

Biodegradation of Imidacloprid, the New Generation Neurotoxic Insecticide

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ABSTRACT: Imidacloprid (1-[(6-chloro-3-pyridinyl)-methyl]-*N*-nitro-2-imidazolidinimine), a chloronicotinyl insecticide used to control biting and sucking insects, is very persistent in the soil with a half-life often greater than 100 days. Although a few soil metabolites have been reported in the literature, there are few reports of biodegradation of imidacloprid. Our objectives were to discover, isolate, and characterize microorganisms capable of degrading imidacloprid in soil. Two soil free stable enrichment cultures (NUS1, and NUS4) in minimal media were obtained that showed maximum degradation of Imidacloprid between 48 – 72 hours after incubation. The degradation was indicated by growth of microorganisms in minimal media, where sole source carbon and nitrogen was Imidacloprid. The degradation product was characterized by High Performance Liquid Chromatography (HPLC), which was found to be 6-Chloronicotinic acid. The two isolates were thus found to metabolize Imidacloprid and were further characterized.

KEYWORDS: Imidacloprid, insecticide, biodegradation, 6-chloronicotinic acid

I. INTRODUCTION

Imidacloprid is a systemic chloronicotinyl insecticide, having first been registered in the UK in 1993 and in United States in 1994. It is possibly the most widely used insecticide of the group[3]. It is used to control sucking insects, soil insects, termites and some species of biting insects. It can be used as seed dressing, as soil treatment and foliar treatment in different crops including rice, cotton, cereals, maize, sugar beet, vegetables, etc.[9]. Typically application rates range from 0.05-0.125 pounds/acre[4]. Imidacloprid field dissipation rates are widely variable, and have been shown to degrade slowly in soil with half-lives exceeding 180 days in non-vegetated soil. Vegetation increased the rate of dissipation of imidacloprid, yielding a range of half-lives from 42-129 days. This effect of vegetation and the identification of a few metabolites may suggest a microbially mediated degradation process that is enhanced through the rhizosphere effect[7].

Biodegradation of pesticides is controlled by the bioavailability of the pesticide to a pesticide-degrading microorganism and the activity of the microorganism. Bioavailability may limit the biodegradation of imidacloprid and its metabolites in soil, resulting in the long half-lives observed [7].

Exposure to Imidacloprid causes symptoms like thyroid lesions, affected reproduction, reduced ability to gain weight, in humans and miscarriages and smaller offspring in pregnant laboratory animals. It is also toxic to birds and causes eggshell thinning. Imidacloprid is responsible for the death of shrimp and crustaceans at less than 60ppb concentration [2,15]. Considering the toxic effects of Imidacloprid in beneficial insects such as bees, earthworm and also to some degree in humans and its potential to leach groundwater, it is essential to remove these chemo-pollutants from the environment[3]. Biological removal of chemo-pollutants becomes the method of choice since microorganisms can use a variety of xenobiotic compounds including pesticides for their growth and they mineralize and detoxify them[12].

Numerous imidacloprid metabolites have been proposed in degradation pathways. Possible microbial metabolites reported in soil metabolism studies include imidacloprid-guanidine, imidacloprid-guanidine-olefin, and imidacloprid-urea[7]. Although there are very few reports of imidacloprid-degrading soil microorganisms. Our objectives were to screen, isolate and characterize the microorganisms capable of degrading imidacloprid in soil.

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II. MATERIALS AND METHODS

Source:

Agricultural soil from Kale, Taluka Karad, District Satara was taken. This soil was exposed to Imidacloprid. For the study Rhizospheric soil was selected.

Screening and isolation:

The soil was taken and was inoculated in Minimal broth with Imidacloprid as the only carbon and nitrogen source. The only microorganisms which were capable of degrading and utilizing Imidacloprid were able to grow in the Minimal broth. This broth was then plated on Nutrient agar to see the colonies. The colonies so obtained were then isolated. Four bacterial isolates were obtained that were named as NUS1, NUS2, NUS3 and NUS4. The microorganisms hence obtained were further used for degradative studies.

Tolerance studies:

The four isolates were inoculated separately into minimal broth containing 0.5% Imidacloprid. To check the tolerance of the obtained isolates in order to carry out imidacloprid biodegradation studies, incubation was carried out at varying temperatures (65°C, 25°C and 5°C) and pH (4, 7 and 10). The cultures were incubated at varying parameters of temperature and pH for 72 hrs. (Different sets of inoculated broth with 0.5% Imidacloprid were assigned for each parameter).

Molecular characterization studies:

The four isolates were inoculated separately into Luria broth containing 0.5% imidacloprid and incubated at 25° C for 24 hrs. Isolation of the plasmid DNA for all the four isolates was carried out by Alkaline Lysis method. Isolated plasmid DNA samples were then analysed on AGE (Agarose Gel Electrophoresis)

Degradation studies:

Using pure Imidacloprid, λ_{max} of Imidacloprid was found to be at 270 nm using U.V spectrophotometer. Thus a standard curve of changing concentration of Imidacloprid vs absorbance was found and constructed. Individual isolates as well as isolates in consortium were inoculated into minimal broth containing 0.5% imidacloprid and changing concentration of it was measured using U.V spectrophotometer and the standard curve for imidacloprid. For all the studies the density of cells were adjusted to 0.1 at 620 nm (10^6 cells per ml) and then used further.

HPLC-studies:

In order to check that whether the pesticide has actually being degraded or not HPLC was done. The sample was prepared in minimal broth with 0.7% of Imidacloprid in it. For each of the four isolate a different broth was used. This was then inoculated with the respective isolates. After the incubation for 72 hours the broth was transferred in microfuge tubes and centrifugation was done. The supernatant was then subjected to HPLC. (Garware Research Institute, Department of Chemistry, University of Pune). The analysis was accomplished using a Water HPLC (Division of Millipore, Milford, MA), which had a Reverse Phase C-18 (RP-18) Symmetry Shield column (Waters Millipore) (3.9mm×150cm) and an ultra violet (UV) detector. Operating conditions were: 30 minutes gradient of acetonitrile (ACN) and acidified (pH 3) ultrapure water [(0 min) 20%/80% ACN:H₂O; (7 MIN) 22%/78% ACN:H₂O; (14 min) 30%/70% ACN:H₂O; (21 min) 40%/60% ACN:H₂O; (23-30 min) 20%/80% CAN:H₂O)], injection volume of 25 μ l, flow at 0.6 ml min⁻¹, and UV detection at wavelength of 247 and 270 nm.

Characterization

All the four isolates which were found to degrade Imidacloprid were characterized. Colony characters of each isolates was seen. Microscopic characterization like Gram staining, motility was performed. Various biochemical tests like sugar utilization / fermentation, IMVIC, amylase, catalase, oxidase was done to identify the isolates. Further identification of the isolates was done by NCCS (National Centre for Cell Sciences), University of Pune, using PCR to amplify a 500bp sequence of 16S rRNA.

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III. RESULTS

In screening and isolation two media were used , namely Nutrient agar and Minimal agar. Nutrient agar was used for primary screening while minimal agar containing 0.5% Imidacloprid was used for isolating microorganisms capable of degrading the pesticide. Increasing concentrations of Imidacloprid ranging from 0.005% to 1% was used in minimal of which 0.5% was found to be the optimum concentration of pesticide supporting the growth of the isolates (OD at 620 nm). Thus this concentration of Imidacloprid was chosen.

Time course of degradation of Imidacloprid by the 4 isolates was done upto 120 hours (OD at 620 nm).

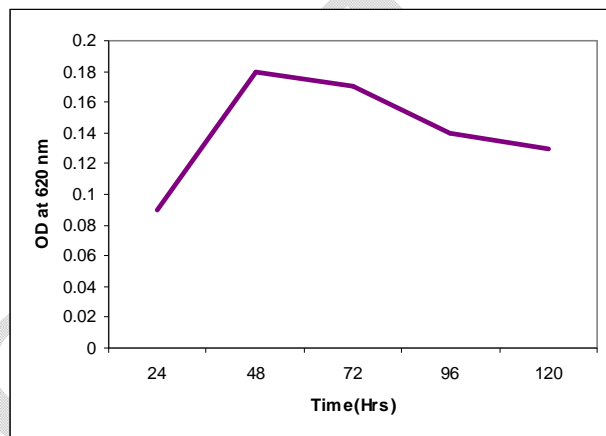
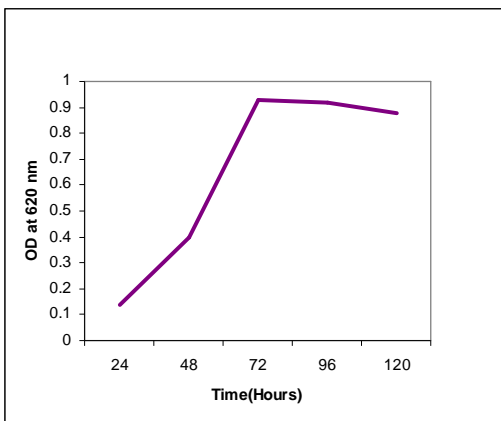


Fig. 1) Growth of isolate NUS1 in Minimal Broth containing

Fig.2)Growth of isolate NUS4 in Minimal Broth containing Imidacloprid Imidacloprid

Tolerance studies: Temperature

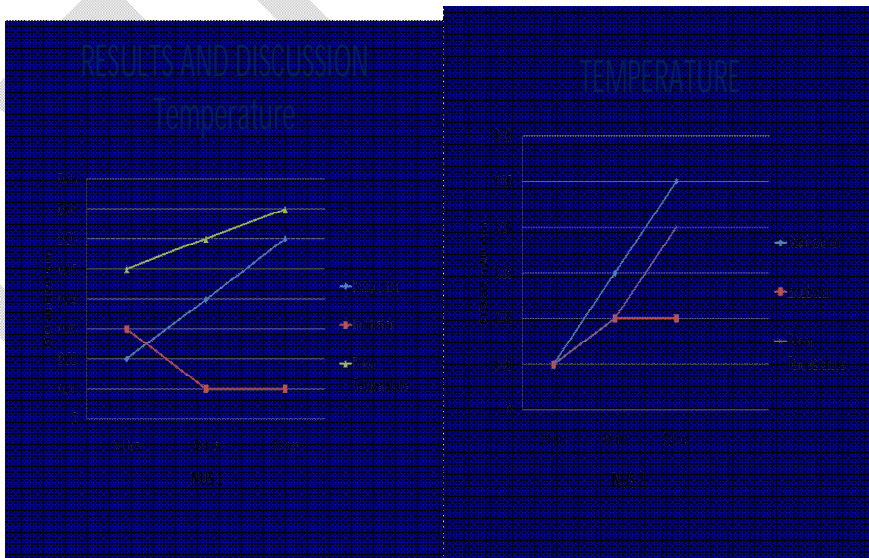


Fig.3 Temperature tolerance study NUS1

Fig.4 Temperature tolerance study NUS2

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(An ISO 3297: 2007 Certified Organization)

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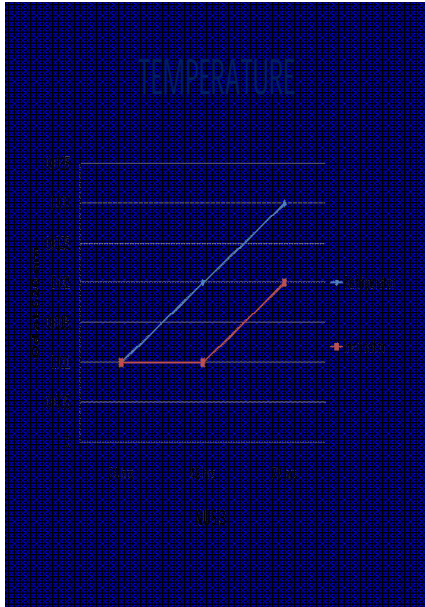


Fig.5 Temperature tolerance study NUS3

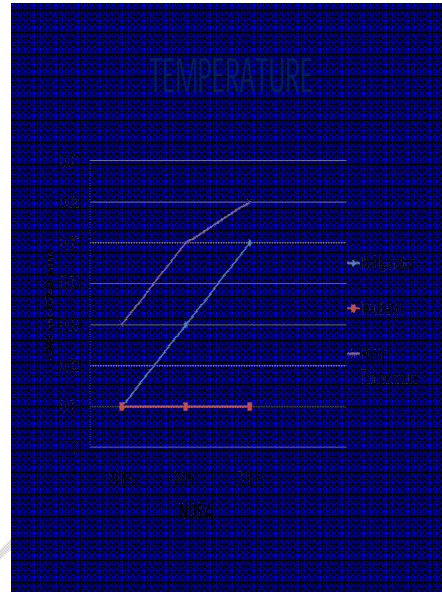


Fig.6 Temperature tolerance study NUS4

For all the four isolates best imidacloprid degradation efficiency was found at room temperature i.e 25°C to 35°C.
Tolerance studies: pH

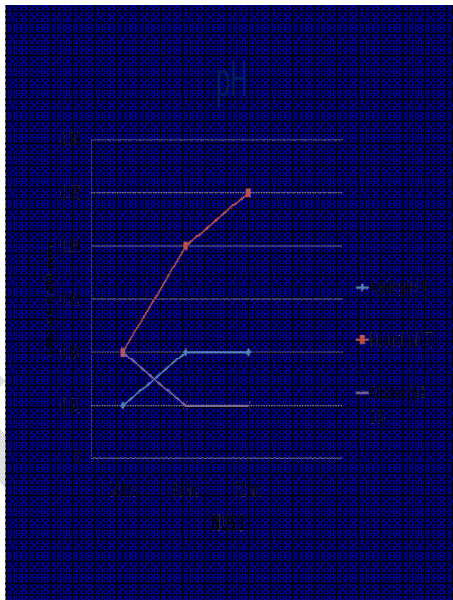


Fig.7 pH tolerance study NUS1

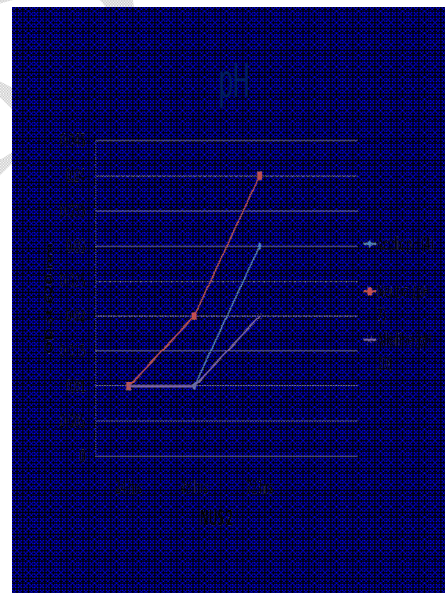


Fig.8 pH tolerance study NUS2

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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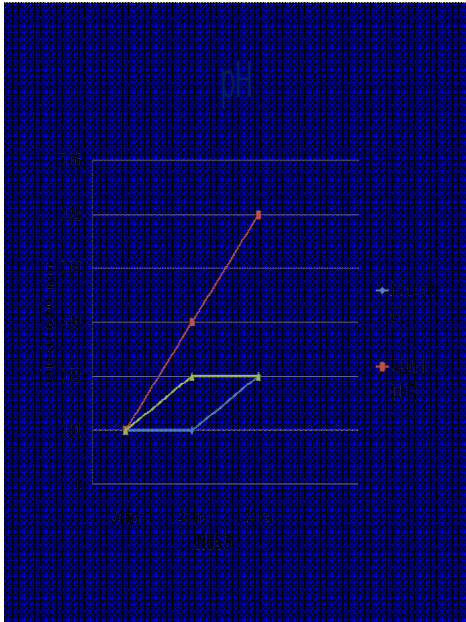


Fig.9 pH tolerance study NUS3

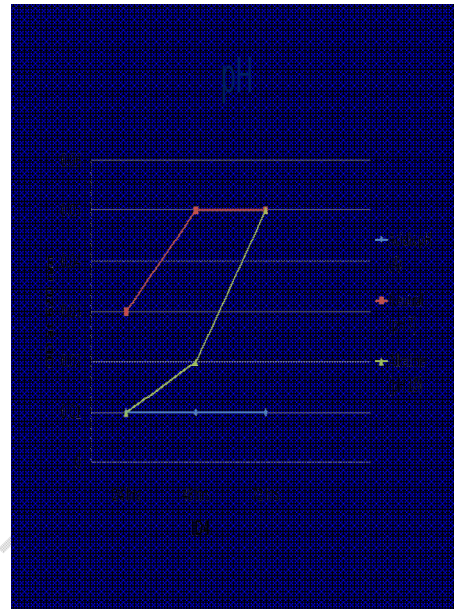


Fig.10 pH tolerance study NUS4

For all the four isolates best imidacloprid degradation efficiency was found at neutral pH i.e 7.

Molecular characterization studies:

Plasmid DNA isolation and gel run was carried out thrice. But no bands were observed. Thus leading to a conclusion that the imidacloprid degradation property of all the four bacterial isolates is a chromosomal encoded property and not plasmid encoded property.

Degradation studies:

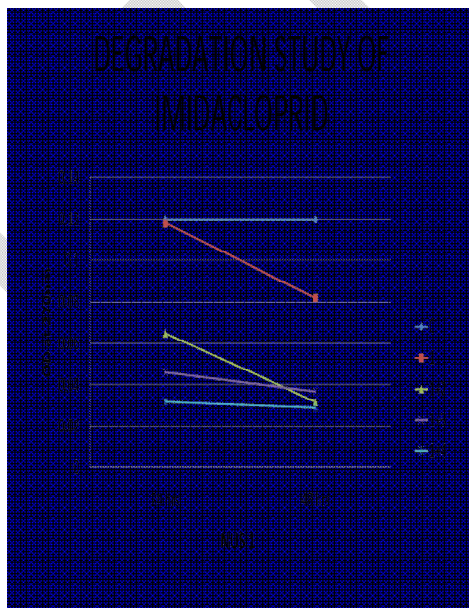


Fig.11 Degradation study NUS1



Fig.12 Degradation study NUS2

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 9, September 2014

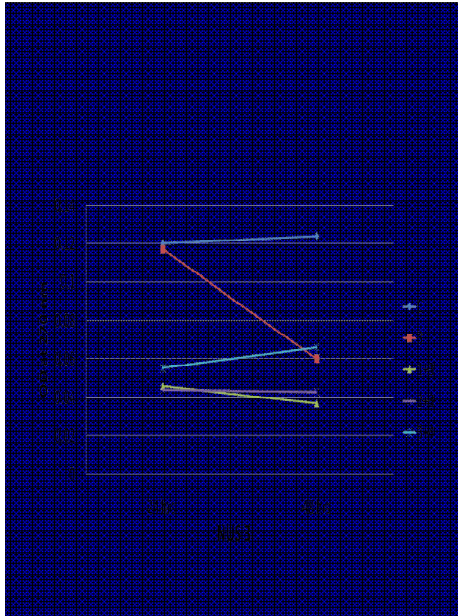


Fig.13 Degradation study NUS1

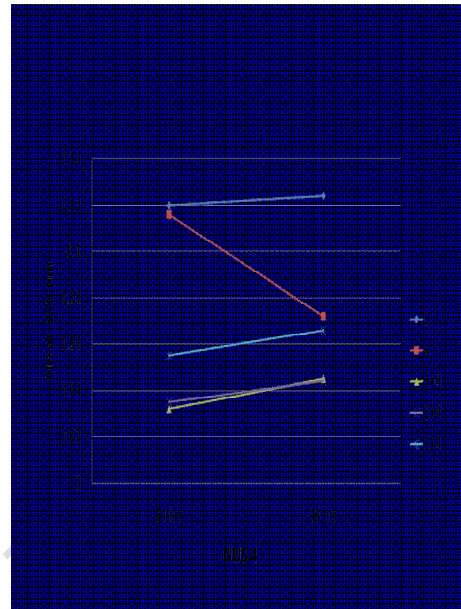


Fig.14 Degradation study NUS2

HPLC-studies:

PRODUCT PROFILE BY HPLC		
Retention Time (min)	Peak Name	Concentration (mg/ml)
10.5	Imidacloprid	100
15.2	Metabolite 1	50
20.8	Metabolite 2	25
25.1	Metabolite 3	10

Characterization:

From Colony and cultural characteristics, biochemical tests and sequencing of 16S rRNA genus level identification of four isolates was done and all four isolates were *Bacillus* sp.

IV.DISCUSSION

It is essential to remove Imidacloprid a chemo-pollutant from the environment. Biological removal of chemo-pollutants becomes the method of choice since microorganisms can use a variety of xenobiotic compounds including pesticides for their growth and mineralize and detoxify them[9]. Bacteria play a significant role in the transformation of pesticides. Even the most persistent pesticides can be metabolized to some extent by bacterial cultures, either by utilization of the compounds as sources of energy or nutrients (carbon, nitrogen, phosphorous or sulphur) or by cometabolism with other

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substrates supporting bacterial growth[12]. Complete mineralization of a chemical is more likely to occur in mixed populations than with single microorganism.

In this study four isolates were obtained from Agricultural soil which able to degrade Imidacloprid. In minimal broth containing imidacloprid maximum growth was seen in between 48-72 hours. The two isolates obtained were able to degrade Imidacloprid upto 1% and showed maximum efficiency at 0.5%. Although individual isolates showed good imidacloprid biodegradation efficiency, in consortium, NUS(1+2) showed the best synergistic effect as far as biodegradation of imidacloprid is considered.

To confirm whether the Imidacloprid was degraded or not, HPLC was performed to detect the degradation product. The product obtained had a retention time of 19 minutes, which correspond to imidacloprid urea that has standard retention time of 6.4minutes[1].

In future, molecular biological studies can throw light on Imidacloprid degrading genes. These studies can also be helpful to know whether there is any involvement of plasmids in degradation of Imidacloprid. Although some reports are available on Imidacloprid degradation by microorganisms, the picture is not very clear on mechanism of degradation of the pesticides. Basic research would throw light on this phenomenon. Constructing recombinant strains for degradation of Imidacloprid and their metabolites would be a challenge. These would be a boon to soil bioremediation processes.

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