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ORIGINAL ARTICLE **OPEN ACCESS**

Screening and Isolation of Imidacloprid Degrading Microorganisms from Pesticide Contaminated soils from Saswad Region in Pune, Maharashtra

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ABSTRACT
Imidacloprid (IMI), a neonicotinoid-class synthetic organic insecticide, is widely used across the world to control both piercing and sucking insect pests. It can persist in soil and contaminate soil, ground water etc. Microbial bioremediation aids in enhancing the soil's health by transforming dangerous pesticide byproducts into less toxic metabolites. The primary screening and isolation of microbes was done by enriching contaminated soil in a carbon-limited liquid Mineral Salt Medium (MSM) containing 100 ppm IMI concentration. Total eleven bacterial strains were isolated and were screened further for their tolerance to proliferate on MSM agar plate containing possible maximum concentration (50,000 ppm) of IMI. Four bacterial isolates VG5, VG7, VG 10 and VG11 were grown fast at highest IMI concentration and were screened further for growth pattern study in full strength MSM medium. Based on the growth pattern and biomass production isolate VG5 was selected as the most efficient strains for the IMI degradation study. Isolates VG5 was characterized on the basis of morphological, cultural and biochemical characteristics as Bacillus sp. by referring Bergey's Manual of Determinative Bacteriology. This potential isolates was identified by 16S rDNA gene sequencing as ***Cytobacillus firmus.***
Keywords: *Bioremediation, Neonicotinoids, Imidacloprid, Bacillus sp., Pesticide hazards*

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INTRODUCTION

The use of pesticides in agriculture is essential since the 45% of the annual food production of the world's agriculture is lost due to pests. According to one of the study on the top agricultural pesticide consumers in the world in 2020, the United States was the highest consumer using 407.8 thousand metric tonnes of pesticides, followed by Brazil with 377.2 thousand metric tonnes (1). India shows much less use of pesticides per hectare than the utilization by other Asian and European countries (2). According to statistics from the Ministry of Chemicals and Fertilizers, ICAR-NIAP, and the non-profit Pesticide Action Network (PAN), Maharashtra has consumed the most pesticides in India during the past five years, followed by Uttar Pradesh, Punjab, and Haryana. For all pesticides used in India, insecticides account for the largest portion.

In the history of contemporary insect management, the discovery of neonicotinoid pesticides marked a turning point (3). These neonics stand out for their capacity to selectively target organisms in an effort to reduce any potential undesirable effects. Imidacloprid [IMI; 1-[(6-chloro-3 pyridynil) methyl] -N-nitro-2-imidazolidinimine] is one of these examples.

Imidacloprid (IMI), a neonicotinoid synthetic organic insecticide, is widely used across the world to control both piercing and sucking insect pests. . Imidacloprid harms the biological nervous system of insects, causes DNA damage, oxidative stress, and mitochondrial malfunction, all of which contribute to biological mortality. According to field research IMI can persist in soil for a half-life of between 27 and 229 days (4, 5). The half-life of imidacloprid in soil varies depending on the type of soil, the usage of organic fertilizers, and the degree to which ground cover is present. Imidacloprid deteriorated more quickly in the presence of vegetation in 48 days as compared to 190 days in the absence of it (6). IMI is photodegraded in soil and has a half-life of 39 days, while field trials showed a half-life of 229 days and laboratory studies found a half-life of 997 days when no light was available (4, 5). Imidacloprid was one of the most widely used pesticides in the globe in 2000, however the European Union restricted its use in 2013 and banned its use on outdoor crops in 2018 (7). Many industry experts mistakenly think

imidacloprid has a low threshold of toxicity. However, it has been discovered to be incredibly dangerous to humans, pollinators, and non-target insects through polluted soil, water, air, and food or through direct exposure during application. Therefore, eliminating imidacloprid residues from the ecosystem is a global concern and top priority.

Since physical and chemical treatments have detrimental effects on the environment, soil, surface and ground water pollution, non-target insects, human health, etc., they have been substituted with biological ones. The soil, which is a great source of biodiversity, contains a large number of useful microorganisms. Bacteria may absorb a variety of resistant contaminants and subsequently degrade them into harmless components. However, there is a paucity of data on imidacloprid's microbial decomposition in Maharashtra. In light of the aforementioned, it is necessary to evaluate the physico-chemical characteristics of the soil and to discover and characterize bacteria that can swiftly degrade imidacloprid and be employed in the future for soil decontamination. The present study was undertaken to isolate different microbes from pesticide contaminated agricultural soil from certain region in Saswad, Maharashtra, India as this region has dearth information on IMI biodegradation till date.

MATERIAL AND METHODS:

All the chemicals used for the preparation of Mineral Salt Media (MSM) and Nutrient agar media were purchased from Himedia, India. The Mineral Salt Broth (MSB) and semi solid Mineral Salt media (MSM) were used for selective isolation and purification of the microorganisms from the pesticide contaminated soils.

Certified reference Standard (98.55% purity) of Imidacloprid was procured from DrEhrenstorfer, Germany. Imidacloprid formulation Admire 70 WG (Buyer Crop Science Limited) was purchased from the local pesticide suppliers.

Collection of soil samples:

Soils having history of repetitive application of imidacloprid was collected from the agriculture fields of Saswad, Dist. Pune, Maharashtra. The soil samples were collected at a 15-20 cm depth in a sterile polythene bags (8). A part of collected soils was taken for physico-chemical analysis and the remaining soil samples were mixed, air dried, sieved through 2mm mesh sieve and stored at 4^0C until further use.

Enrichment and Isolation of bacteria:

10 g of collected and processed dried soil sample was taken in a sterile 250 ml conical flask containing 100 ml of Carbon limited sterile Mineral Salt Broth spiked with 100 ppm IMI formulation Admire 70 WG (w/v) as a sole source of carbon/nitrogen. Flask was incubated for 7 days in a rotary shaker maintained at 120 rpm at 35±2^{0}C. After 7 days, isolation of bacteria was done on carbon limited sterile MS agar plates containing 100 ppm of imidacloprid. The pure cultures of all the recovered isolates were maintained on nutrient agar plates and slants.

Screening of potent bacterial isolates for their tolerance at different concentrations of imidacloprid on MSM agar plates: Isolates were grown on MSM agar media containing a range of imidacloprid (100-50,000 ppm) concentrations with and without glucose. Plates were incubated at 35±2^{0}C temperature for 3- 4 days. Isolates showing maximum growth on highest concentration of IMI were selected for further studies.

Study on growth pattern of the acclimatized bacterial isolates: The growth pattern of bacterial isolates which have shown maximum growth at highest concentration of imidacloprid (50,000 ppm) was evaluated in 100 ml of MSM broth containing glucose (0.2g%) and imidacloprid (100 ppm). One ml of each bacterial isolate (1.0 O.D) was inoculated in separate conical flasks containing MSM broth and flasks were incubated in orbital shaker and at static conditions at 35±2^{0}C. The growth of these isolates was monitored at regular intervals by measuring absorbance @ 600 nm using spectrophotometer (Jasco V-630). Among these isolates the better growing isolates were chosen for further studies.

Morphology and biochemical identification: On the basis of growth pattern, the better growing isolates were selected for morphological, cultural and biochemical characterization. Morphology was studied on nutrient agar plates in terms of size, colour, consistency, opacity, and elevation. Microscopic examination was done for Gram staining, size, and shape and motility. Isolates were also characterized on the basis of different biochemical tests including Indole production, Methyl red Test, Voges-Proskauer Test, Citrate utilization Test, Catalase Test, Oxidase Test, Starch hydrolysis Test, Gelatinase Test and Sugar fermentation Test. The results were compared with the characteristics described in Bergey's Manual of Determinative Bacteriology (1994).

Molecular characterization of the isolate:

Overnight grown bacterial cells were processed to obtain pure genomic bacterial DNA. The PCR was performed using the Eppendorf Gradient Master cycler system with a cycle of 94^{0}C for 3 min; 35 cycles of denaturation, annealing and extension at 94^{0}C, 60^{0}C for 30 seconds and 72^{0}C for 90 seconds each

respectively; and final extension at 72^{0}C for 5 min and the mixture was held at 4^{0}C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice using 70% ethanol and dissolved in Tris-HCl (10 mM, pH 8.0). The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the nearly complete gene (9). The sequencing reaction and template preparation were performed and purified in accordance with the directions of the manufacturer (Applied Biosystems; ABI prism BigDye terminator cycle sequencing manual). Samples were run on an ABI3730XL Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the accompanying DNA Sequence Analyzer computer software (Applied Biosystems). The sequence was compared with the EzBioCloud database available at www.ezbiocloud.net/.

Physico-chemical analysis of soil sample:

Before processing, soil was analysed for its different physico-chemical parameters (moisture content, pH, Electrical conductivity, Organic carbon, available nitrogen, available phosphorous, available potassium) by using standard procedures (10, 11, 12).

RESULT AND DISCUSSIONS

Enrichment and Isolation of bacteria: Eleven visually appeared morphologically different microbial isolates were isolated from the agriculture fields having history of repeated use of imidacloprid application located at Saswad, Taluka Purandar, Dist. Pune, Maharashtra. Eleven bacterial isolates retrieved from imidacloprid contaminated soils were considered for further studies and purified on MSM agar plates spiked with same concentration of IMI. Bacterial isolates were designated as VG1, VG2, VG3, VG4, VG5, VG6, VG7, VG8, VG9, VG10 and VG11. Bacterial isolates were maintained on nutrient agar plates, slants and stored in refrigerator. Bacterial cultures were also preserved in 20% glycerol in nutrient broth until further use.

Screening of potent bacterial isolates for their tolerance at different concentrations of imidacloprid on MSM agar plates:

Nine of the eleven isolates were successfully grown up to 1000 ppm imidacloprid concentration, seven isolates grew upto 2000 ppm concentration of IMI whereas five isolates VG3, VG5, VG7, VG10 and VG11 were shown growth at highest imidacloprid concentration (up to 50,000 ppm) in 3-4 days (Table 1; Fig.1). Whereas bacterial isolates VG5, VG7, VG10 and VG11 were shown maximum growth within 24 to 48 hrs at this concentration. Based on the screening results, acclimatized bacterial isolates VG5, VG7, VG10 and VG11 were selected for further experiments. *Xanthomonas*, *Bacillus* and *Microbacterium* sp. tolerated concentration of endosulfan upto 130-172 µg/ml whereas *Achromobacter*, *Pseudomonas* and *Microbacterium* sp. grew well up to 110-150 µg/ml of imidacloprid (13). *Pandoraea* sp. could utilize and degrade up to 10-200 µg/ml of lindane (14). Whereas decrease in the growth of *Pseudomonas* strain was observed at 30 µg/ml and higher concentration of lindane (15).

Study on growth pattern of the acclimatized bacterial isolates: Bacterial isolates showing maximum growth at highest concentration of imidacloprid in 24 to 48 hrs were selected to study their growth pattern in MSM broth containing 0.2 g % glucose and imidacloprid @100 PPM concentration. Broths were monitored at regular intervals by measuring absorbance at 600 nm. Among selected bacterial isolates, VG5 and VG10 were growing better under shaking and static conditions (Fig. 2). Based on biomass, VG5 was selected for further degradation studies and was characterized.

Morphology and biochemical identification: The VG5 strain was evaluated for further study and was identified by using standard morphological, biochemical and physiological tests as per Bergey's Manual of Determinative Bacteriology. Morphological characterization of bacterial isolates (VG5) was performed on the basis of their colony color, surface, shape, margin, consistency, opacity along with elevation on Nutrient Agar medium. Isolate VG5 was found to be of off white colored colony with irregular shape, undulate margin and rough on the surface and sticky consistency beneath. Colony of VG5 was also marked as opaque with flat elevation (Table 2). Gram staining and endospore staining of VG5 showed Gram positive rods and green coloured endospores by malachite green endospore staining.

The biochemical tests indicated in Table 3 were used for the presumptive identification of isolate. The results were also compared with the characteristics described in Bergey's Manual of Determinative Bacteriology (1994).

Molecular characterization of the isolate: PCR amplification and sequencing of the 16S r DNA gene:

The 16S rDNA sequence of VG5 was curate by using EzBioCloud tool. The database is available at (www.ezbiocloud.net/.). The bacterial isolate designated as VG5, showed 100% similarity with ***Cytobacillus firmus*** NBRC15306 in EzBioCloud data base (Accession number-BCUY01000205). The 16S rDNA nucleotide gene sequences of VG5 were deposited in NCBI GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the accession numbers (**MN907480**).

Phylogenetic position of ***Cytobacillus firmus*** strain VG5 and those of related bacterial species of genus found in the Gene Bank database is shown in Fig. 3.

Physico-chemical analysis of soil sample: Pesticides degradation in the soil environment depend upon the physico-chemical properties of soil like, soil organic matter and soil minerals, soil pH, moisture etc. Soil characterization plays important role for dissipation of imidacloprid. Similarly the diversity of microorganisms in composite soils plays an important role for microbial processes and the persistence of pesticides (16). The mean pH (distilled water suspension) of the soil determined was 7.14 was found to conform to the pH used for the degradation of IMI in MSM. The some of the physico-chemical properties of soil sample collected from the agriculture fields from Saswad are listed in Table 4.

DISCUSSIONS

In both urban and rural areas, pesticides are applied broadly across large geographic areas. They endure in the environment for a very long time as well. Pesticide residues of different kinds have been found in drinking water (17). It's crucial to have a timely and effective bioremediation. Thus, the current study's objective is to find native bacteria in soil samples from Saswad for bioremediation of pesticides. Eleven visually appeared morphologically different microbial isolates were isolated from the agriculture fields of Saswad. After screening at different stages, among selected bacterial isolates, VG5 was screened out to be the best growing strain at IMI highest concentration and under shaking and static conditions. Using common morphological, biochemical, and physiological tests in accordance with Bergey's Manual of Determinative Bacteriology, the VG5 strain was assessed for further investigation and identified as ***Cytobacillus firmus*** strain VG5.

Many studies reported earlier isolated pesticide degrading microorganisms from contaminated soil. *Achromobacter* spp. and one as *Diaphorobacter* sp. isolated from pesticide contaminated farmland soil (18). *Acinetobacter radioresistens*, *Pseudomonas frederiksbergensis*, *Bacillus pumilus*, *Serratia liquefaciens*, *Serratia marcescens*, *Burkholderia gladioli* among bacteria and *Aspergillus niger*, *Ganoderma austral*, *Trichosporon* , *Verticillium dahaliae* among fungus were isolated from soil (19). *Kocuria assamensis* isolated from chlorpyrifos and malathion contaminated agricultural soil (20). Enterobacter cloacae isolated from DDT contaminated soil (21). *Pseudomonas*, *Rhodococcus* and *Achromobacter* were isolated from a biopurification system (22). *Hymenobacter latericoloratus* CGMCC 16346 isolated from water environment to remediate imidacloprid contaminated surface water (23).

The main factors influencing the persistence of neonicotinoid insecticides in the environment are soil type, organic matter/organic carbon (OM/OC) composition, pH, sunlight, temperature, and groundwater circulation (24, 25). By competing with pesticide molecules for sorption sites, dissolved organic carbon (OC) can reduce the sorption of neonicotinoids on the surface of the soil (26). In contrast, a few papers stated that soil with high organic matter content has a high imidacloprid sorption rate (27). The soil utilized in this study had a high level of organic matter, which was crucial for IMI absorption. In low organic matter soil, its leaching from soil to ground water may be expected.

There is no information till date about *Cytobacillus firmus* strain VG5 isolates of bacteria that can effectively cure soil polluted with chemicals. Therefore, this research has enormous potential for pesticide bioremediation with this strain.

CONCLUSIONS

The key elements affecting the persistence of neonicotinoids in the environment are the soil type, organic matter/organic carbon (OM/OC) content, pH, sunlight, temperature, and groundwater circulation. Soil samples collected from Saswad region were characterized for their organic carbon and minerals content which helps in understanding the surroundings of microbes and pesticide dissipation. On the basis of screening, among eleven bacterial isolates, isolate VG5 was screened and selected for the imidacloprid degradation studies and was characterized and identified as *Cytobacillus firmus* strain VG5. Further one has to study the potential of this strain to degrade imidacloprid under laboratory conditions in liquid media as well as ex-situ and in-situ in soil.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

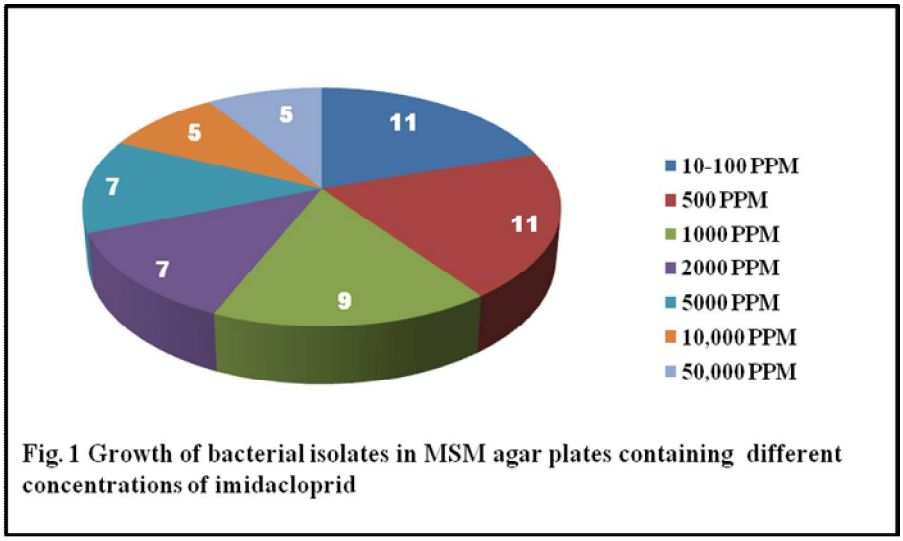

Fig. 1 Growth of bacterial isolates in MSM agar plates containing different concentrations of imidacloprid

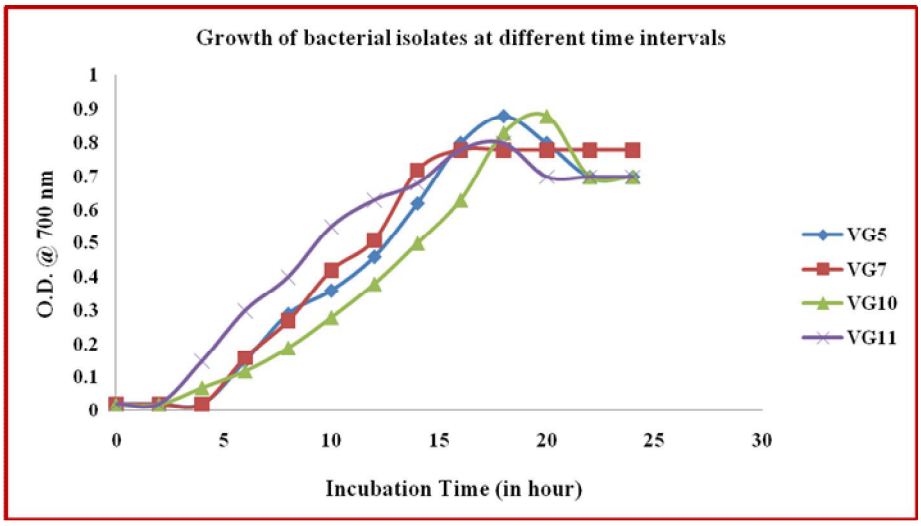

Fig. 2 Growth of bacterial isolates at different time intervals

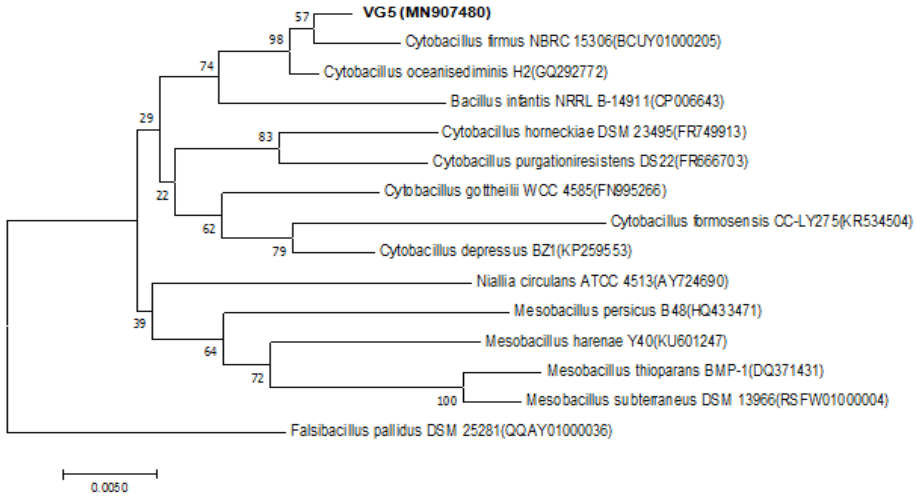

Fig. 3 Neighbor-joining tree derived from 16S rDNA gene sequences of the bacterial *Cytobacillus firmus* strain VG5 (shown in bold) and the closely related strains. Bootstrap values at branch points are expressed as percentage of 1000 replicates. EzBioCloud accession numbers are shown in brackets. The scale bar represents (0.0050) indicates substitutions per nucleotide position

Table 1 Growth of bacterial isolates on MSM agar plates spiked with different concentrations of imidacloprid							
	Different Concentrations of Imidacloprid in ppm						
Isolates	**10-100**	**500**	**1000**	**2000**	**5000**	**10,000**	**50,000**
VG 1	+	+	+	-	-	-	-
VG 2	++	++	++	++	+	-	-
VG 3	++	++	++	++	++	+	+
VG 4	+	+	-	-	-	-	-
VG 5	++	++	++	++	++	++	++
VG 6	+	+	-	-	-	-	-
VG 7	++	++	++	++	++	++	+
VG 8	+	+	+	+	+	-	-
VG 9	+	+	+	-	-	-	-
VG 10	++	++	++	++	++	++	++
VG 11	+	++	++	++	++	+	++
(+) = moderate Growth (++) = Maximum Growth (-) = No growth							

Table 2 Morphological characterization of bacterial isolate	
Colony character	**VG5**
Size (mm)	1-2mm
Shape	Irregular
Color	Off white
Margin	Undulate
Consistency	Rough
Opacity	Opaque
Elevation	Flat
Gram staining	Gram positive rods
Endospore staining	sporulating
Motility	Motile

Table 3 Biochemical Characterization of bacterial isolate VG5	
Characteristics	**VG5**
Biochemical Tests	
Indole	**-**
Methyl red	**-**
Voges-Proskauer	**-**
Citrate utilization	**+**
Catalase	**+**
Oxidase	**-/v**
Starch hydrolysis	**+**
Gelatin hydrolysis	**+**
Urease	**-**
Nitrate reduction	**+/v**
O-F test	
Sugar Fermentation	
Glucose	**+/v**
Fructose	**-**
Lactose	**-**
Maltose	**+**
Mannitol	**-/+**
Sucrose	**-**
No growth= (-), Minimum growth= (+), Moderate growth= (++), Maximum growth= (+++)	

Table 4 Physico-chemical properties of soil sample:		
Parameters	**Optimum level**	**Soil (Saswad)**
Moisture (%)	50- 70	54
pH	6.51- 7.50	7.14
E.C. (dSm^{-1})	<1.00	0.27
Organic carbon (%)	0.41- 0.60	**0.76**
Available nitrogen (Kg / ha)	281- 420	204.28
Available Phosphorous (Kg / ha)	14.01- 21.00	**22.65**
Available Potassium (Kg / ha)	151- 200	**604.48**

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