ISOLATION AND IDENTIFICATION OF IMAZETHAPYR (HERBICIDE) DEGRADING SOIL MICROORGANISM FROM PUNJAB REGION (INDIA).

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A thesis submitted in fulfilment of the requirements for the award of the degree of Master of Technology (Biotechnology)

To

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Under The Guidance Of

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(April, 2017)

DECLARATION

I declare that this thesis entitled "Isolation and identification of Imazethapyr (herbicide) degrading soil microorganism from Punjab region (India)." is the result of my research work carried out at Lovely Professional University, Phagwara under the guidance of Dr.Digvijay Singh. The thesis has not been accepted for any degree and is not synchronously submitted in candidature of any other degree.

Signature :…….

Name of Candidate : Parishmita Gogoi

Date : 15th December 2016

CERTIFICATE

This is to certify that Parishmita Gogoi has completed M.tech dissertation II titled "Isolation and identification of Imazethapyr(herbicide) degrading soil microorganism from punjab region (India)." under my guidance and supervision. To the best of my knowledge, the existing work is the result of her original investigation and study. No part of the dessertation has ever been submitted for any other degree or diploma at any university.

The dessertation is suitable for the submission and the partial fulfilment of the conditions for the award of M.tech Biotechnology.

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ABBREVIATIONS

%	-	Percentage
µ	-	Micro
h	-	Hours
L	-	liter
M	-	molar
min	-	minutes
ml	-	milliliters
^{o}C	-	degree Celsius
pH	-	potential hydrogen
v	-	volume
w	-	Weight
approx	-	approximately
Fig	-	Figure

ABSTRACT

Leaching out of synthetic chemical pesticides, fertilizers and herbicides to surface and water level is at the root of environmental pollution and accounts for severe environmental and social dispute all over the world. Present studies investigated the type of microorganisms that have been continuously exposed to different herbicide and pesticides concentrations in the soil. Still, these microorganisms are surviving in such conditions; it means that they have the potential to degrade substances and use those compounds as their energy source that harms our environment. Bacteria which belongs to the genus *Pseudomonas*, *Bacillus*, *Rhodococcus*, and many more are capable of degrading large number of petroleum products, herbicides, pesticides, industrial effluents, xenobiotic compounds etc. The best among the six isolates of imazethapyr degrading bacteria was isolated from imazethapyr contaminated soils and identified by biochemical and 16s rRNA gene amplification techniques to be *Bacillus licheniformis.*

Keywords: Herbicide**,** Biodegradation, Imazethapyr, *Bacillus*, Biochemical test.

INTRODUCTION

There is a tremendous boost in usage of herbicides and synthetic pesticides, throughout the world since the middle of last century. Around 30% of agricultural product is lost by virtue of pests. Herbicides and pesticides are believed to prohibit, control and damage pests. In spite, of their utility in agriculture, their extensive uses in midst of producing, processing, storeing, marketing and transporting of the agricultural products leads to environmental pollution. These environmental concerns about the effect caused by pesticides and herbicides toxicity have encouraged strict regulation to protect ecosystem. 2,4- dichlorophenoxyacetic acid (2,4-D), dichlorodiphenyltrichloroethane (DDT) and 2,4,5- trichlorophenoxyacetic acid (2,4,5-T), pentachlorophenol, polychlorinated biphenyls, and plasticizers are examples of halogenated aromatic compounds. Their durability and toxicity are the root of worry for surrounding aura and health issues related to public. The position, number of halogens and halogenated aliphatic compounds are significant in deciding both mechanism of biodegradation and the rate of degradation (Mulligan, 2005). Among the biological approaches the most adequate and profitable option to clean pesticide-contaminated sites is considered as the usage of microorganisms with degradable property.

In present scenario, waste produced by pesticides and herbicides are treated by the use of physico-chemical methods that are not adequate as well as least effective. As an outcome of which, pesticides and herbicides residue reside in water and soil, causing toxic effect to biota and thus disturbs the food chain directly or percolate down to the water table (CFTRI, 2003). As per some of the data collected by World Health Organization (WHO), shows that only 2 - 3% of herbicides and chemical pesticides are adequate in controlling, prohibiting, and killing pests, although the rest of it remains in the soil surface (EPA, 2005). Thus, the outer soil surface which are rich in residual pesticides and herbicides causes toxicity to the environment. The waste generated during process of manufacturing pesticide and herbicides is very complex which also effects the environment.

Indirect accumulation of the pesticides and herbicides in higher trophic level organisms (i.e. mammals) may cause problems in the health over time due to the increasing levels of toxicity of those compounds within the body (Gavrilescu, 2005; Hussain *et al.,* 2009). The pesticides are accumulated in food products and water supplies causing harm to biota, therefore it is essential to develop secure, favorable and efficiently feasible process for pesticide degradation (Zhang and Chiao 2002). One of the method used to remove these pesticide and herbicide accumulation from contaminated sites is to accomplish the capacity of microorganisms. This is a substitutive strategy for treating these pollution. It is efficient, least hazardous, cost-effective, adaptable and eco-friendly and is known to be as **bioremediation** (Finley *et al.,* 2010).

Microorganisms brings a dormant wealth in biodegradation. These organisms are able to cut-down the concentration of xenobiotics and is precisely related to their permanent variation to surrounding where these compounds subsist. Moreover, for enhancing the activity of microorganisms having the favourable properties, required for biodegradation are genetically modified (Schroll *et al.,* 2004). The areas polluted by pesticides are decontaminated by use of biological methods as they have less side-effects. These methods take into account the thousands of microorganisms present in the earth that for survival pursue for alternatives to eradicate the pesticides that are used against them. Complex and efficient metabolic pathways are followed by many native microorganisms that degrade toxic substances released into the environment. Inspite of the lengthy metabolic pathways, it is accepted as most viable substitute for separating the herbicide and pesticide residue from soil (Diaz, 2004; Schoefs *et al.,* 2004; Finley *et al.,* 2010).

Imazethapyr is a herbicide containing imidazole compound. It is used when there is preemergence, cracking, preplant incorporated and postemergence. Imazethapyr reduces the elevation of three branched-chain aliphatic amino acids namely valine, leucine and isoleucine, by prohibiting the action of aceto-hydroxyacid synthase (enzyme used in biosynthetic pathways of the three amino acids), thus controlling weed. The prohibition leads to disruption in synthesis of protein hindering the synthesis of DNA and cell growth. Some broadleaved weeds like velvetleaf, barnyardgrass, panicums, cocklebur, smartweed, pigweeds, mustard, jimsonweed, crabgrass, seedling johnsongrass, nightshade, lambsquarters, foxtails, etc and grasses are

controlled by this herbicide. Crops including soybeans, alfalfa, dry and edible beans, peanuts, peas and imidazolinone resistant/tolerant corn are tolerant to it. Further research of Imazethapyr is being carried out on leguminous crops. It has a formulation of aqueous concentration. It may be used in combination to other herbicides.

Residues of herbicides can cause harm to environment and pollute it. They have negative impact on living organisms. Furthermore, the cytological mechanisms of agricultural plants are effected by use of these chemicals (Çali, 2008). Commercial formulation of imidazolinone imazethapyr (IMZT)-based herbicide was named as Pivot H® (10.59% IMZT). Its letha, acute and sublethal toxicity was studied. Pérez-Iglesias *et al*., 2015 evaluated it upon *Hypsiboas pulchellus* tadpoles, their findings featured the properties that endanger nontarget living species when exposed to IMZT.

REVIEW OF LITERATURE

2.1 Herbicide and Pesticide utilize in crop production

Prior to evolution of synthetic pesticide and herbicide, use of naturally-occurring substances like pyrethrum and arsenic were practiced by farmers to control pest and diseases. Synthetic pesticides and herbicides were used widely after World War II (Lewis *et al.*, 1988). During that time pesticides and herbicides were created as weapons of war. Use of pesticide and herbicide was credited with increased yield of crops by reducing natural threats to production. By the mid-1950s their use became an integral part of agricultural practices. Following World War II, pesticides were anticipated as a "green revolution" with help of which the food production increases. Advancement in production and use of synthetic pesticides in our biota has increased due to the intensive nature of modern agriculture practices (Bhatnagar, 2001). Compounds used widely as pesticides, plasticizers and petroleum additives are synthetic organophosphorus (Dragun *et al.,* 1998). Half of insecticides used worldwide are made up of these compounds. Annual statistical data reports that, in agricultural crops almost over 5.1 billion ib of organophosphates are used (EPA, 2001). Since 10,000 BC weeds have been a major problem in agronomy (Avery, 2006). It has become one of the cause behind the limiting production of crops. One of the damage caused by weeds i.e. damage 6 is responsible for about $75.6 billion or a loss of 13.2% of production annualy (Oerke *et al.,* 1994). The earnest pest complex are considered as weeds as they have constant property, while insects and disease pathogens are sporadic in nature (Gianessi and Sankula, 2006). Weeds causes quantitative damage of the crops because of clash with water, antagonism and to light nutrients. Weeds causes qualitative indirect damages to crop yield and reduces and contaminate the seeds (Zvonko, 2007). Weed is controlled mechanically in agriculture by the use of herbicide. They overcome the problems caused by weeds. Herbicides are more adequate and cost effective way of weed control than hoeing, hand pulling and cultivation. Nowadays many types of ureas, also known as substituted ureas, tested for the property of herbicide and are used (Ware and Whitacre, 2004). These urea contains linuron, fenuron-TCA, diuron, tebuthiuron, siduron and

monuron. These urea based herbicides are selective or non selective weed killers. Donaldson and Kiely, 2002 find out that these herbicides work by inhibiting photosynthesis.

2.2 Categorization of pesticides

Currently various formulations (around 1000) and metabolites of pesticides are used around the globe (Kamla-raj, 2010). All substances that can prohibit, damage and confront pest organisms are present in pesticide. Some of the well-known pesticides includes insecticides, herbicides, fungicides and rodenticides (Robert, 1995). Growth regulators, surface disinfectants, plant defoliants and some swimming pool chemicals are least known pesticides in the market. Pesticides are also classified based on their chemical structure, the pests they control, their mode of action or how/when they work (Hajslova *et al.,* 1999). They have same kind of structures, same characteristics and also same type of mode of action. The effective ingredients in pesticide may be inorganic or organic (Robert, 1995). Pesticides having inorganic property are free from having carbon group and thus are extracted from mineral ores that are present on earth. On the other hand pesticides having organic property includes carbon group in their chemically formed structure. Various compounds has been used to create most of the organic pesticides, few of them are also eradicated from plant source and are named as botanicals (Ward *et al.,* 1993). Classification of pesticide also based on by virtue of what or when they work. Direct contact pesticides also known as "Contact pesticides" control a pest by, spraying directly kills the insect or when they come in contact with the surfaces spread with a residual contact insecticide. Adequate surface area is enclosed with a contact herbicide to kill weed foliage (Vander Hoff and Van Zoonen, 1999). Absorption of systematic pesticide occurs either by pest or plant. According to level of hazards it causes to human beings pesticides are grouped (WHO, 1990). The most toxic pesticides to the biota are considered as insecticide succeded by herbicides and fungicides. However, a few of the herbicides are far more hazardous in comparison insecticides (Kumar 2010).

2.3 Challenges in utilization of pesticide

Pesticides ecological effect are different and they are mostly integrated to one another (U.S.EPA, 2002). Pesticides that are easily soluble in water comes out of the destination into

streams and ground water. Chemicals that are soluble in fat are absorbed by some organisms like fish, insects and other animals, outcome of which is detected in the food chains and thus disturbs it (Yadav, 2007). A few of the hazardous pesticides as per the WHO categorization are (a) fungicides: benomyl, captan, mercury, copper, and pentachlorophenol (b) insecticides: DDT, parathion, diazinon, dieldrin and aldicarb and (c) herbicides, 2-4-D, paraquat, atrazine and glyphosate. The toxicity index of organophosphorus compounds are high and have a large number of continual ecological effects which are frequently unrecognized by spontaneous observers, still causes immense adverse effect to surrounding (David *et al*., 2005). These compounds have quite same structures and so, same mode of toxicity which works by reducing red blood cell membranes and the breakdown of acetylcholine in synapses (Lotti, 2002). The toxic effect of the organophosphorous pesticides are shown in both targeted and non-targeted animals (Galloway and Handy, 2003). Annually due to use of these compounds approximately 3 million reports of poisoning and 300,000 reports of human deaths are recited (Bird *et al.*, 2008). Reports of adverse effect of organophosphate pesticides causing death to the population of Kenya are sited (Kimani and Mwanthi, 1995). Organophosphorus compounds also have reports of causing nervous and muscular dieases (Ragnarsdottir, 2000). A selective N-methoxy-Nmethyl named as Linuron [3-(3,4-dichlorophenyl)-1- methoxy-1-methyl urea] is a substitution to phenylurea herbicide which has a toxic effect to aquatic organisms (Orme and Kegley, 2006). Action of herbicide is further quite stagnant in soil, drainage and leaching resulting in the movement of the compound to various water bodies present nearby and in one intense case reported, linuron was found in a drinking-water well with concentrations upto 2,800 μg liter-1 (Caux *et al.*, 1998). It acts as an androgen receptor antagonist and an endocrine disruptor affecting the male reproductive system (McIntyreet *et al.*, 2002).

2.4 Removal of environmental contaminants by bioremediation

Bioremediation is the process which employs the use of microorganisms for degrading or detoxifing the pesticide, herbicide or any other similar compound present in soil. Reduction of concentration and adverse effect of various contaminants at location is known as *In situ* bioremediation (Watanabe *et al.*, 2001). It is a cost-effective, environment-friendly, adaptable and valuable cure blueprint, and has recently being used immensly (Rakesh *et al.*, 2005). In

March 1989 bioremediation is used in biological clean up in the Gulf of Alaska where approx 41,000 m^3 of crude oil spreaded to 2000 km of sea shore and contaminated it (Margesin and Schinner, 1999). Neoteric scientists, modified the chemical structures of pesticide, these reconstructions are often responsible for effectivity of pesticide and its persistance in the surrounding (Muller and Korte, 1976). The Pesticides basically depends upon chemical structure and functional groups.The change in functional group basically leads to different categories of pesticides. The chemical structure of pesticide determines it bioavailability and solubility (Buyuksonmez *et al.,* 2000). Esterase and phosphatase are the example of enzymes responsible for breakdown of natural compounds developedby microorganisms (Lemmon and Pylypiw, 1992). Garcinuno *et al.,* 2006 reported that mono- and di-oxygenases are some other categories of enzymes, degrades pesticide by bringing a conformational change in the structure thus oxidizing the pesticide and making it more susceptible for degradation by increasing its solubility in water. Thus increases bacteria bioavailability.

2.5 Soil microbiology

The soil is colonized by diverse group of microbes. Microbiology of soil aims on the soil viruses, actinomycetes, bacteria, protozoa, and fungi. Consistently it also involves studying of mites, nematodes, and other microarthropods. All these microbes and soil animals are collectively considered as the soil biota. They work in soil and helps the plants in making their food. In the summary of modern soil microbiology assimilation of microbiology to perception of soil science, chemistry, and ecology in knowing the role of soil microbes. Microorganisms plays a key role in maintaining a balance on earth. The elements transforms there forms in the process called elemental cycles. Nitrogen fixation is carried out by the soil microorganisms. The earth is surrounded approximately by 80% of nitrogen gas (N_2), which is available for plants in form of ammonia (NH_3). It is made either by nitrogen fixation carried out by microbes or by fertilizers manufactured by human. Microbes present in soil also facilitate denitrification. It is a process in which nitrogen is retured to the biota by converting NO_3 to N_2 or nitrous oxide (N_2O) gas. Microbes recycles sulfur, iron, phosphorus, and many micronutrient trace elements. Microorganisms take into account a number of natural substances which indicates diversity in their mode of degradation. Various types of manmade compounds pollutes the environment by

the human actions. Few of these hazardous or highly toxic substances degradation occur due to action of soil microbes. Thus the environmental contamination causes by pesticides and herbicide are cured with the ability of the microbes to detoxify them. This is a recent advancement in science, a process known as bioremediation.

2.6 Bergey's manual

Bergey's manual is a manual. It is used to identify microbe as to their genus and species. Some examples of the identification flow chart of Bergey's Manual are cited below.

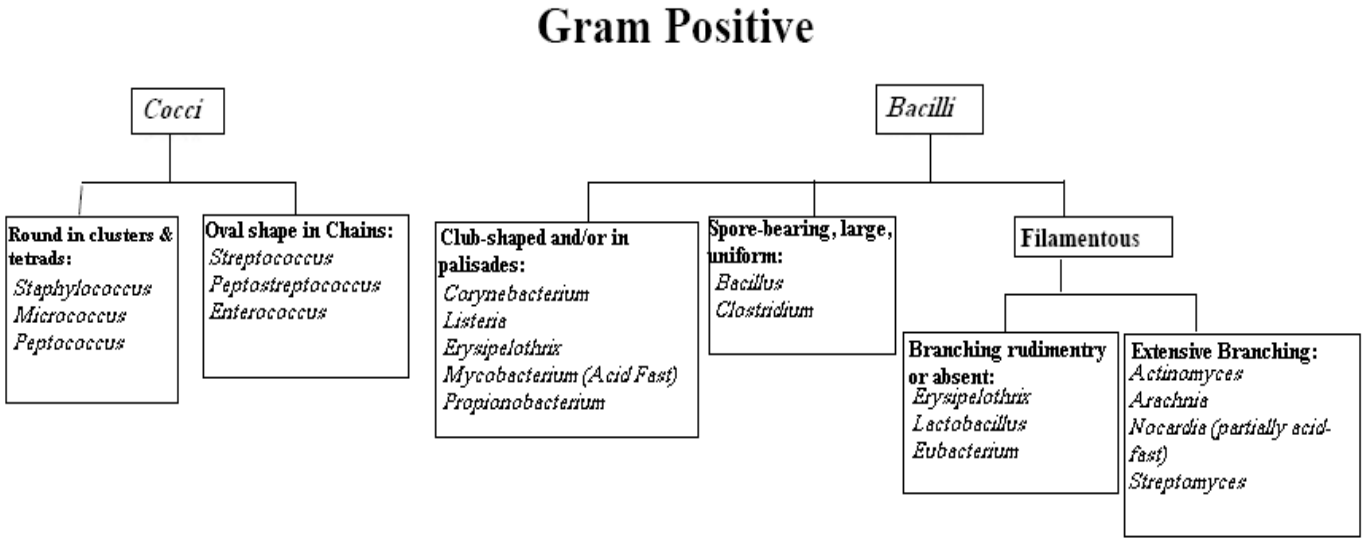

Fig 2.1: Gram posivite bacteria

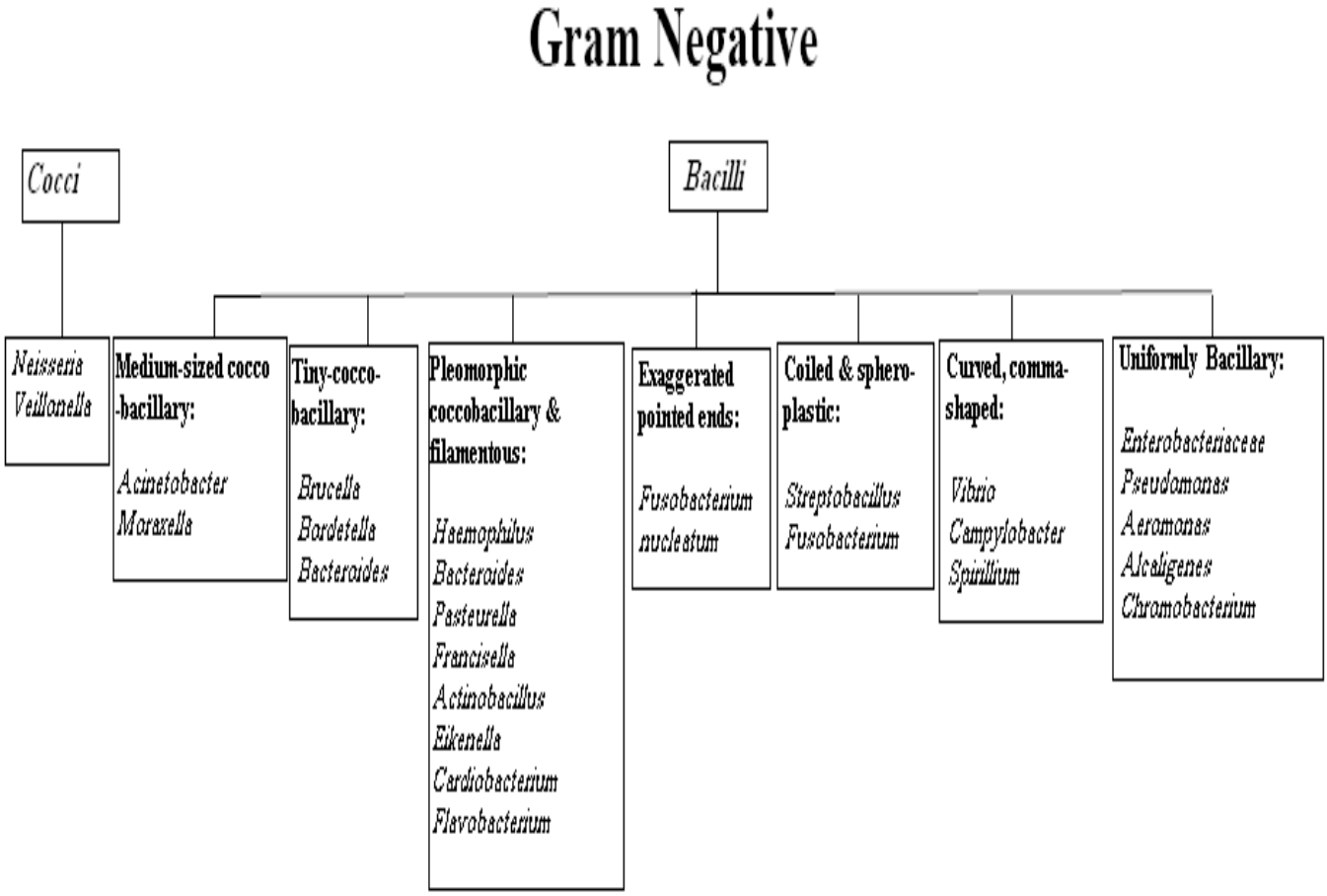

Fig 2.2: Gram negative bacteria

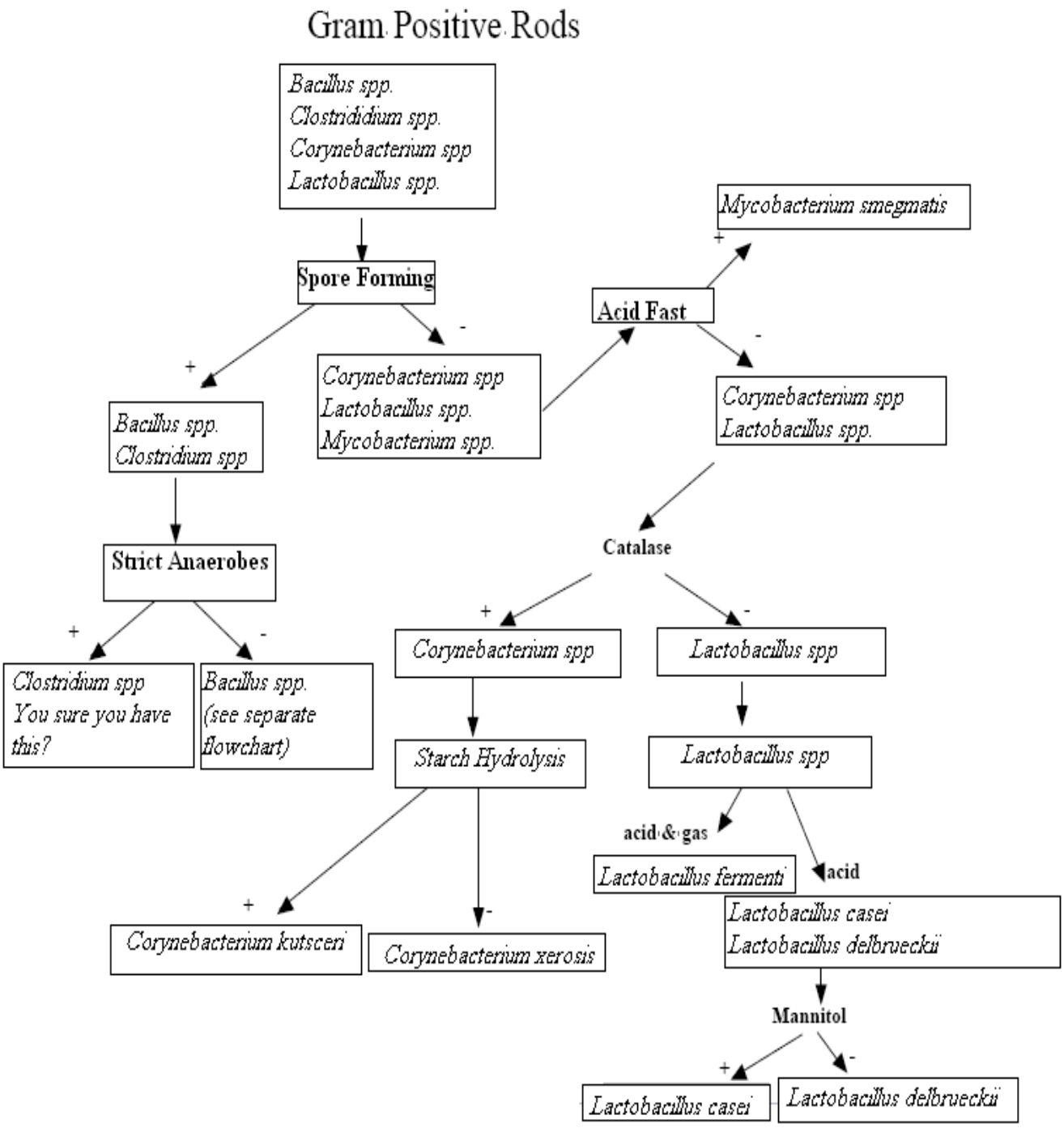

Fig 2.3: Gram Positive Rods ID Flow Chart

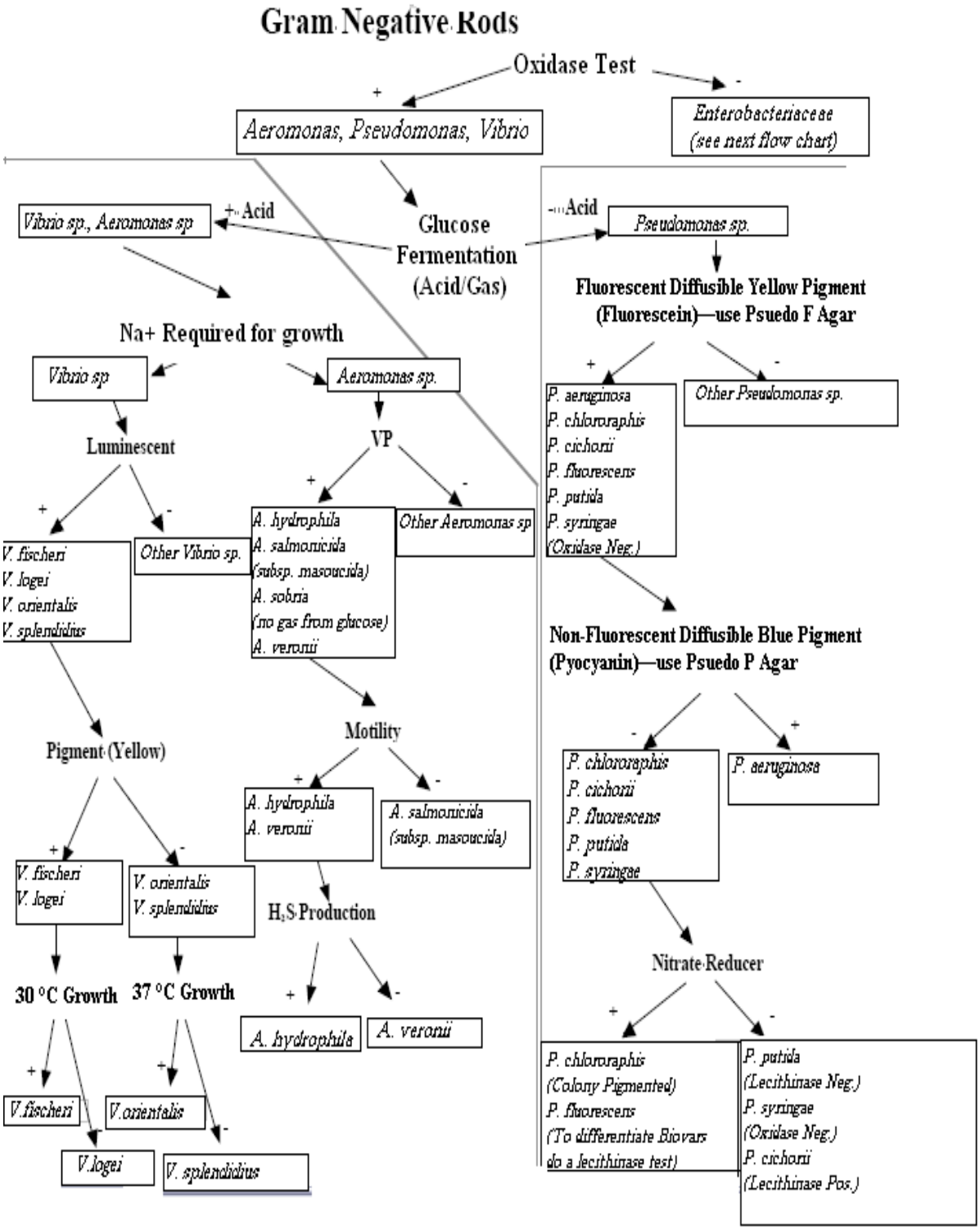

Fig 2.4: Gram Negative Rods ID Flow Chart

MATERIALS AND METHODS

3.1 Site of collection

Two provinces of Punjab namely Jalandhar and Phagwara formed areas of study. Information regarding the crop grown, agrochemical, herbicides and pesticides that are applied in fields, the span of uses and their consequences on the form of crop cultivated is gathered.

3.2 Collection of soil samples

Sampling was obtained by randomly collecting sample from six farms from the two provinces. For individual provinces, plots were selected on the basis of the category of crop cultivated in that field. 200g of surface mineral soil (5 cm deep) was collected from each filed of the two provinces. The soil samples were collected in sterile polythene bags. For control sample was collected from 100m of the fields where no horticultural activities were carried out. The samples were brought to laboratory and stored at 36^{o}C.

Fig 3.1: Collection of sample from site

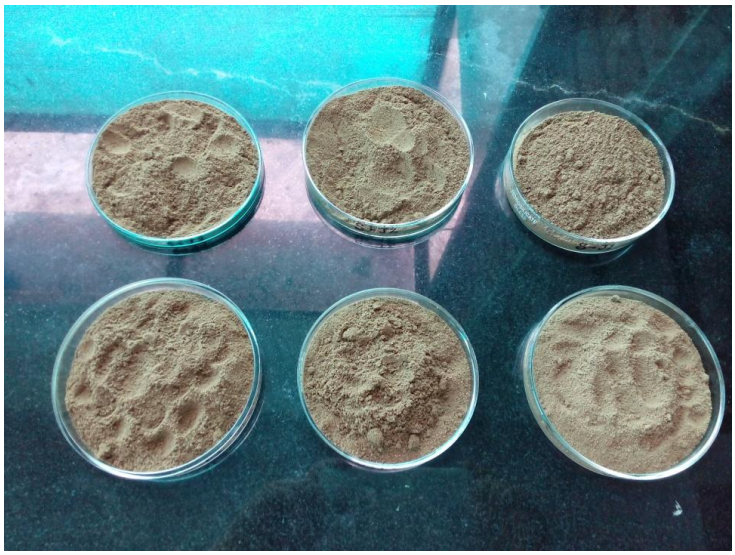

Fig 3.2: Collected samples

3.3 Isolation of the sample

The soil sample was sieved through a sieve having pores less than 2mm. Then the sample was air dried at temperature less than 37^{o}C. During the process of drying the sample was kept in the incubator (temp-36^{o}C).

Fig 3.3: Sieving of soil sample

3.3 MSM for isolation of imazethapry degrading bacteria

Boon *et al., 2000* has given that Mineral salts medium (MSM) was prepared using different types of salts. The medium constitute of 1,419.6 mg of Na_2HPO_4, 1,360.9 mg of KH_2PO_4, 98.5 mg of $MgSO_4$, 5.88 mg of $CaCl_2 \cdot 2H_2O$, 1.16 mg of H_3BO_4, 2.78 mg of $FeSO_4 \cdot 7H_2O$, 1.15 mg of $ZnSO_4 \cdot 7H_2O$, 1.69 mg of $MnSO_4 \cdot H_2O$, 0.38 mg of $CuSO_4 \cdot 5H_2O$, 0.24 mg of $CoCl_2 \cdot 6H_2O$, 0.10 mg of MoO_3, and 3.2 mg of EDTA suspended in 1 liter of distilled water. 3 gms of soil sample was suspended in flasks measuring 250ml which have 50ml of MSM enriched with imazethapyr (50 mg/l). Incubated for 7 days at less than 37^oC.

3.4 Plating of the imazethapry suspended sample

Mineral agar was used as media in the plating. 26.6 gm of mineral agar was suspended in 1000 ml of distilled water and was autoclaved. Then media was poured into the plates allowed to solidify. Loopful of the bacterial growth from the flasks containing MSM supplemented with imazethapyr were streaked onto the mineral agar plates. The plates were kept for incubation at 37^oC for 2 days and growth of microorganisms were observed. There was one control, which was not inoculated with soil sample but only medium with the pesticides imazethapyr was taken (Sorensen and Aamand, 2003)

3.5 Different concentration of imazethapyr

Single colony from the streaked plates was taken and further observation was done for growth of bacteria at various concentration of imazethapyr. In the mineral agar 250 mg/l and 300 mg/l of imazethapyr respectively were suspended accordingly and streaking was performed. Plates were then incubated at 37^oC for two days and the growth for the bacteria was observed.

3.6 Sub culturing to obtain pure colonies

After the growth of the bacteria was observed at various concentration of imazethapyr sub culturing was performed by taking a loop full of bacterial growth from the single colonies

obtained and further streaked into new plates of mineral agar. Sub culturing was carried out thrice and the pure colonies were obtained.

3.7 Gram staining of isolated bacteria

Various methods were used for the staining of the isolated bacteria depending upon the bacteria that was in focus. Gram staining characterize the bacteria into two types: Gram positive and Gram negative bacteria. Gram positive bacterias are either cocci or vibrios or bacilli. *Staphylococci, Streptococci, Pneumococci* etc are gram positive pathogenic bacterias. And the Gram negative bacterias are either bacilli or cocci type. *E.coli, Klebsiella, Salmonella spp, shigella*, etc are commonly encountered gram negative pathogenic bacteria. **Table 4.1** represents the chemicals used during the performance of the gram staining technique

Table 3.1: Chemicals required for gram staining technique.

S.No	Chemicals name	Amount required(drops)	Time(second)
1	Crystal violet (Primary stain)	3-4	60
2	Grams Iodine (Mordant)	3-4	60
3	Ethyl Alcohal (Decolourizer)	3-4	5-10
4	Safranin (Secondary Stain)	3-4	45

Following was the procedure for gram staining :

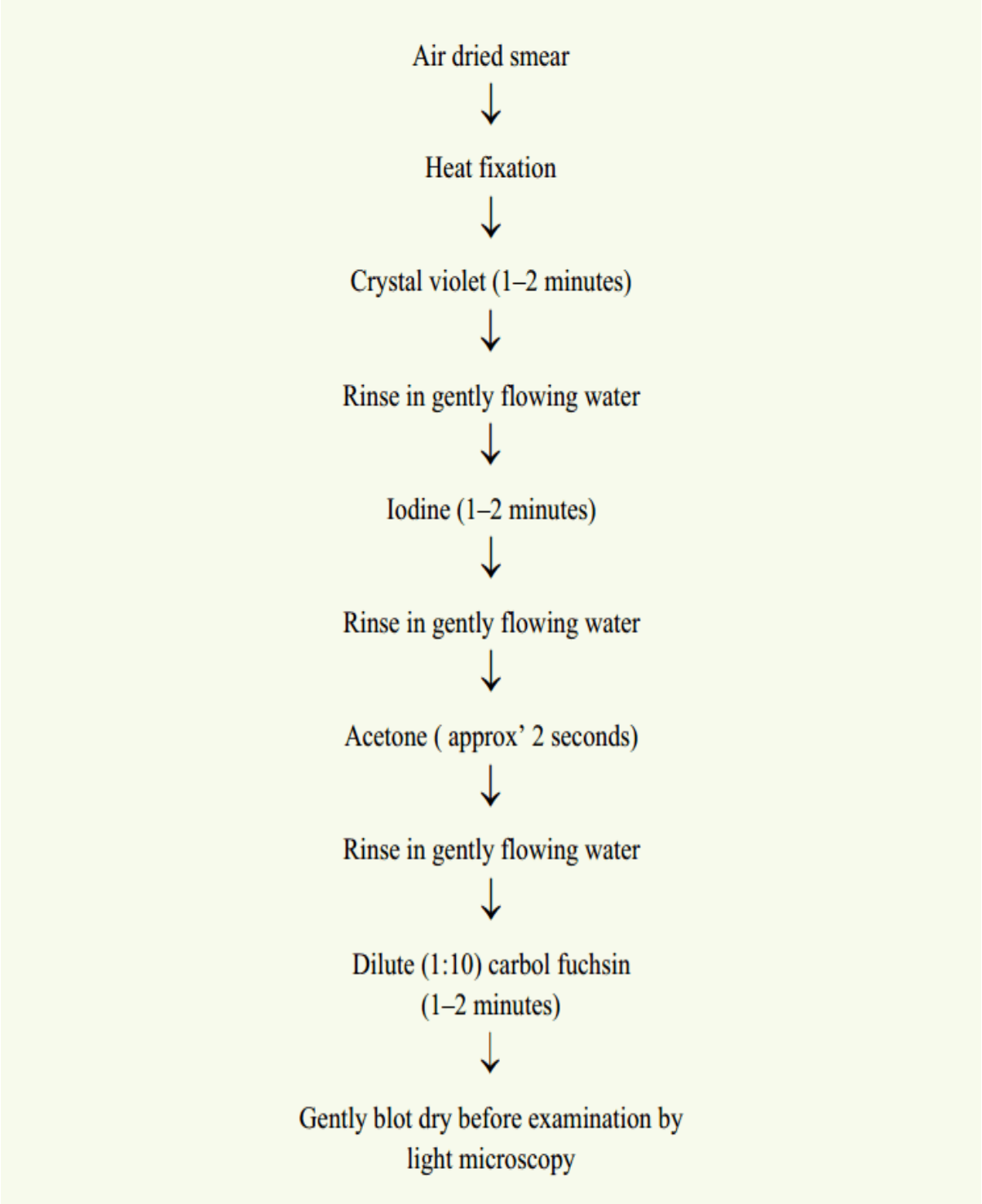

Fig 3.4: Flowchart representation of gram staining technique

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3.8 Endospore staining

Structurally endospores are dormant and tough survival which are produced by a very few types of bacteria, mostly the genera Clostridium and *Bacillus*. Endospores are kind of "bacteria seeds", which are made through a process called sporulation to survive in extreme environmental conditions, such as high temperatures, desiccation, chemicals, pH changes and lack of food. Production of endospore is a crucial differentiation of some bacteria, which allows them to prevent harsh climatic conditions i.e. desiccation, exposure to chemicals, intense heat, etc. Endospores were discovered in the 1800s (John Tyndall introduce a procedure to destroy them with recurrent heat), although the stain processes for identifying them was not developed till the early twentieth century. Of all living organisms bacterial endospores are the most resistant structures, and are able to survive in this latent dehydrated state for more than hundreds of years (even some are reported at thousands of years). Endospores are not for reproduction: One spore formation takes place inside the vegetative cell. On germination of the spore, there will be production of one vegetative. The stimulus for sporulation varies according to desiccation, chemicals, nutrient depletion, heat, etc. As a spore formation takes place inside the vegetative cell, the spore wall chemically starts to change and thicken. Due to sporulation method the spore's stainability changes , making them resistant to staining dyes, and therefore a gimmick steaming increases the primary dye's penetration. Malachite green is used as the primary dye which is a comparatively weakly binding dye that binds to the spore wall and cell wall. In fact, if this dye is washed well with water, the dye comes out of the cell wall, how so ever it does not come out of the spore wall once the dye is locked inside. Therefore there is no need of a decolourizer in this type of staining: depending on the binding of the dye malachite green and the permeable charateristics of endospore and the cell wall. . The steaming allows the malachite green to permeate through the low-permeability spore wall. Many chemicals consists the spore wall like keratin, protein, and calcium, but deep inside the wall is peptidoglycan. The outer portion of the endospore wall is made up of keratin thus making it resistance to the dye. The bacteria is heated to make the spore wall extra permeable to the malachite green, and then it gets attached to the peptidoglycan. After it gets in, primary dye malachite green cannot penetrate out as by cooling the smear, the overlying spore wall becomes less permeable.

Following was the procedure for Endospore staining :

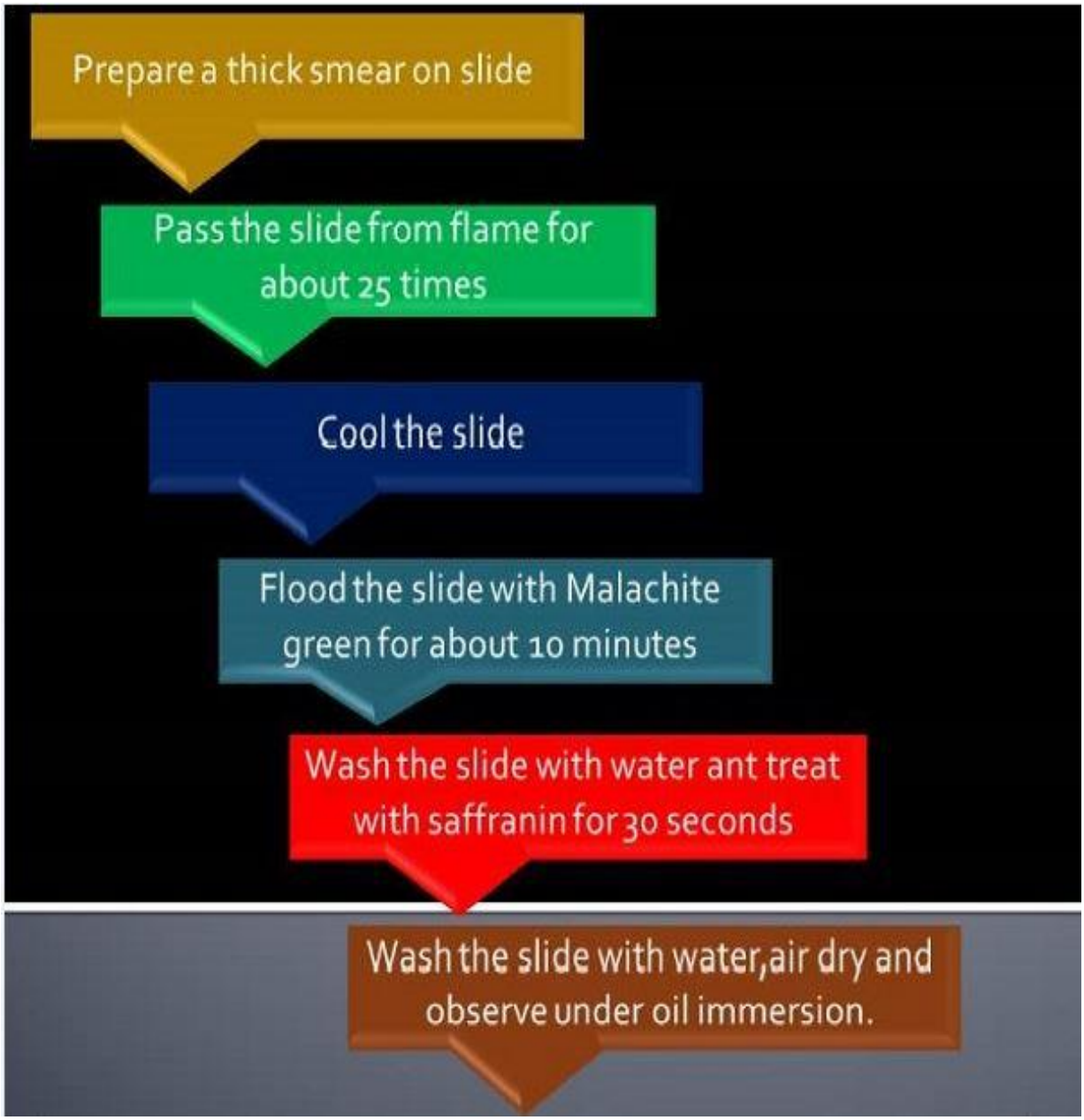


Fig 3.5: Flowchart representation of Endospore staining

3.9 Acid fast staining

This differential staining method was first established by Ziehl, then was modified by Neelsen. Thus the acid fast staining method is further known as **Ziehl- Neelsen staining** techniques. In 1883 Neelsen made use of Ziehl's carbol-fuchsin and heated and then decolorized it with an acid alcohol, and after that did counter staining with methylene blue. The

main objective of the staining method is to characterize bacteria into two groups namely acid fast group and non-acid fast groups. This staining process finds it's use in the identification of those microorganisms which cannot be stained by Gram staining or by simple method, mainly the microorganisms of genus *Mycobacterium*, which are resistant and are detected only by acid-fast staining. When staining of smear made, is done with carbol fuchsin, the lipoidal material gets solubilized which is found in the Mycobacterial cell wall however on raising the temperature, carbol fuchsin further enters through lipoidal wall and moves inside the cytoplasm. Resulting in the red colour appearance of cells. Then with help of decolorizing agent the smear is decolourized with i.e. 3% HCL in 95% alcohol but the cells which are acid fast don't get decolourized as they are resistant to the agent and does not allow the decolorizing solution to penetrate in their cell wall due to the presence of a massive amount of lipoidal material . The non-acid fast microorganisms does not have the lipoidal material present in their cell wall because of which they gets easily decolorized, and the cells are rendered colourless. After that the smear is stained with methylene blue which is acts as a counterstain, methylene. Only those cells which are decolorized absorbs the counter stain and take the colour and blue colour appears while acid-fast cells retains the red colour.

Table 3.2 Reagent used for Acid fast staining

S.No	Application of	Reagent	Cell Colour for acid fast	Cell Colour for non acis fast
1.	**Primary dye**	**Carbol fuchsin**	Red	Red
2.	**Decolorizer**	**Acid alcohol**	Red	Colourless
3.	**Counter stain**	**Methylene blue**	Red	Blue

Following was the procedure for Acid fast staining :

Fig 3.6: Flowchart representation for Acid fast staining

3.10 Biochemical tests for unknown bacteria:

Various tests were performed for the identification of unknown bacteria.

3.10.1 Catalase test

The catalase test helps to detect, presence of catalase enzyme in bacteria. It is important to differentiate between *Micrococcaceae* giving positive catalase test and *Streptococcaceae* giving negative catalase test. Whereas it is primarily helpful to make differentiation in between genera, it is even important to do speciation of some gram positive bacteria such as Gram positive bacteria *Aerococcus urinae* from Gram negative bacteria *Aerococcus viridians* and Gram negative organisms like *Campylobacter fetus*, *Campylobacter jejuni*, *Campylobacter coli* all Gram positive from other *Campylobacter* species. The enzyme catalase is produced by many aerobic bacteria and by most of those bacteria which are facultatively anaerobic. The function of the catalase enzyme is that it helps in detoxifying hydrogen peroxide (H_2O_2), and this formed from the superoxide radical by the help of superoxide dismutase.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Following was the procedure for catalase test:

Inoculate bacteria into a glass test tube

Keep it in incubator at 37 degree Celsius


after 24 hours

Add a drop of hydrogen peroxide into test tube

If bubbles are formed the organism is catalase positive/if not,negative

Fig 3.7: Flowchart representation of catalase test

3.10.2 Motility test

An organism's ability to move by itself is referred to as motility. Motility is somewhat similar to chemotaxis, which refers to the ability to orientate along certain chemical gradients. Eukaryotic cells can move with the help of different types of locomotor organelles like cilia, flagella and pseudopods. Prokaryotes with the help of propeller-like flagella which is the uniqueness of bacteria or through special fibrils which produce motility in gliding form. Mostly all spiral bacteria and about half of the bacilli bacteria are motile, and about none of the cocci shaped bacteria are motile. The capability for true motility is present in wide variety of cells, but is not present in all of them. The bacteria with no motility are called non-motile. Although, when present in an aqueous environment, these no-motile bacteria appears to move erratically. This erratic movement is caused due to Brownian movement which is the result of the bacteria being bombarded by the random motion of the water molecules which cause them to move. Most of the motile bacteria move by using their flagella which is a thread like locomotor appendage that extends outwards from the plasma membrane and cell wall. There may be one flagellum in motile cells, but mostly there are several. Each flagellum has very rigid and helical structure and the actual motility is the result of the rotation of the flagellum. Motility with the help of flagella is specifically important for identifying the microorganisms, for example Bacilli shaped bacteria are motile except anthrax, which is capsular, so flagella can be produced to enable them to escape from danger and to migrate from less to more favourable environment. The same case is seen in Clostridia which are motile except that *Clostridium perfringens* has a capsule. It has

been suggested that motility is an important feature for colonization and therefore pathogenic characteristic of *Helicobacter pylori.* The positions of the insertion of the flagella into the bacterial cell are the features of a genus. For example, in **monotrichous** cells there is only a single flagellum and if the flagella is located at an end, it is called polar flagellum. The **SIM medium** (Sulphide Indole Motility medium) is mainly used for this purpose which combines differential medium which is a test for checking three different parameters Sulphur Reduction, Production of indole and Motility. The soft consistency of the media helps in the migration of motile bacteria readily through them that causes cloudiness. The non-motile bacteria only grows in soft agar tube and only in the area of inoculation. But motile bacteria will grow along the stab area and will also come outside the stabbed area. So, if growth is in a distinct zone along the stab negative result is detected. A positive test is shown if at the top and bottom of the stab there is diffuse or cloudy growth.

Following was the procedure for motility test:

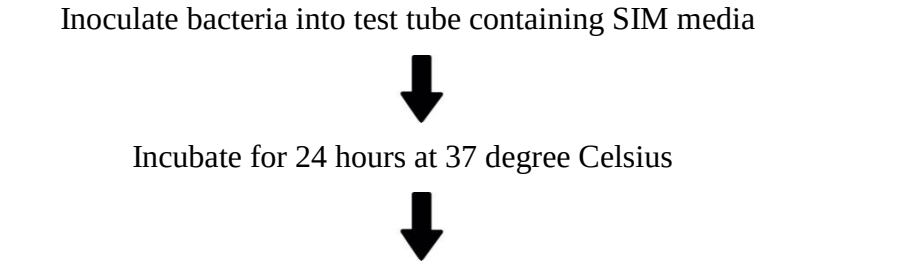


Fig 3.8: Flowchart representation of motility test

3.10.3 Starch hydrolysis test

The main motive of enzyme test is to make sure if the microorganism will make use of starch which is a posh saccharide that is made up of aldohexose which acts as a carbon source and energy needed for growth. Usage of starch can be achieved by an accelerator known as alpha-amylase. For this test starch agar medium is used. The medium may be a agar and starch is accessorial to that. Starch containing medium is used. When doing immunisation and whole night long incubation, iodine chemical agent is needed to see the existence of starch. Iodine chemical agent form complexes with starch and makes dark-blue colour in the matter.

Following was the procedure for Amylase test:

An inoculum from a pure culture is streaked on a sterile plate of starch agar

↓ incubate at 37 degree Celsius for 24 hours

Iodine reagent is added to flood the growth

↓

Presence of clear halos surrounding colonies is positive for their ability to digest the starch

Fig 3.9: Flowchart representation of starch hydrolysis test

3.10.4 Salt concentration

Microorganisms vary widely in their salt tolarence.In a hypertonic(high solute content) environment,all cells lose water by osmosis and become shrivelled,the phenomenon known as plasmolysis. Its effect on ceel reproduction is inhibitory. In a hypotonic(low solute content)environment,water is taken by the cells and become swollen,phenomenon called plasmoptysis.There is no harmful effect on microorganisms of hypotonis environment while in such environments animal cells undergo lysis which causes their death. Most natural environment of high osmolarity contain high concentration of salts, precisely sodium chloride. Microorganisms which are capable of growing in this type of environment are called halophiles. Bacteria may be categorized into four groups depending on their capacity to grow at various sodium chloride concentrations, non halophilic(those capable of growing on media containing less then 2% sodium chloride), halophilic(those capable of growth on a medium containing above 2% salt).Halophilic may further be of three types: slightly halophilic(grow at concentration between 2 and 5%),moderately halophilic(capable of growth at salt concentration upto 10%) and extremely halophilic(capable of growth at salt concentration more than 20%).

Following was the procedure for salt concentration test:

Inoculate bacteria into petri plate containing nutrient agar medium.

↓

Keep it in incubator at 30 degree Celsius for 2-5 days.

3.11 Bacterial Identification-16 S rRNA Sequencing

3.11.1 DNA Extraction

- Lysis/homogenization: Suspend 1-3 colonies aseptically to lysis cells grown in monolayer. They are then mixed with 450 µl of "B Cube" lysis buffer in a 2ml micro centrifuge tube and lyse the cells by repeated pipetting.
- 4 µl of RNAse A and 250 µl of "B Cube" neutralization buffer is added.
- Content is vortexed and tubes are incubated for 30 minutes at 65^{o}C in water bath. Mix DNA sample by inversion.
- Tubes are centrifuged for 15 minutes at 14,000 rpm at 10^{o}C.
- Viscous supernatant is transferred into a fresh 2ml micro centrifuge tube without disturbing the pellet.
- 600 µl of "B Cube" binding buffer is added to content and is mixed thoroughly by pipetting. Then content is incubated for 5 minutes in room temperature.
- Transfer 600 µl of content to a spin column placed in 2ml collection tube.
- Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
- Reassemble the spin column and the collection tube then transfer the remaining 600 µl of the lysate.
- Centrifuge for 2 minutes at 14,000 rpm and discard flow-through.
- 500 µl "B Cube" washing buffer I is added to spin column and centrifuged at 14,000 rpm for 2 minutes and discard flow-through.
- Reassemble the spin column and 500 µl "B Cube" washing buffer II is added and centrifuged at 14,000 rpm for 2 minutes and discard flow-through.
- Transfer the spin column to a sterile 1.5 ml microcentrifuge tube.
- Add 100 µl of "B Cube" Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filter.
- Tubes are incubated for 5 minutes at room temperature and centrifuge at 6000 rpm for 1 minute.
- Repeat above mentioned two steps for complete elution. Buffer remaining in the microcentrifuge tube contains DNA.

- Measurements of the DNA concentrations were done by running aliquots on 1% agarose gel.
- DNA samples were stored at -20^{o}C until futher use.

3.11.2 PCR Protocol

Polymerase chain reaction (PCR) is a technique that uses primers to amplify specific cloned or genomic DNA sequences by making use of a very unique enzyme. It uses enzyme DNA polymerase which leads to the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. The enzyme adds nucleotides to 3' end of a custom-designed oligonucleotide during annealing. Annealing occurs to a longer DNA template. Hence, DNA polymerase treat the oligonucleotide like a primer for elongation of its 3' end to develop an double stranded DNA with extended region.

Composition of the Taq Master Mix

1. Taq DNA polymerase is supplied in 2X Taq buffer
2. 0.4 mM dNTPs
3. 3.2 mM $MgCl_2$
4. 0.02% bromophenol blue

PRIMER DETAILS

Table 3.3: Primer details

Primer Name	**Sequence Details**	**Number of Base**
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

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 following thermal cycling conditions.

1. Denaturation

   DNA template is heated to 94^{o}C for 3 minutes. Heating cleft the weak hydrogen bonds which holds DNA strands together in a helix. Thus, separation of strands occurs and results into single stranded DNA.

2. Annealing

   The denatured mixture is allowed to cool down from 94^{o}C for 30 sec or 50^{o}C for 60 sec or 72^{o}C for 60 sec. Thus allowing the primer to bind/anneal to template DNA containing the complementary sequence.

3. Extension

   Heating of the reaction is then done at 72^{o}C for 10 mins, optimal temperature for enzyme DNA polymerase. The enzyme extends the primers and with the help of target DNA as a template result in adding nucleotide to the primer in sequential manner.

Purification of PCR Product

Unincorporated PCR primers and dNTPs from PCR products are removed using Montage PCR clean up kit. PCR product was sequenced using 27F/1492R primers. ABI PRISM® BigDyeTM Terminator Cycle was used for sequencing reactions. Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme).

3.11.3 Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

3.11.4 Bioinformatics protocol

- The 16s rRNA 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 and was succeeded by multiple sequence alignment.
- The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The aligned sequences obtained were cured using program Gblocks 0.91b. This Gblocks removes poorly aligned positions and divergent regions (Talavera and Castresana 2007). Finally, program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85as Substitution model.
- PhyML was exhibited to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was displayed to be at least as accurate as other existing phylogeny programs using simulated data,while being one order of magnitude faster. The Tree Dyn 198.3 program was used foe tree rendering. (Dereeper *et al*.,2008)

RESULTS AND DISCUSSION

Frequency of herbicides application on agricultural fields survey revealed the usage of various formulation of herbicides. The study demonstrated that, different herbicide constitution used by farmers in two provinces of Punjab (Jalandhar and Phagwara) sampled a number of herbicides.

4.1 Imazethapyr soils enrichment cultures

The soil sample was inoculated into MSM media suspended with imazethapyr. Growth of bacteria was observed even though the presence of imazethapyr was there. This indicates that the bacteria that grows is capable of degrading the imazethapyr concentration in the media and thus was able to show its growth.

4.2 Culture and isolation of imazethapyr degrading bacteria

Using improved cultures steps bacterial strains were isolated from soil samples collected from the two different provinces. The isolation process is a method of isolating single colony from the mixture of colonies. This process was performed with the help of streaking method to obtain pure cultures. The soil sample was added to MSM media suspended with imazethapyr to obtain solution sample before transferred into minimal agar plate. The sample prepared was streaked on to minimal agar plates, fused and also single colonies of bacteria were observed. It is important that the numbers of colonies developing on the plates are not being too large. So, to obtain the appropriate colony number, sub culturing of the colonies was performed.

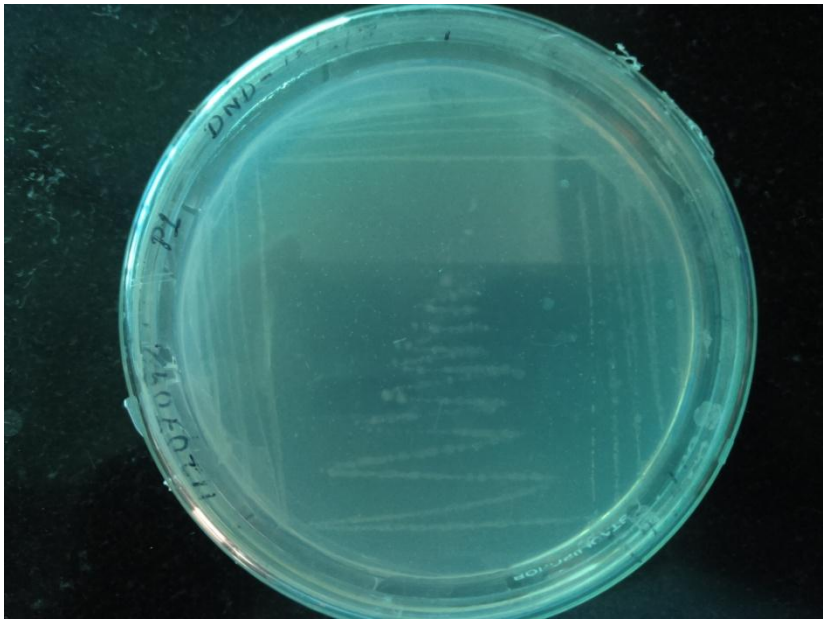


Fig 4.1: Streak plate of soil sample on minimal media

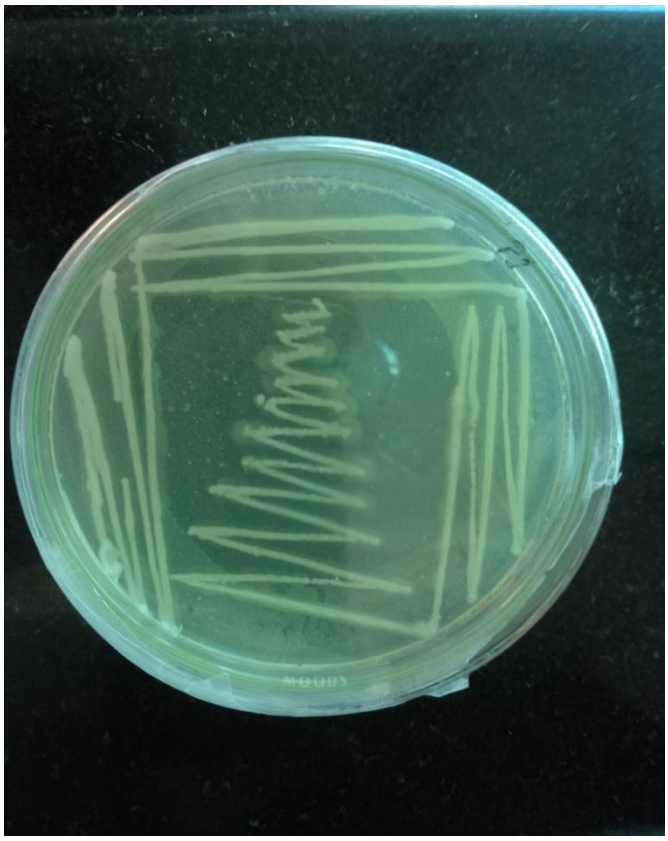
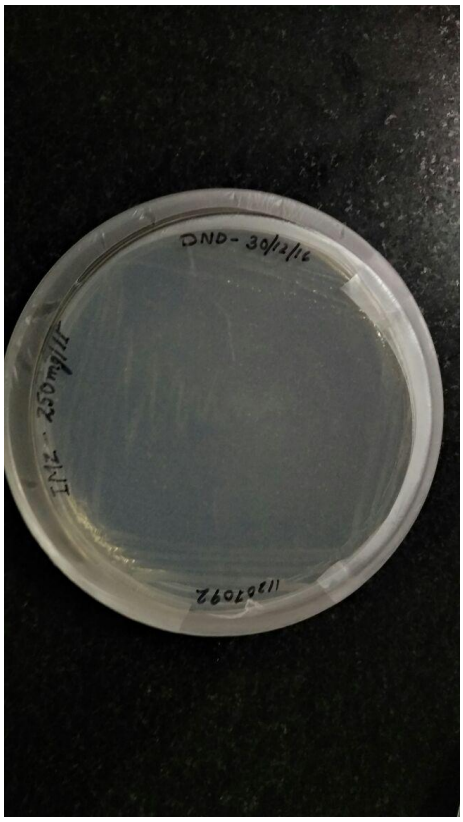


Fig 4.2: Streak plate of soil sample on nutrient agar media

4.3 Imazethapyr degradation by soil bacteria

The bacteria isolated is said to be degrading imazethapyr herbicide as growth was observed in different concentrations of it. Imazethapyr degrading isolates were monitored at different concentration. Two concentration were taken 250 mg/l and 300 mg/l. Growth was observed at both the concentration but it occurs to decrease at 300 mg/l. Growth of the bacteria varies at different concentration. As a result when concentration increases the growth of bacteria trends to decrease which implies that bacteria are able to degrade herbicide until certain level. So from this we can conclude that the bacteria present in sample is able to degrade imazethapyr herbicide.



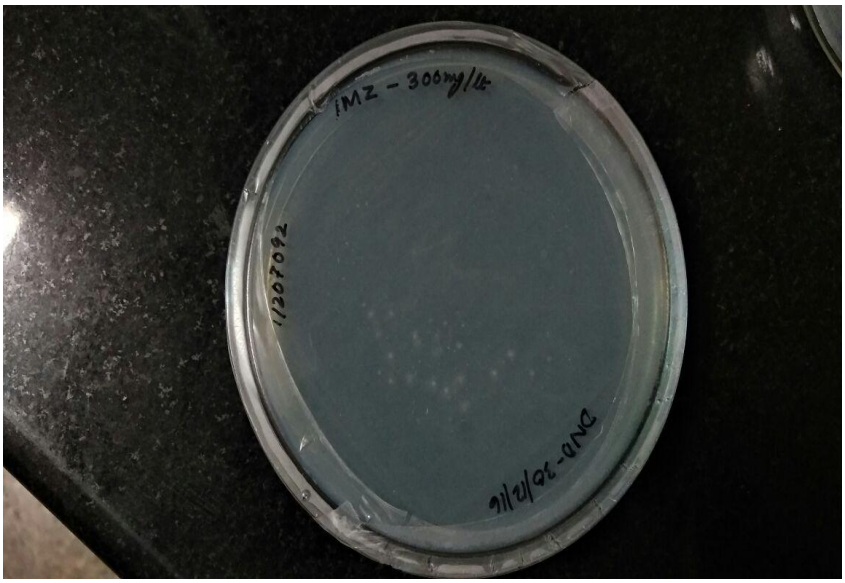


Fig 4.3: a) Streak plate having 250mg/l concentration of imazethapyr. b) Streak plate having 300mg/l concentration of imazethapyr

4.3 Results of staining

Gram staining is done from the obtained pure culture to identify bacteria is gram negative or gram positive. We observed Gram positive bacteria. Endospore staining was performed to characterize bacteria as endospores and non-endospores. Then acid-fast staining was performed to distinguish bacteria into two categories namely acid fast group and non-acid fast groups. The acid-fast staining method finds it's use in the detection of those microorganisms which cannot be stained by simple or Gram staining method , which are non-tolerable and may be only identified by acid-fast staining. Table 4.1 represents the bacterial shape and also the results of gram, endospore and acid-fast stains.

Table 4.1 Results of staining

NAME OF BACTERIA	SHAPE	GRAM STAIN	ENDOSPORE STAIN	ACID-FAST STAIN
Bacillus	Rod	+ve	+ve	+ve

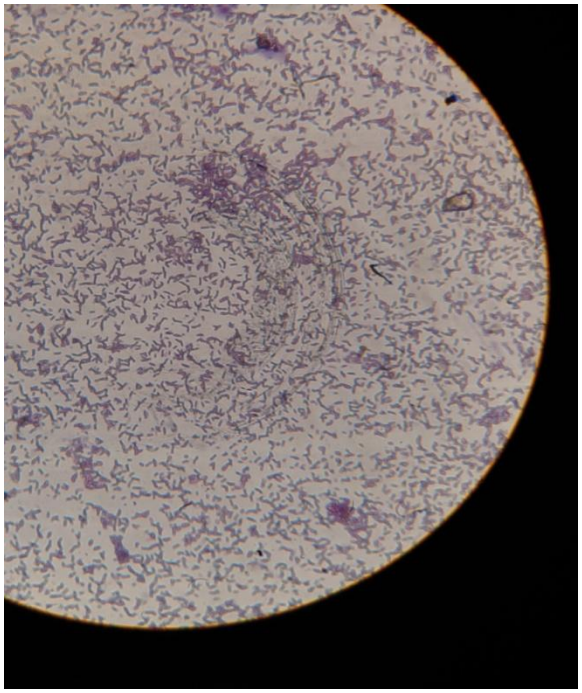


Fig 4.4: Result of gram staining

4.4 Results of biochemical analysis

A series of biochemical tests were performed. Biochemical tests were used for the detection of bacteria species depending on the variations in the biochemical activities of bacterial isolate obtained. Bacterial morphology varies from one species to the other. The variations in carbohydrate metabolism, protein metabolism, fat metabolism, formation of certain enzymes, capability to use a definite compound help them in the identification by the biochemical tests. Table 4.2 represents results of various biochemical test.

Table 4.2 Results of biochemical test

NAME OF BACTERIA (genus)	**CATALASE TEST**	**MOTILITY TEST**	**STARCH HYDROLYSIS TEST**
Bacillus	+	+	+

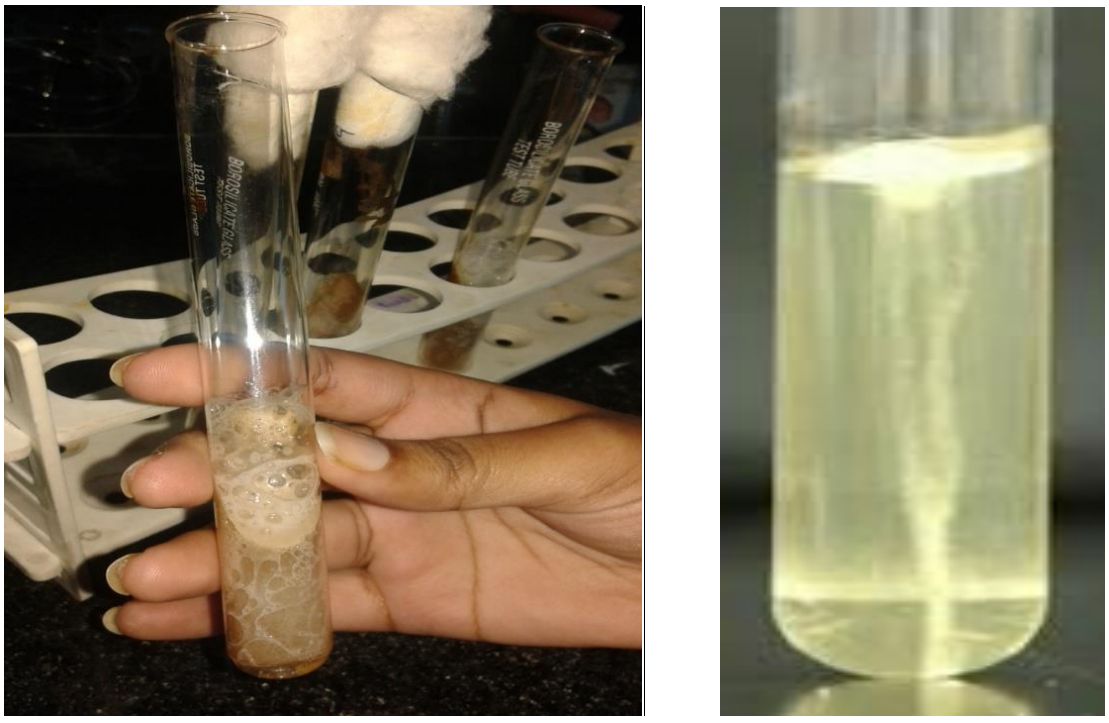
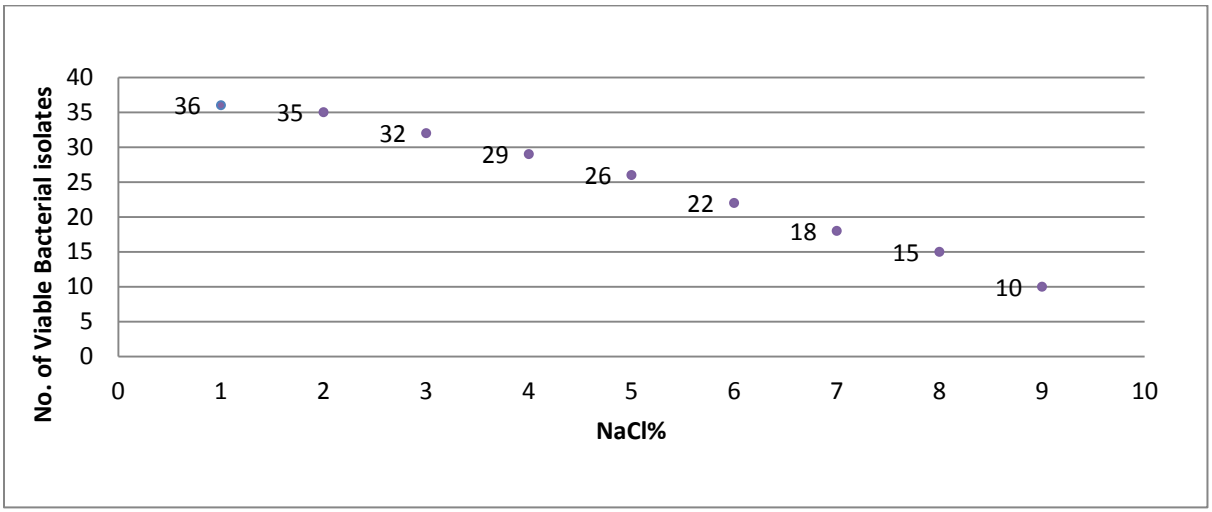


Fig 4.5: (a) Represents positive catalase test for *Bacillus* (b) Represents positive motility test for *Bacillus*

4.5 Result of Salt Concentration

Most natural environment of high osmolarity contain high concentration of salts, precisely sodium chloride. Microorganisms which capable of grow in this type of environment are known as halophiles. The importance of this test is that which bacteria can survive in which environment, for example if bacteria is slightly halophilic then it will not grow in high concentration soils. Regarding the halophillic nature, the bacteria isolate have the ability to tolerate 0-3% NaCl concentration. The population decreased after 7% concentration.There was no growth obtained after 9% .The detailed results of the halophilic nature of the bacterial isolates are presented in Graph 4.1.



Graph 4.1: Represents halophilic nature of *Bacillus*

Graph shows that the population decreased as the salt concentration increased. At 1%, 36 colonies were observed. As we increased the concentration of NaCl after 6%, the growth decreased to 5 colonies. After 9% almost no growth was observed.

4.7 Sequence data in FASTA format

1. **Bac 970R:** The following sequence represents the sequence that is obtained after using the reverse primer (1492R) to the sample.

>160808-031_G13_Bac_907R.ab1 1177

ACGTGAGTCTCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGA
AACCCTCTAACCTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCC
TGTTCGCTCCCCACGCTTTCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCG
CCACTGGTGTTCCTCCACATCTCTACGCATTTACCCTACACGTGGAATTCCACTCTCC
TCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGTTGAGCCGGGGGCTTT
CACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGACAACG
CTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTT
AGGACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAG
TTTTACGATCCGAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCA
TTGCGGAAGATTCCCTACTGCTGCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA
GTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATGTCGCCTTGGTGAGCCGTTACC
TCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCGAAAGCCACCTT
TTATGATTGAAACCTGGCGGTTCATCCAACCATCCGGAATAACCCCCGGTTTCCCGA
ATTAATCCCATCCTAACGGGCGGGTAACCCACTGGTAACCCACCCGCCGGCCGTTAA
CCAAAGGAAGCAGCTTCCCGCCGGCCGGTTCAACTTGCAGGAATAAGGAAGCCCGC
CGGCGTTCGTCTGAGCGAGTAAAAACCCTAAAAAACGGCGCTTTCGCTGGGGGTGG
GAGGGGCCCCTTGCTGCAAACCATAAAGCAAAATATTTACGGAGAATTCTCCCCCCT
TAGCATAATAAAAGGGGGGGGCCCCCCGGATGGGAAAGGAAGGGGGGGGTTTGT
GGTGTGGGTTACGAGGCCCCCCCACGAAGAGGAAGGGTGTGGGTCTCTCCCCCGCC
GGCGGGCCGTTTTGTTCTGGCTCGCCCCGACCTTCTCGGTCGGCGGGTGGGTTTGGTT
TGGCTCGGGTTGTTTCTGTCAGG

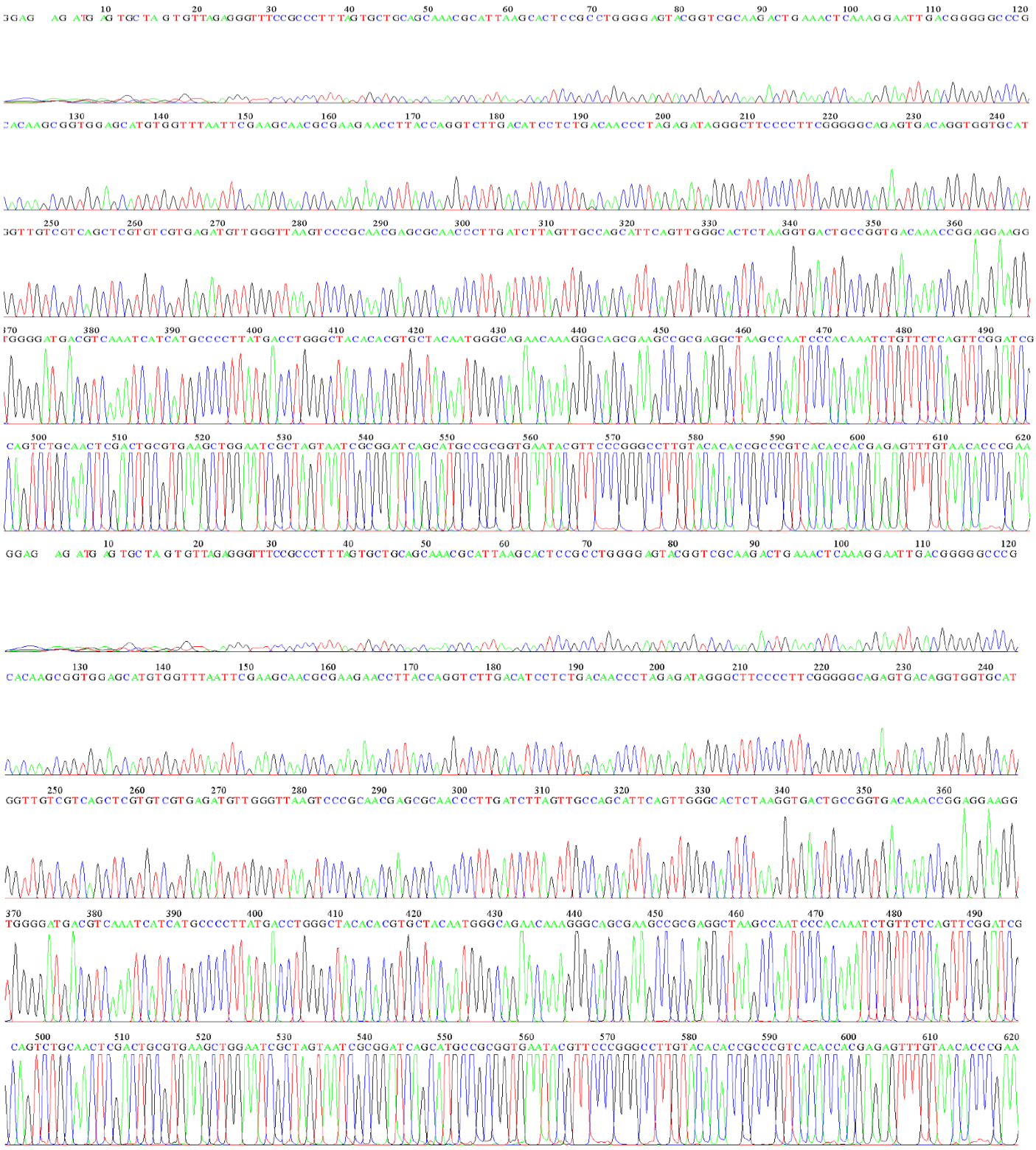
2. **Bac 785F:** This sequence is the result obtained after using the forward primer (27F) to the sample.

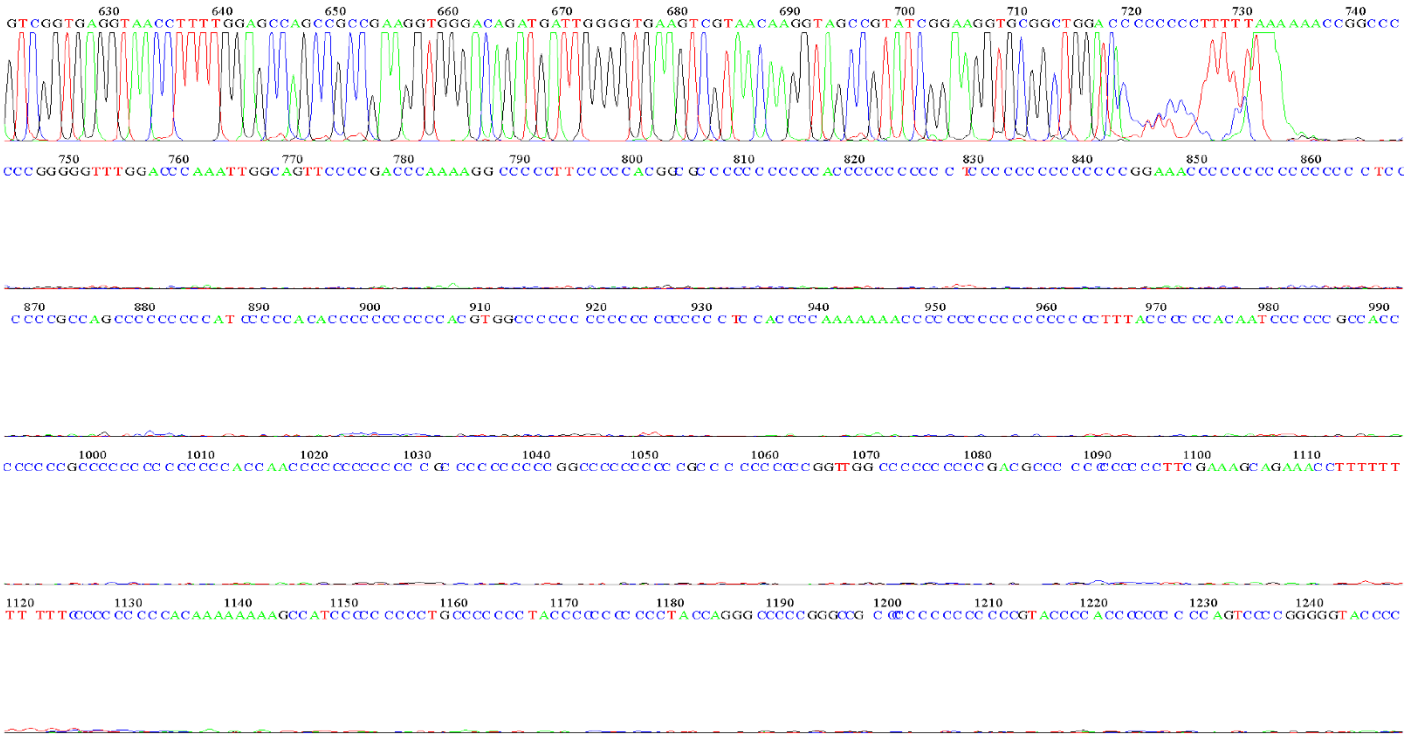
>160808-031_Bac_785F.ab1 1257

GGAGAGATGAGTGCTAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCT
TACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCA
GAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCC
GCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTC
GACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAG
TCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTG
AAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGACCCCCCCCTTTTTAAAA
AACCGGCCCCCCGGGGGTTTGGACCCAAATTGGCAGTTCCCCGACCCAAAAGGCCC
CCTTCCCCCACGGCGCCCCCCCCCCCACCCCCCCCCCCTCCCCCCCCCCCCCCGGAA
ACCCCCCCCCCCCCCCCCTCCCCCCGCCAGCCCCCCCCCATCCCCCACACCCCCCCCC
CACGTGGCCCCCCCCCCCCCCCCCCCTCCACCCCAAAAAAACCCCCCCCCCCCCCCC
CCTTTACCCCCCACAATCCCCCCGCCACCCCCCCCGCCCCCCCCCCCCCCACCAACC
CCCCCCCCCCCCGCCCCCCCCCCCGGCCCCCCCCCCCGCCCCCCCCCCGGTTGGCCCCCC
CCCCGACGCCCCCCCCCCCCTTCGAAAGCAGAAACCTTTTTTTTTTCCCCCCCCCCA
CAAAAAAAAGCCATCCCCCCCCCTGCCCCCCCTACCCCCCCCCCCTACCAGGGCCCC
CGGGCCGCCCCCCCCCCCCCCGTACCCCACCCCCCCCCCAGTCCCCGGGGGTACCCC
CCCCCCC

File: Bac_907R.ab1 Signal G:2728 A:3764 C:8709 T:499 Sample: Bac_907RLane: 57 Base spacing: 15.327656 1177 bases in 14313 scans

File: Bac_785F.ab1 Signal G:2704 A:3503 C:5817 T:3539 Sample: Bac_785F Lane: 59 Base spacing: 15.249888 1257 bases in 15089 scans





4.8 BLAST result of Aligned sequence data

Contig sequence is the sequence received after comparing the two sequences received after applying forward and reverse primers. The contig sequence contains the best possible sequence after the use of primers. Thus, following is the sequence of DNA obtained from the sample.

>contig

CCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAA
CACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCT
CCCCACGCTTTCGCGCCTCAGCGCAGTTACAGACCAGAGAGTCGCCTTCGCCACTG
GTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCT
TCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTC
ACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAAC
GCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTG
GTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAA
CAGAGTTTTACGATCCGAAAACCTTCATCACTCACCGGCGTTGCTCCGTCAGACTT
TCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGCGTGTCTC
AGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGA

GGTTCCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCTGAAA
GCCACCTTTTATGATTGAAACCTGGCGGTTCATCCAACCATCCGGAATAACCCCCG
GTTTCCCGAATTAATCCCATCCTAACGGGCGGTAACCCACTGGTAACCCACCCGCC
GGCCGTTAACCAAAGGAAGCAGCTTCCCGCCGGCCGGTTCAACTTGCAGGAATAAG
GAAGCCCGCCGGCGTTCGTC

Result: *Bacillus licheniformis*

4.9 Phylogeny tree

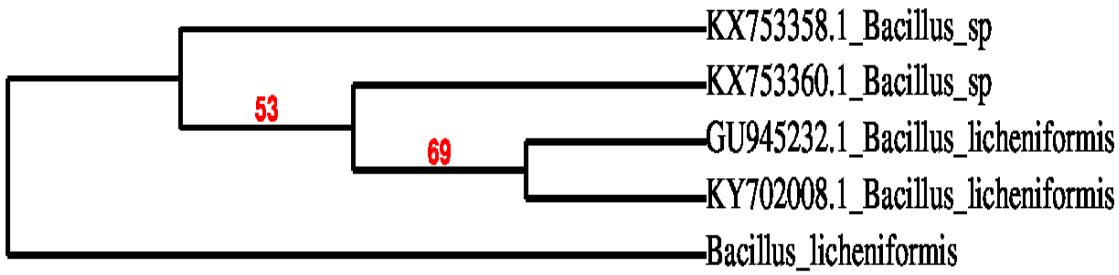


Fig 4.7: Phylogenetic tree

Phylogenetic tree in the (figure no: 4.7)53 and 69 are the bootstrap value. They are values of non-parametric test of how well the data support the nodes of a given tree. The sample is closely related to *Bacillus* species and is also closely related to *Bacillus licheniformis*.

CONCLUSIONS

The study has concluded that agricultural fields under horticultural production applies different herbicides formulations. In current study, Imazethapyr (herbicide) degrading isolates were obtained from six different horticultural farms of Jalandhar and Phagwara region of Punjab. Samples were sieved, dried and were then inoculated into MSM media suspended with Imazethapyr and incubated for seven days. This prepared sample is then streaked into mineral agar and growth of microorganisms were observed. Bacterial colonies obtained were grown on different plates containing different concentration of Imazethapyr. It was found out that resulted bacteria were able to degrade Imazethapyr to a certain level as the concentration was increasing its growth was observed to be decreasing. Later for the isolation of pure and single colonies sub culturing was carried out thrice in the mineral agar. The best bacterial sample out of the six was selected and further staining, biochemical test and sequencing was performed for that sample. To identify the bacterial isolate three different staining methods were carried out. Firstly, gram staining was done and the result came out to be gram positive bacteria. Secondly, endospore staining was carried out which was also positive and endospore was present. Subsequently, acid-fast staining was performed and the sample was found out to be acid-fast bacterial group. For identification of unknown bacteria three different biochemical test were performed. Catalase test, motility test and starch hydrolysis test. They showed positive results confirming the resulting bacteria to be *Bacillus licheniformis*, and have the enzyme catalase present in it. It is motile and is capable of digesting the starch. The salt concentration test was also carried out in order to find out the halophilic or non-halophilic nature of the bacteria. *Bacillus licheniformis* was found out to be halophilic bacteria. 16S rRNA sequencing was performed for the identification of the bacteria. The sequencing method contains DNA extraction, PCR, Purification of PCR product, Single-pass sequencing and different bioinformatics tools were also used. Bioinformatics analysis gave the sequence of the bacteria in FASTA format, sequence with Chromatogram, BLAST result of aligned sequence data. The reverse and forward primer used were respectively Bac_970R and Bac_785F. The peaks received by usage of these primers were of good quality. Two resultant sequences were obtained after the application of the primers. Then these sequences were contiged to obtain the best sequence. Thus contig sequence was found out to be the

sequence similar to *Bacillus licheniformis*. The bioinformatics summary report contains the Phylogeny tree. Phylogeny tree has 53 and 69 are the bootstrap value. They are values of non-parametric test of how well the data support the nodes of a given tree. Thus, the soil bacteria which is responsible for the degradation of Imazethapyr (herbicide) is *Bacillus licheniformis*, which has a history of commonly found in soil. It is reported to be bacteria with feather degradation property. *B. licheniformis* has the capacity to degrade the organophosphate compounds, Malathion and Quinalphos. The isolate Bacillus was found to be most potent against imazethapyr.

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