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Isolation and Identification of Imazethapyr (Herbicide) Degrading Soil Microorganism from Punjab Region (India)

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Abstract

Leaching out of synthetic chemical pesticides, fertilizers and herbicides to surface and water level is at the root of environmental pollution. It 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. Soil samples were collected from wheat ranch from different sites in Punjab region. Isolation of these microorganisms in pure culture is being done by adopting streaking method. Biochemical and molecular tests were also carried out for identification process of the isolated microorganisms.

Keywords: Isolation; Streaking method; Biochemical test, molecular 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, storing, 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 [12]. 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 [5]. 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 [13]. 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 [6], [8]. 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 methods for pesticide degradation [22]. One of the method used to remove these pesticide and herbicide accumulation from contaminated sites is to exploit the ability of microorganisms. This is a substitutive strategy for treating these pollutants. It is efficient, least hazardous, cost-effective, adaptable and eco-friendly and is known to be as **bioremediation** [4].

Microorganisms provide a potential wealth in biodegradation. These organisms are able to cut-down the concentration of xenobiotics and is precisely related to their permanent adaptation to surrounding where these compounds subsist. Moreover, for enhancing the activity of microorganisms having the favourable properties, required for biodegradation are genetically modified [15]. 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 [1], [4] and [14].

Imazethapyr is an 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 i.e. 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 barnyardgrass, cocklebur, panicums, smartweed, pigweeds, nightshade, mustard, velvetleaf, jimsonweed, crabgrass, seedling johnsongrass, 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. Commercial formulation of imidazolinone imazethapyr (IMZT)-based herbicide was named as Pivot H® (10.59% IMZT). Its letha, acute and sublethal toxicity was studied [23]. 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.

METHODOLOGY

Material and methods

Sample collection

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.

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±2 ^{o}C.

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±2 ^{o}C. During the process of drying the sample was kept in the incubator (temp-36^{o}C).

MSM for isolation of imazethapyr degrading bacteria: Boon *et al., 2000* has given that Mineral salts medium (MSM) was prepared using different types of salts. The medium constitutes 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±2 ^{o}C.

Plating of the imazethapyr 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^{o}C±2 ^{o}C 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 [18].

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±2 ^{o}C for two days and the growth for the bacteria was observed.

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.

Biochemical staining and tests for unknown bacteria: Gram staining, endospore staining, acid fast staining, catalase test, starch hydrolysis test and motility test were conducted on bacterial isolates following standardized microbiological protocols.

Bacterial Identification-16 S rRNA Sequencing: 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).

RESULTS

The soil samples were 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.

The bacteria isolated is said to be degrading imazethapyr herbicide as growth was observed in different concentrations of it (Fig. 1). Imazethapyr degrading isolates were monitored at different concentration. Two concentrations 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 as clearly shown in Fig. 2.

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. Therefore, from this we can conclude that the bacteria present in sample is able to degrade imazethapyr herbicide. Better growth was shown by **B3** bacterial isolate namely among six other isolates.

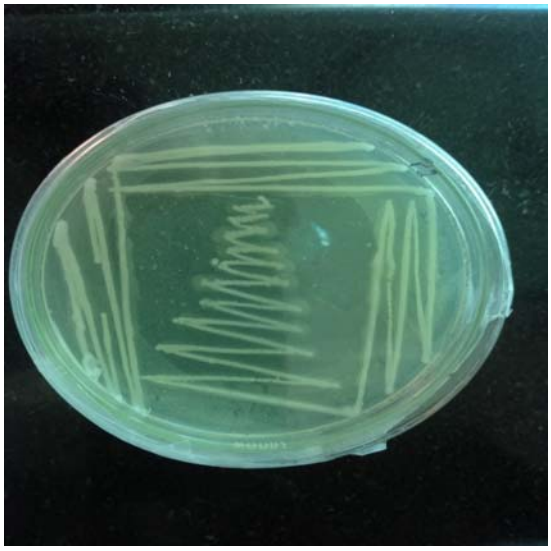


Fig 1: Streak plate of soil sample on nutrient agar media

Fig. 2b): Streak plate having 300mg/l concentration of imazethapyr.

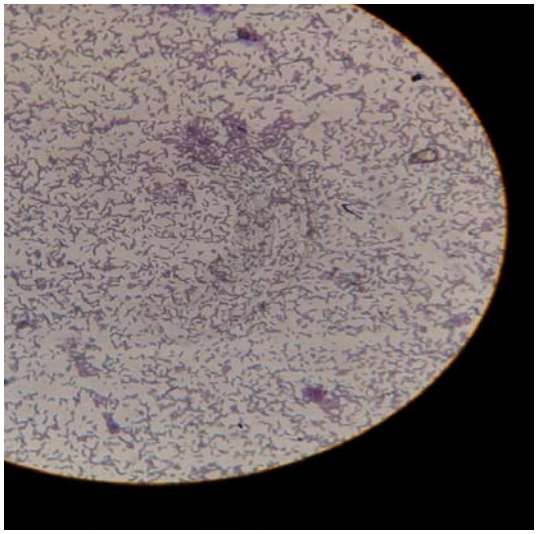
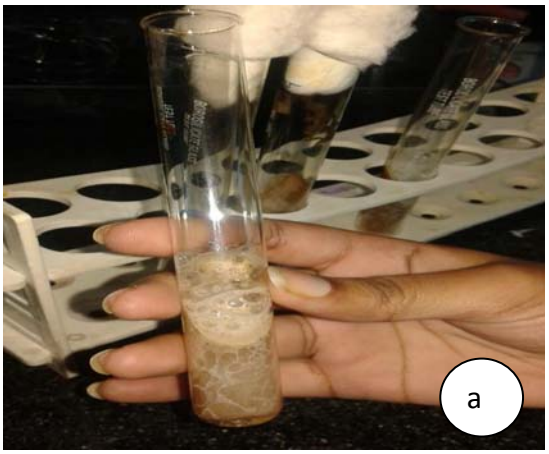


Fig 3: Result of gram staining



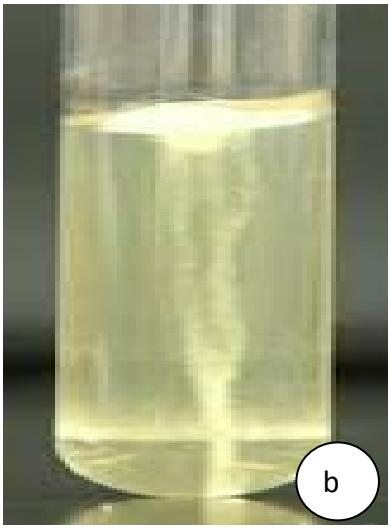


Fig 4: (a) Represents positive catalase test for *Bacillus*
Fig 4 (b) Represents positive motility test for *Bacillus*

Staining of the pure cultured of B1 TO B6 samples had identified the bacteria to be rod shaped, gram positive and endospore forming bacteria belongs to *Bacillus* genus (Table 1 and Fig. 3).

Table 1: Table showing results of biochemical characterization for all the soil samples.

S.NO.	Sample	Shape	A	B	C	D	E	F
1	B1	Rod	+	+	+	+	+	+
2	B2	Rod	+	+	+	+	+	+
3	B3	Cluster	+	-	-	+	+	+
4	B4	Rod	+	+	+	+	+	+
5	B5	Rod	+	+	+	+	+	+
6	B6	chain	+	-	+	-	+	+

***A- Gram Stain, B- Endospore Stain, C- Acid-Fast Stain, D-Catalase Test, E- Motility Test, F- Starch Hydrolysis Test**

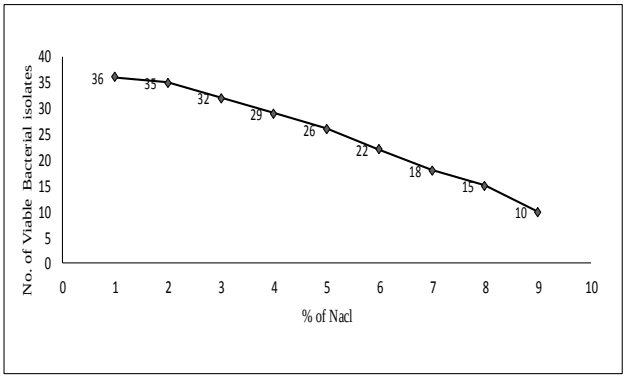


Fig 5: Graph represents halophilic nature of *Bacillus*

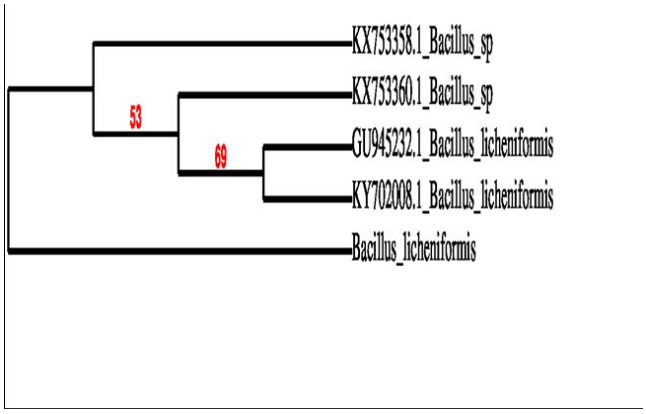


Fig 6: Phylogenetic tree

The isolated bacteria have the ability to tolerate 0-3% NaCl concentration. The population decreased after 7% concentration. There was no growth obtained after 9%.

Fig 5 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.

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
CCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCA
CTAAAGGGCGGAAACCCTCTAACACTTAGCACT
CATCGTTTACGGCGTGGACTACCAGGGTATCTA
ATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGC
GCAGTTACAGACCAGAGAGTCGCCTTCGCCACTG
GTGTTCCTCCACATCTCTACGCATTTCACCGCTA
CACGTGGAATTCCACTCTCCTCTTCTGCACTCAA
GTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAG
CCGGGGGCTTTCACATCAGACTTAAGAAACCGCC
TGCGCGCGCTTTACGCCCAATAATTCCGGACAA
CGCTTGCCACCTACGTATTACCGCGGCTGCTGGC
ACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCG
TCAAGGTACCGCCCTATTCGAACGGTACTTGTTC
TTCCCTAACAACAGAGTTTTACGATCCGAAAAC
CTTCATCACTCACCGGCGTTGCTCCGTCAGACTT
TCGTCCATTGCGGAAGATTCCCTACTGCTGCCTC
CCGTAGGAGTCTGGCGTGTCTCAGTCCCAGTGT
GGCCGATCACCCTCTCAGGTCGGCTACGCATCG
TCGCCTTGGTGAGGTTCCTCACCAACTAGCTAAT
GCGCCGCGGGTCCATCTGTAAGTGGTAGCTGAA
AGCCACCTTTTATGATTGAAACCTGGCGGTTCAT
CCAACCATCCGGAATAACCCCCGGTTTCCCGAA
TTAATCCCATCCTAACGGGCGGTAACCCACTGG
TAACCCACCCGCCGGCCGTTAACCAAAGGAAGC
AGCTTCCCGCCGGCCGGTTCAACTTGCAGGAATA
AGGAAGCCCGCCGGCGTTCGTC

Result: *Bacillus licheniformis*

4.9 Phylogeny tree

Phylogenetic tree in the (Fig. 6) 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 B3 is found to be closely related to *Bacillus* species and is also closely related to *Bacillus licheniformis*.

Discussion

In the present study, six different samples were collected from agricultural fields in Punjab area. Among them, the isolates were shown to belong to the genus *Bacillus*. The isolate selected for further characterization in this study, namely isolate B3, showed the greatest similarity to members of the order Bacillales and *Bacillus* genus. The results obtained in this study were in agreement with earlier reports that indicated the involvement of different species of *Enterobacteriaecae* and *Bacillacae* in the degradation of organophosphorous insecticides like chlorpyrifos [17], phosphonate [10] and glyphosate [2] and herbicides also. Singh et al. (2004) reported that *Enterobacter* strain B-14 used chlorpyrifos as a source of carbon and phosphorous. Sethunathan and Yoshida (1973) isolated a *Flavobacterium* sp. that could use parathion as source of phosphorous but not diazinon as carbon source.

The main focus of the present study was to isolate potential imazethpyr-degrading bacteria and to study their growth response in the presence of different concentrations of the herbicide. Among the bacterial isolates, B3 has maximum hydrolyzing capability, as was evidenced from the broader hydrolysis zone observed on mineral salts agar, compared to the other isolates. Growth experiments conducted had shown that *Bacillus licheniformis* is able to grow in the

presence of high concentrations of imazethpyr and utilized it as energy source. Similar observations were reported regarding utilization of chlorpyrifos and other herbicides as carbon or energy source by bacteria isolated using an enrichment procedure [21]. Some organophosphorous insecticides such as diazinon, chlorpyrifos, ethion, parathion, fonofos, malathion and gusathion are susceptible to microbial hydrolysis and serve as carbon sources for the growth of pure and mixed cultures of *Flavobacterium sp*., *Pseudomonas* sp. and *Arthrobacter* sp. [3], [7]. The soil used for the present study had been exposed to imazethpyr for three years. The tolerance of the organism might be due to previous exposure or due to its ability to hydrolyze the supplemental substrate. Successful removal of pesticides by the addition of bacteria had been reported earlier for many compounds, including coumaphos [11], ethoprop [9] and atrazine [19].

CONCLUSION

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.

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