide as a primer & elongate its 3` end in order to generate an extended region of double stranded DNA (ds DNA) if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide.

Composition of the Taq Master Mix

• Taq DNA polymerase is supplied in 2X Taq buffer

• 0.4mM dNTPs,

• 3.2mM MgCl2 and

• 0.02% bromophenol blue.

Primer details

Primer details	Sequence details	Number of base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Table 3.3: Primer details for 16S r RNA Sequencing of bacteria

Add 5 µL of isolated DNA in 20 µL of PCR reaction solution (1.5 µL of Forward Prime and Riverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

1. Denaturation

The DNA template is heated to 94°C for 3 minutes, results in the breakage of weak hydrogen bonds that hold the DNA strands together in a helix, permit the strands to separate creating single stranded DNA (ss DNA).

2. Annealing

3. The mixture is cooled to anywhere from 94℃ for 30 sec, 50℃ for 60 sec, & 72℃ for 60 sec., allowing the primers to anneal to their complementary sequence in the DNA template.

3. Extension

The reaction is then heated to 72° C for 10 mins, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Purification of PCR Products

Separation of unincorporated PCR primers & dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced by using 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

3.7.1.3 Sequencing protocol

Execution of single-pass sequencing was being done on each template using 16s rRNA universal primers mentioned below. Purification of the fluorescent-labeled fragments was being done from unincorporated terminators by using an ethanol precipitation protocol. The samples were then, subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems) after their resuspension in distilled water.

3.7.2 Fungal Identification via 18S rRNA Sequencing

3.7.2.1 DNA Extraction

1) Lysis/homogenization: Cells grown in monolayer should be lysed by suspend 1-3colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml microcentrifuge tube and lyse the cells by repeated pipetting.
2) Add 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer.

3) Vortex the content and incubate the tubes for 30 minutes at 65˚C in water bath. Tominimize shearing the DNA molecules, mix DNA solutions by inversion.
4) Centrifuge the tubes for 15 minutes at 14,000 rpm at 10 ˚C.
5) Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 mlmicro centrifuge tube without disturbing the pellet.
6) Add 600 µl of "B Cube" binding buffer to the content and mix thoroughly by pipettingand incubate the content at room temperature for 5 minutes.
7) Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.
8) Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
9) Reassemble the spin column and the collection tube then transfer the remaining 600 µl ofthe lysate.
10) Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
11) Add 500 µL "B Cube" washing buffer I to the spin column. Centrifuge at 14,000 rpm for2 mins and discard flow-through
12) Reassemble the spin column and add 500 µl "B Cube" washing buffer II and Centrifugeat 14,000 rpm for 2 mins and discard flow-through
13) Transfer the spin column to a sterile 1.5-ml microcentrifuge tube
14) Add 100 µl of "B Cube" Elution buffer at the middle of spin column. Care should betaken to avoid touch with the filter.
15) Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000 rpm for 1min.
16) Repeat the above mentioned step 14 and 15 for complete elution. The buffer in themicrocentrifuge tube contains the DNA.
17) DNA concentrations were measured by running aliquots on 1% agarose gel.
18) The DNA samples were stored at -20°C until further use.

3.7.2.2 PCR Protocol

Composition of the Taq Master Mix

•Taq DNA polymerase is supplied in 2X Taq buffer

•0.4mM dNTPs,

•3.2mM MgCl2 and

•0.02% bromophenol blue.

Primer Details

Primer details	**Sequence details**	**Number of base**
ITS1	TCCGTAGGTGAACCTGCGG	19
ITS4	TCCTCCGCTTATTGATATGC	20

Table 3.4: Primer details for 18S rRNA Sequencing of bacteria

Add 5 µL of isolated DNA in 20 µL of PCR reaction solution (1.5 µL of Forward Primer & Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

1.Denaturation

The DNA template is heated to 94°C for 3 minutes. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2.Annealing

The mixture is cooled to anywhere from 94℃ for 30 sec, 50℃ for 60 sec, and 72℃ for 60 sec. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3.Extension

The reaction is then heated to 72° C for 10 mins, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Purification of PCR Production

Separation of unincorporated PCR primers & dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced by using

27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

3.7.2.3 Sequencing protocol

Execution of single-pass sequencing was being done on each template using 16s rRNA universal primers mentioned below. Purification of the fragments, that were labelled with fluorescent, was being done from un-incorporated terminators by using a protocol of ethanol precipitation. The samples were then, subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems) after their resuspension in distilled water.

3.7.1.4 Bioinformatics protocol

1) The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

2) The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3) PhyML was reported to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. For tree rendering, the program Tree Dyn 198.3 was used.(Dereeper *et al*., 2008).

3.8 Preparation of Heavy Metal Solution

The 1000 ppm stock solutions of Cu, Zn and Hg were made in double distilled water using Copper (II) Sulphate pentahydrate (CuSO4.5H20) (Titan Biotech), Mercuric Chloride (HgCl2) (Qualikems), Zinc Sulphate heptahydrate (ZnSO4.7H20) (Central Drug House). The 50, 100, 400 & 800 ppm solutions of these heavy metals was prepared from 1000 ppm stock solution. And, added to nutrient broth & potato

dextrose broth (that were sterilized at 121ºC for 15 min at 15 lbs/psi.) in order to make its concentration 50, 100, 400 & 800 ppm solution.

3.9 Heavy Metal uptake by Bacterial isolate from liquid media

Bacterial isolate, *Bacillus subtilis*, isolated from deltamethrin-contaminated soil, was further evaluated for its toleration to heavy metals derived from deltamethrin pyrethroid at higher concentrations of respective heavy metals (Cu, Zn & Hg). Sterilized nutrient broth (at 121ºC for 15 min at 15 lbs/psi.) containing 50, 100, 400 & 800 ppm concentrations of each Cu, Zn & Hg heavy metals was dispensed in 100 ml lots to conical flasks of 250 ml. Bacterial inoculum of 24 hours old bacterial culture of *Bacillus subtilis* was taken & inoculated into conical flasks containing 50, 100, 400 & 800 ppm concentrations of each Cu, Zn & Hg heavy metals. A control without HM is maintained for each HM media. The conical flasks were then, incubated at 37℃ for time period of 24 hrs. The bacterial growth, after incubation, is measured turbidometrically at an interval of 48 hrs using uv-vis spectrophotometer (Double beam SL 210) at 645nm, 540nm & 485nm of Cu, Zn & Hg respectively, readings were taken & analyzed for bacterial adaptation to different heavy metals. All the experiments were conducted in triplicate & avg. values were taken for analysis.

3.10 Heavy Metal uptake by Fungal isolates from liquid media

Fungal isolates, *Aspergillus flavus* & *Penicillium citrate* isolated from deltamethrin-contaminated soil, were further evaluated for its toleration to heavy metals derived from deltamethrin pyrethroid at higher concentrations of 50, 100, 400 & 800 ppm of respective heavy metals (Cu, Zn & Hg). Potato dextrose broth (PDB) containing 50, 100, 400 & 800 ppm concentrations of each Cu, Zn & Hg heavy metal was dispensed in 100 ml lots to conical flasks of 250 ml. & sterilized at 121ºC for 15 min at 15 lbs/psi. Fungal inoculums of 24 hours old fungal cultures of *Aspergillus flavus* & *Penicillium citrate* were taken & inoculated into conical flasks containing 50, 100, 400 & 800 ppm concentrations of each Cu, Zn & Hg heavy metals. Un-inoculated flasks containing potato dextrose broth (PDB) broth of 50, 100, 400 & 800 ppm concentration of different heavy metals served as control. All the flasks were then put on shaker incubator at 28℃ for 96 hrs. at 150 rpm. Harvesting of fungal growth was being done after 96 hrs. of incubation by filteration process using whatman filter no. 42, followed by rinsing of harvested fungal biomass with (dd/w)

double distilled water 3-4 times & dried at 80°C for 18 hrs. in hot air oven. After drying the fungal biomass, it was weighed & estimation of concentration of heavy metals was being done by digestion process, using nitric acid & perchloric acid in 3:1. Further, filteration of digested fungal biomass was being done using whatman filter no. 42 & filterate volume was made upto 50 ml in the volumetric flask. Estimation of heavy metal concentration in digested fungal biomass of *Aspergillus flavus* & *Penicillium citrate* was being done using uv-vis spectrophotometer (Double beam SL 210) at 645 nm, 540nm & 485nm of Cu, Zn & Hg respectively.

All the experiments were conducted in triplicate & avg. values were taken for analysis. Calculations of heavy metal uptake by fungal biomass of *Aspergillus flavus* & *Penicillium citrate* was being done using the following equation:

$$Q_e = (Ci - Cf)V / 1000M$$

Where, Q_e is conc. of HM accumulated by fungal biomass in (mg/g), *Ci* is initial conc. of HM (mg/L), *Cf* is the final conc. of HM (mg/L), *V* (L) is the volume of the aqueous medium & M is the dry weight (g) of the fungal biomass.

3.11 Maintenance of bacterial and fungal pure cultures

Pure cultures of *Bacillus subtilis, Aspergillus flavus and Penicillium citrinum* were maintained on nutrient agar slants & kept at 4°C. Cultures were prepared by streaking a single colony onto the slant agar surface by using a sterilized inoculating loop followed by incubation at 37°C for 24 hrs. prior to storage in 4°C. Glycerol stock cultures of *Bacillus subtilis, Aspergillus flavus* &*Penicillium citrinum* were prepared at the end of the project, by taking mixture of 0.5 ml of overnight broth culture & 1.5 ml of 60% sterilized glycerol and stored at -20°C.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Physiochemical properties of soil sample

The physiochemical properties of soil sample used for isolation of microbial species were analysed from soil testing laboratory of Punjab Agricultural University (PAU) Ludhiana, and are presented in table no. 4.1.

SOIL TEST REPORT			
Test	**Results**		**Remarks**
Presence of small elements in the soil	Type of soil	Mara	White color in the field, Using good/canal water
	Alkaline parts	7.8 (ok)	
	Salty substances	0.92 (white color)	
	Zooid carbon category (percent)	0.990 (more)	
	Phosphorus (kg/acre)	26.3 (too much)	
	Potash catergory (kg/acre)	360 (much)	
	Zinc	3.26	Is not the lack of small elements
	Iron	36.10	
	Manganese	6.28	
	Copper	2.36	

Table 4.1: Soil test report

4.2 Morphological and Biochemical Characterization of Deltamethrin resistant Bacteria

On the basis of morphological & staining characteristics of bacterial isolate, isolated on nutrient agar & soyabean casein digest agar, bacterial isolate was microscopically observed as gram negative rod shaped bacteria exibiting opaque/dull white color, dull surface refraction, medium sized flat & irregular colonies with lobate margins. The bacterial isolate was assumed to belong to the family of *bacillus spp.*, that was further conformed by various biochemical tests followed by molecular characterization by 16S rRNA gene sequencing & finally, conformed to be ***Bacillus subtilis.***

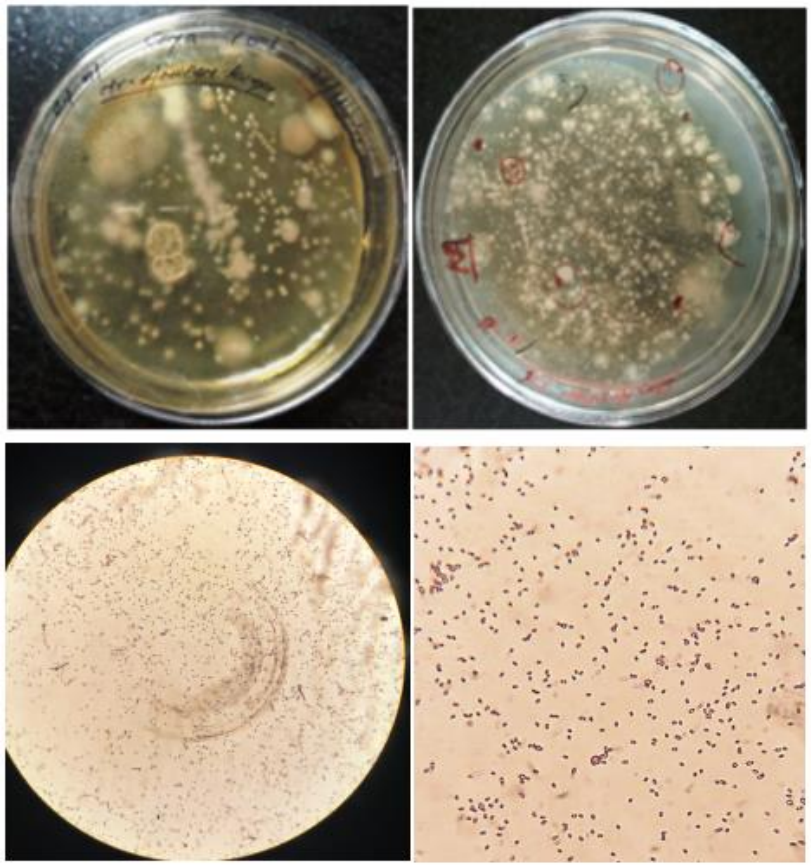

Figure 4.1: Morphological and microscopic observations of *Bacillus subtilis.*

Biochemical Test	Observations	Results

Motility Test		**+ve :** ***Bacillus subtilis*** **(**Motile; medium gets turbid) **-ve:** ***Staphylococcus aureus*** **(**Non-motile; no turbidity)
Indole Test		**+ve :** ***E.coli*** (Formation of cherryred ring) **-ve:** ***Bacillus subtilis*** (Reagent layer remains yellow)
Citrate Test		**+ve:** ***Bacillus subtilis*** (Media changes to prussian blue) **-ve:** ***E.coli*** (Color remains green)

Urease Test		**+ve:** ***Staphylococcus aureus*** **(**Color changes to pink**)** **-ve:** ***Bacillus subtilis*** (No change in color)
Methyl Red (MR) Test		**+ve:** ***E.coli*** (Change in color: yellow to red) **-ve:** ***Bacillus subtilis*** (No change in color)
Voges-Proskauer (VP) Test		**+ve :*Bacillus subtilis*** (Change in color; red color) **-ve:** ***E.coli*** (No change in color)

Starch Hydrolysis Test		**+ve :** ***Bacillus subtilis*** (Transparent clear zones around colony) **-ve:** ***Staphylococcus aureus*** (No clear zones)
Catalase Test		**+ve:** ***Bacillus subtilis*** (Immediate bubbling) **-ve:** ***Streptococcus pyregenes*** (No bubbling)

Table 4.2: Biochemical test results of *Bacillus subtilis.*

4.3: Molecular Characterization of Bacterial isolate by 16S rRNA Gene Sequencing

On the basis of molecular characterization of bacterial isolate by 16S rRNA Gene Sequencing, isolate was conformed as ***Bacillus subtilis.***

4.3.1 Sequence of *Bacillus subtilis*

CCAAATCTTCTTCCTTCATTCCATGCTGTCGAGCGGACAGATGGGAGCTTG
CTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCT
GTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTT

TGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGA
TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCA
ACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGCC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT
CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGA
GGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAGCGTGCGGAG
CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACT
CCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCATCGCGAGAACT
TACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCG
GGGGCAGAGTGACAGGTGGTGCATGCTGTCGTCAGCCGTGTCGTGAGATG
TTGGGTTAGTCCCGCAACGAGCGCATCCCTTGATCTTAGTTGCCAGCAATT
CAGTGGTACTCTAGGTGACTTGCCGGTGACATACGAGAGTGGGATGACGT
CAATCATCATGCCCTTATGACTGGCTACACACCTCTACATTGACGGACATG
GCATCGTAACCGCGGAGGTTAAGCCAATCCCACAAACTCTGG

(Courtesy: Yaazh Xenomics)

4.3.2 Phylogeny Tree of *Bacillus subtilis*

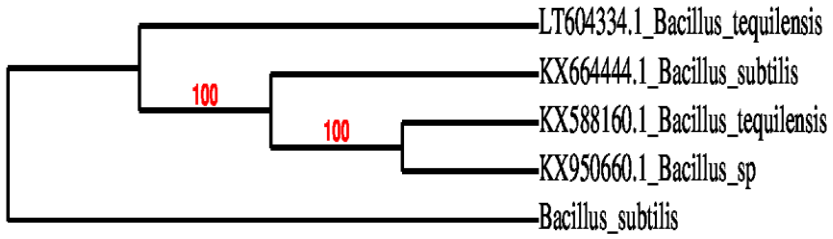

Figure 4.2: Phylogeny Tree of *Bacillus subtilis*

4.4 Morphological and Biochemical Characterization of Deltamethrin resistant Fungi

Based on the microscopic analysis & morphological characteristics of fungal isolates, isolated on sabouraud dextrose chloramphenicol agar and rose bengal agar medium, fungal isolates were assumed to be their beloging to the family of *Aspergillus spp. and Penicillium spp.* Colonies characteristics and microscopic analysis of *Aspergillus spp*.

were observed as woolly, yellow-brown to olive colored, granular in shape, floccose texture exhibiting long conidiophores, rough just beneath the globose vesicle and circumferentially raised phialides. It was further conformed by molecular characterization by 18S rRNA gene sequencing & finally, conformed to be ***Aspergillus flavus.***

Figure 4.3: Morphological and microscopic observations of *Aspergillus flavus*

Similarly, colonies characteristics & microscopic analysis of *Penicillium spp.* were observed as green/ grayish-green with a white lining, velvety to powdery surface varied colors of exudates on the surface, reverse side shows a pale cream to yellow color or more intense reddish-brown. It was further conformed by molecular characterization by 18S rRNA gene sequencing & finally, conformed to be ***Penicillium Citrinum.***

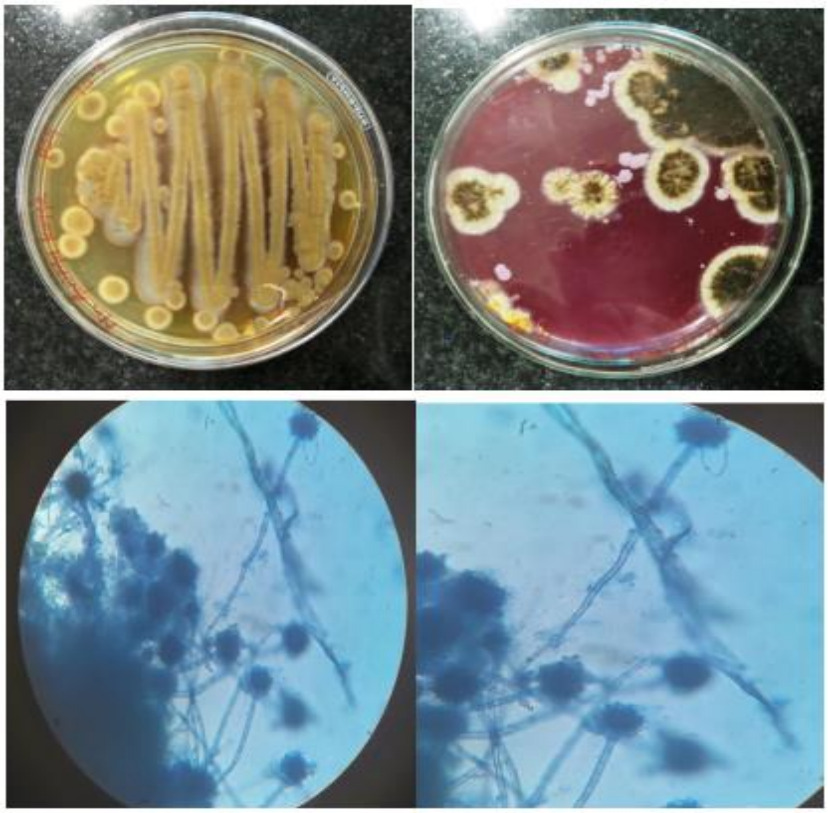

Figure 4.4: Morphological and microscopic observations of *Penicillium citrinum*

4.5: Molecular Characterization of Fungal isolates by 18S rRNA Gene Sequencing

On the basis of molecular characterization of fungal isolates by 18S rRNA Gene Sequencing, isolates were conformed as ***Aspergillus flavus*** and ***Penicillium citrinum.***

4.5.1 Sequence of *Aspergillus flavus*

AGTTTACTGATTGCGATACAATCAACTCAGACTTCACTAGATCAGACAGA
GTTCGTGGTGTCTCCGGCGGGCGCGGGCCCGGGGCTGAGAGCCCCCGGCG
GCCATGAATGGCGGGCCCGCCGAAGCAACTAAGGTACAGTAAACACGGG
TGGGAGGTTGGGCTCGCTAGGAACCCTACACTCGGTAATGATCCTTCCGC
AGGTTCACCTACGGAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCC
CACCCCCCCCGGTTTTTTGGACCCTAGTTGGTTTCGGGGGCCCCCATTTAT
GGGCCGGGGGGCTTTAGCCCGGGCCGGGCCCCCGGGGAAAAC

(Courtesy: Yaazh Xenomics)

4.5.2 Phylogenetic tree of *Aspergillus flavus*

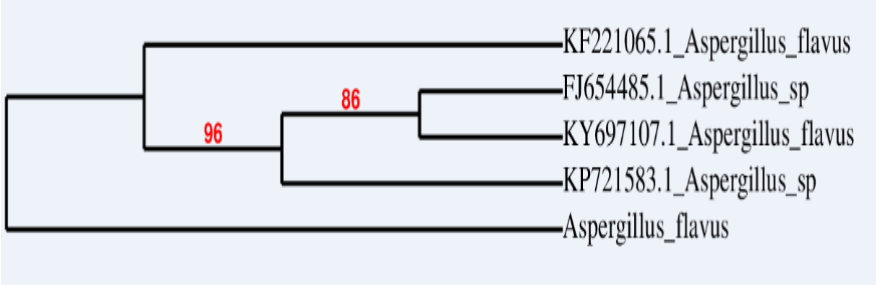

Figure 4.5 : Phylogenetic tree of *Aspergillus flavus*

4.5.3 Sequence of *Penicillium citrinum.*

TTAGAATCGTTGATGAGTTTTACTAATTTCGTTATAGGTCTCAGACTGCAA
CTTCAGACAGCGTTCAGGGGGGCCGTCGGCGGGCGCGGGGCCCGCCGAG
GCAACATAGGTTCGGGCAACACGGGTGGGAGGTTGGGCCCCGAGGGGCC
CGCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAG

(Courtesy: Yaazh Xenomics)

4.5.4 Phylogenetic tree of *Penicillium citrinum.*

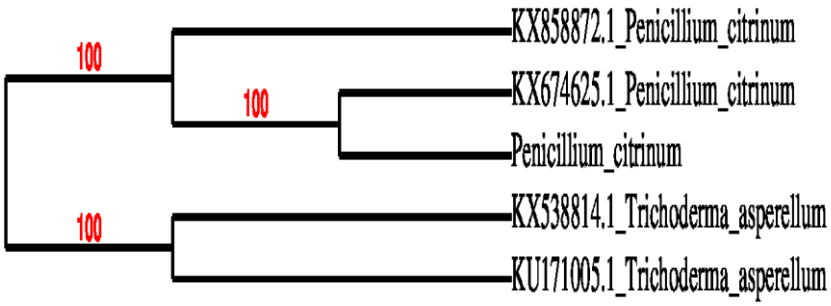

Figure 4.6 : Phylogenetic tree of *Penicillium citrinum.*

4.6 Calibration curves of Copper, Zinc & Mercury

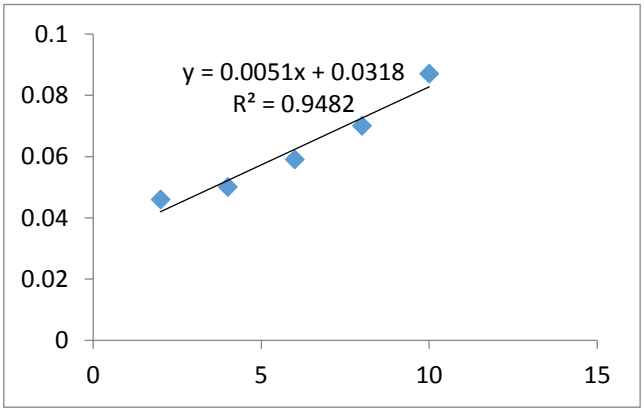

Figure 4.7: Standard curve of copper (Cu).

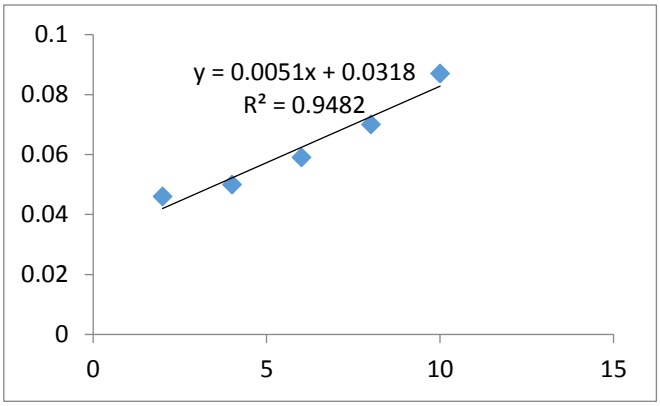

Figure 4.8: Standard curve of zinc (Zn).

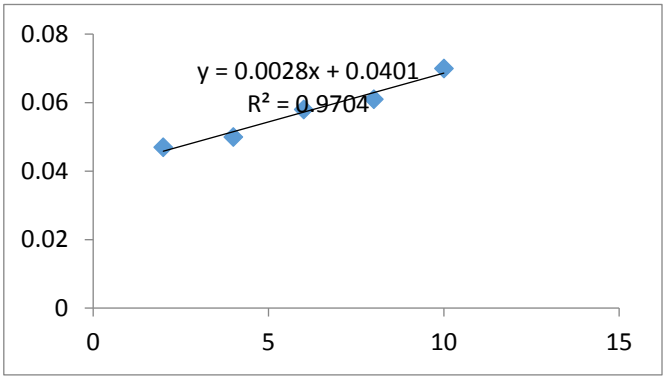

Figure 4.9: Standard curve of mercury (Hg)

4.7 Bacterial adaptation to heavy metals in the liquid media

Bacillus subtilis has shown maximum adaptability to copper (Cu) at 50 ppm concentration followed by zinc (Zn) & mercury (Hg). At 50 ppm concentrations of Cu, Zn & Hg, *Bacillus subtilis* acquire the absorbance values of 0.9901, 0.9500 and 0.3647 respectively; at 100 ppm concentrations, 0.877, 0.9204 and 0.1689; at 400 ppm concentrations, 0.7456, 0.4050 and 0.1210; & at 800 ppm conc. 0.4385, 0.1879 and 0.0775 respectively has been gained by *Bacillus subtilis*. The graphical representation of absorbance vs different concentrations of heavy metals (Cu, Zn & Hg) is depicted as follows:

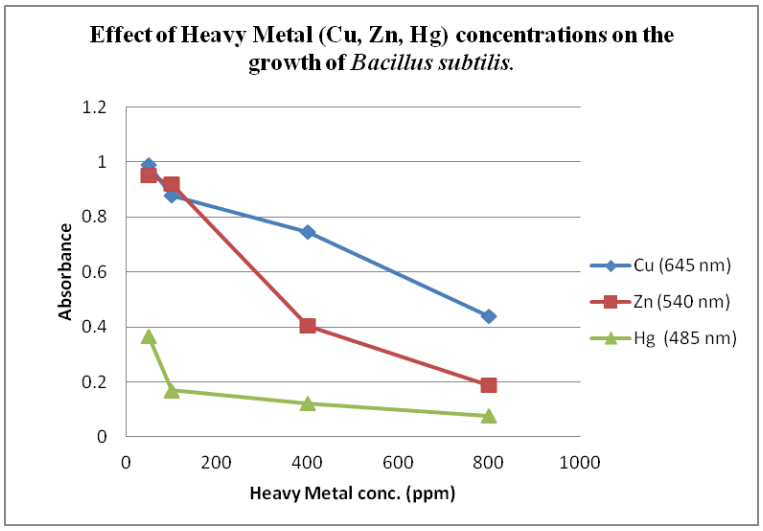

Figure 4.10: Graphical representation of the effect of different concentrations of heavy metals on the growth of *Bacillus subtilis.*

4.8 Cell dry weight of fungal biomass of *Aspergillus flavus and Penicillium citrinum* in the liquid media

After incubation of 96 hours at 28℃ and 150 rpm, fungal biomas *(Aspergillus flavus and Penicillium citrinum)*, containing different concentrations (50, 100, 400 & 800 ppm) of heavy metals (Cu, Zn & Hg), was dried in hot air oven at 80℃ for 18 hrs. & weighted. Dried biomass weight (g) of *Aspergillus flavus*, at 50ppm conc. of Cu, Zn and Hg was noted as 0.22, 0.22 and 0.23g respectively; at 100 ppm conc. 0.28, 0.32 nd 0.25 respectively; at 400 ppm conc. 0.19, 0.29 and 0.20g respectively; & 0.20, 0.28 and 0.35g at 800 ppm conc. respectively.

Figure 4.11: Fungal biomass of *Aspergillus flavus* after 96 hrs. of incubation,with different conc. of Cu, Zn & Hg heavy metals, at 28 ºC at rotary shaker (150 rpm)

Figure 4.12: Filteration of biomass of *Aspergillus flavus* and digestion of dried biomass with nitric acid and perchloric acid (3:1)

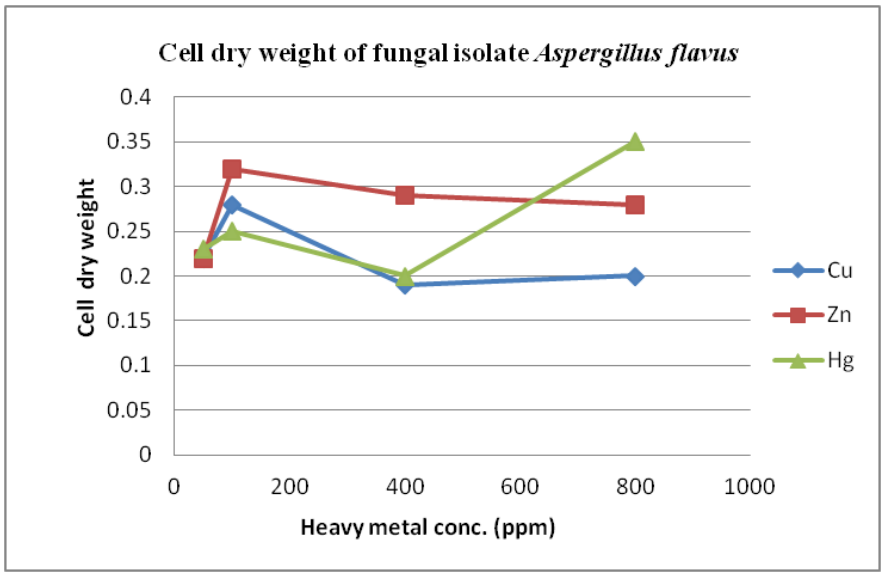

Figure 4.13: Cell dry weight (g) of fungal biomass of *Aspergillus flavus.*

Similarly, dried biomass weight (g) of *Penicillium citrinum*, at 50ppm conc. of Cu, Zn and Hg was noted as 0.34, 0.01 and 0.44g respectively; at 100 ppm conc. 0.44, 0.01 nd 0.25g respectively; at 400 ppm conc. 0.37, 0.17 and 0.10 respectively; & 0.35, 0.20 and 0.12g at 800 ppm conc. respectively.

Figure 4.14: Fungal biomass of *Penicillium citrinum* after 96 hrs. of incubation, with different conc. of Cu, Zn & Hg heavy metals, at 28 ºC at rotary shaker (150 rpm).

Figure 4.15: Filteration of biomass of *Penicillium citrinum* and digestion of dried biomass with nitric acid and perchloric acid (3:1)

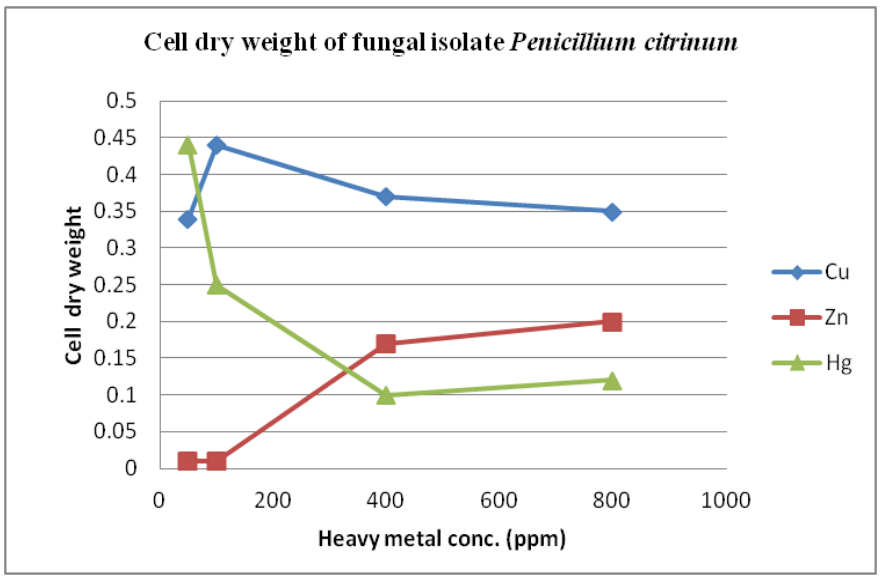

Figure 4.16: Cell dry weight (g) of fungal biomass of *Penicillium citrinum.*

4.9 Effect of Biosorption capacity (q_e) of *Aspergillus flavus* and *Penicillium citrinum* on different heavy metal (Cu, Zn, Hg) concentrations

Fungal enzymes tend to degrade the HMs by incorporating them in their metabolic pathways & by utilizing them as their carbon and energy source. Two fungal isolates were isolated from deltamethrin contaminated soil, designated as *Aspergillus flavus* & *Penicillium citrinum,* were further tested for their tolerance to higher concentrations of Cu, Zn and Hg heavy metals. Higher biosorption capacity (q_e) of *Aspergillus flavus* was observed for copper (Cu) at 400 and 800 ppm followed by Zn at 800 ppm concentration. At 50ppm conc. of Cu, Zn and Hg, biosorption capacity (q_e) of the *Aspergillus flavus*,was observed as 23.03, 21.80 and 21.50 mg/g respectively; at 100 ppm conc. 34.05, 29.70 and 39.70 mg/g respectively; at 400 ppm conc. 209.40, 137.10 and 199.80 mg/g respectively; & 398.90, 284.16 and 228.40 mg/g at 800 ppm concentration respectively.

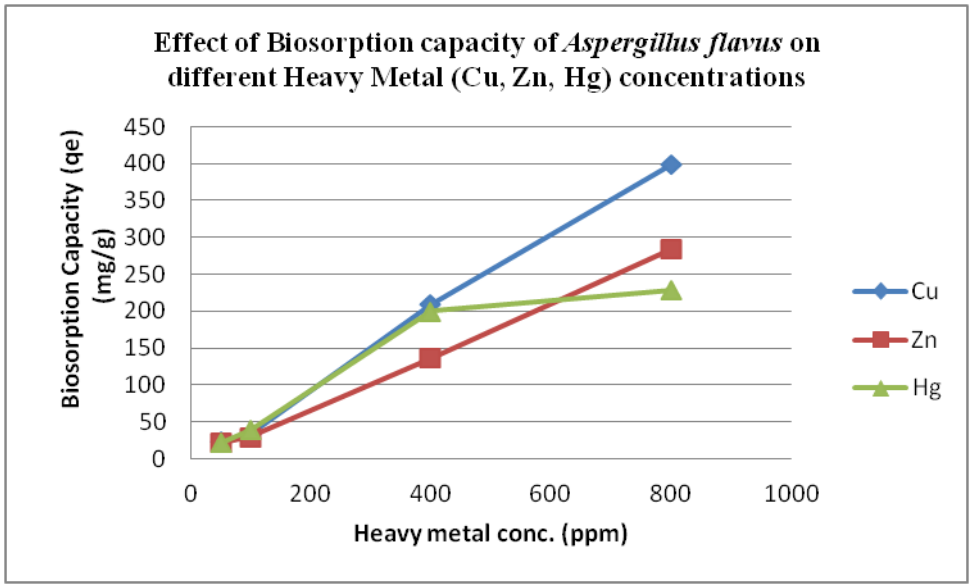

Figure 4.17: Graphical representation of the biosorption capacity (q_e) of *Aspergillus flavus* at different heavy metal concentrations.

Higher biosorption capacity (q_e) of *Penicillium citrinum* was observed for Cu at 50 ppm followed by Hg at 800 ppm concentration. At 50ppm conc. of Cu, Zn and Hg, biosorption capacity (q_e) of the *Aspergillus flavus*,was observed as 14.70, 501.70 and 11.05 mg/g respectively; at 100 ppm conc. 22.70, 1001 and 39.70 mg/g respectively;

at 400 ppm conc. 108.13, 235.40 and 399.80 mg/g respectively; & 228.60, 400.08 and 666.50 mg/g at 800 ppm concentration respectively.

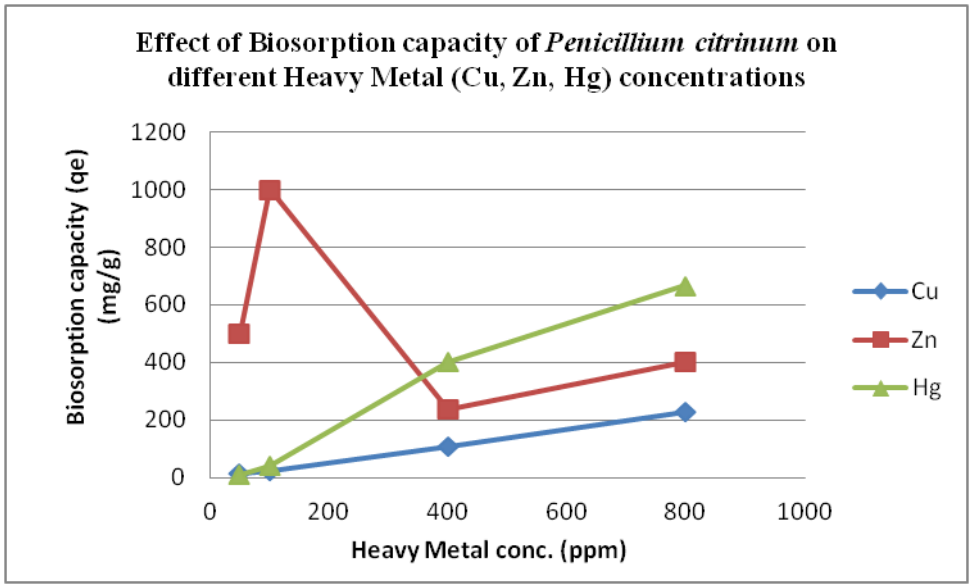

Figure 4.18: Graphical representation of the biosorption capacity (q_e) of *Penicillium citrinum* at different heavy metal concentrations.

CHAPTER 5

CONCLUSION

Due to the complexity involved in the conventional methods for remediation of soil, the use of microbes has arisen as a time-saver for bioremediation. The data presented in this project highlights the great potential of *Bacillus subtilis*, *Aspergillus flavus* & *Penicillium citrinum* to degrade deltamethrin pyrethroid insecticide. The high absorption capacity of respective bacterial and fungal isolates made them well suited for removal of heavy metal present in very low or diluted concentrations from bioremediation of polluted sites. Bioremediation is highly economical, ecofriendly process & sustainable to rid of the pollution problem. Thus, we can conclude that *Bacillus subtillis, Aspergillus flavus* and *Penicillium citrinum*, being ubiquitous, showed the potential to be used as biosorbents for the biosorption of higher concentratoions of different heavy metals in the environment. However, awareness is required to farmers in context of usage of pyrethroid pesticides that pesticides are necessary & should be employed within recomended limits set by EPA, in order to diminish reported fatal risks to non-target species including animals, humans & to the ecosystem.

CHAPTER 6

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