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Biodegradation of imidacloprid in liquid media by an isolated soil bacteria *Cytobacillus firmus* strain VG5

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ABSTRACT

Imidacloprid (IMI), a neonicotinoid-class synthetic organic insecticide has the potential to have a negative influence on ecosystems and human health, and it has been identified as an emerging pollutant in all parts of the world in recent years. Microbial degradation is an efficient, clean and environmentally acceptable technique over physical and chemical methods for the bioremediation of organic contaminants like IMI. Our aim was to evaluate the ability of soil isolate *Cytobacillus firmus* strain VG5 to degrade IMI in MSM medium with glucose as a co-substrate. In liquid media, degradation was initially confirmed by nitrate reduction test. VG5 reduced IMI into guanidine with the removal of NO_2^- group and showed up to 87% degradation in 7 days. Spots with different Rf values on TLC sheet were identified by HPLC and GC-MS/MS techniques as imidacloprid guanidine and 6-chloronicotinic acid based on respective standard spectra and m/z ratio. Strain VG5 showed potential to reduce pesticide pollution and further studies should be conducted to understand the degradation mechanism of IMI in soil under *in- situ* conditions.

Keywords: *Bioremediation, Neonicotinoids, Imidacloprid, Bacillus sp., Pesticide hazards*

Introduction

Imidacloprid (IMI), 1-[(6-chloro-3-pyridinyl)-methyl]-N-nitro-2- imidazolidinimine is a first-generation commercial neonicotinoid. Neonicotinoid insecticides have specialised effect against an insect's nervous system because they are extremely selective agonists of nicotinic acetylcholine receptors (nAChR). IMI is consumed more frequently all around the world due to its great insecticidal efficacy and low mammalian toxicity (Sabourmoghaddam *et al*., 2015; Liu *et al*., 2002). It is extremely water soluble, leachable, and persistent and harms ecosystem functions (Pietrzak *et al.*, 2019). Environmental hazards grows over time due to the long half-lives of IMI in the environment (e.g., 9–1250 days in soil) and its prolonged exposure to non target organisms (e.g., bees) (Goulson, 2013, Zhu *et al.*, 2019; Strobl *et al*., 2021; Main *et al*., 2021). The field dissipation rates of insecticide are highly variable, and it has been found to degrade slowly in soil, with half-lives longer than 180 days in non-vegetated soil (Anhalt *et al*., 2007; Sharma *et al*., 2014). The rate of imidacloprid dissipation was accelerated by vegetation. Therefore it is imperative to clean up imidacloprid residues from the ecosystem.

The use of biological approaches has replaced physical and chemical methods due to their negative impacts on the environment, soil, surface and groundwater, non-target insects, and human health.

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The microbial dissipation of pesticide is dependent on the bioavailability of pesticides to microorganisms and microbial activity. The earlier findings demonstrated the contribution of microorganisms in pesticide removal in soil. *Leifsonia* sp., *Pseudomonas* sp., *Bacillus* sp., *Ochrobactrum* sp., *Enterobacter* sp., etc. for the degradation of imidacloprid isolated from various types of soil under different crop environment (Anhalt *et al*., 2007; Pandey *et al*., 2009; Sharma *et al*., 2014; Hu *et al*., 2013; Herner *et al*., 2014). Since microbes convert xenobiotics into less toxic forms, many scientists have demonstrated the degradation pathways of microbial degradation of imidacloprid. The qualitative and quantitative analysis of imidacloprid and its metabolic products were reported in the earlier literatures with a wide choice of analytical techniques such as TLC, HPTLC (Phugare *et al*., 2013; Shubair, 2011; Chandegaokar *et al*., 2009; Sherma, 2005; Rezic *et al*., 2005; Ugbeye *et al*., 2003). In the present study bacterial isolate *Cytobacillus firmus* strain VG5 recovered from the pesticide contaminated soils was evaluated for their potential to degrade imidacloprid under *in-vitro* conditions. However, as of this writing, no literature has been discovered describing imidacloprid degradation by *Cytobacillus firmus*. This work was the first to establish the degradation of imidacloprid by the *Cytobacillus firmus* strain VG5.

Materials and Methods

A primary standard solution of 10,000 ppm of imidacloprid (Admire 70 WG) was prepared by adding 0.143g in 10 ml of methanol and solution was sonicated to dissolve. This stock solution was used to prepare 100 ppm working standard of imidacloprid. Analytical standards in the range of 10 PPM to 100 ppm were prepared for UV spectrophotometer calibration. Imidacloprid was detected at wavelength range between 268.8 nm to 270 nm. Calibration curve (linear graph) was drawn to determine the concentration of imidacloprid by linear graph line equation Y= m X + C. The value of X obtained was equivalent to residual imidacloprid concentration.

Biodegradation study of imidacloprid in liquid media: This study was carried out in 250 ml Erlenmeyer flasks containing 100 ml of full strength MSM medium with glucose (0.2 g % w/v) to enhance biodegradation process. The media was amended with imidacloprid @ 100 mg/L concentration and inoculated with 3% of 24 h old culture (OD600 nm = 1.0) of VG5. All the biodegradation experiments were carried out in triplicate. All the flasks were incubated at 30±2 ºC in shaker incubator at 120 rpm. At regular intervals 2 ml of sample withdrawn was centrifuged and syringe filtered through 0.22 µm filter paper. Each filtered aliquot was extracted with ethyl acetate (1:1 v/v). Organic phase was collected and pesticide concentration was determined at 270 nm by spectrophotometer. Formation of metabolites was detected initially by performing TLC and identified by HPLC and GC-MS/MS.

Thin layer chromatography (TLC)

Thin layer chromatography was prepared for the initial detection of the metabolites of imidacloprid from processed broth by referring methodology of Phugare *et al*., 2013; Farouk *et al*., 2013; Chandegaonkar *et al*., 2009 with some modification.

About 10 µl of the recovered samples were spotted on silica coated TLC plate along with control and imidacloprid standard at a distance of 1.5 cm from the bottom edge of plate. The loaded plate was run in a pre saturated TLC chamber with ethyl acetate: n- hexane (6:4) as a mobile phase. TLC plate was removed and spots were dried at room temperature. The spots were examined in UV chamber at 254 nm and 270 nm. Spots were developed by putting the plate in iodine crystal chamber or the spots were sprayed with 5% *p*-dimethylaminobenzaldehyde solution, heated at 100 °C in oven for 10 min and then cooled to room temperature (Chandegaonkar *et al*., 2009).

R.F value was calculated by using formula:

R.F = Distance travelled by the solute / Distance travelled by the Solvent

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) analysis was carried out on Zorbax SB C18 5u (4.6*150 mm) column by using acetonitrile as a solvent system with flow rate of 1.0ml/min and PDA (Photo Diode Array) as a detector (270 nm). Generic acidic- Mobile phase A- Water: Acetonitrile: formic acid (95: 05: 0.1) and Mobile phase B- Water: Acetonitrile: formic acid (10: 90: 0.08) was used as mobile phase for 30 min.

Results and Discussion

Biodegradation study of imidacloprid in liquid media

For biodegradation study, to enhance microbial

growth and to fasten degradation of imidacloprid in media, the liquid media was prepared with 0.2g% of glucose with the addition of 3% inoculums (1.0-$O.D_{600nm}$). In the present study we analysed the degradation of imidacloprid and its different metabolites obtained after degradation by using various analytical techniques UV spectrophotometry, TLC, HPLC and LC-HRMS.

UV spectrophotometry analysis

Conventional spectrophotometry method was selected for the detection of imidacloprid at 270 nm (Guzsvány *et al*., 2009) and 6-chloronicotinic acid at 224 to 230 nm wavelengths. Periodically collected and processed samples were extracted for imidacloprid and its metabolites by using ethyl acetate (1:1 v/v). The extracted solvent phase was dried by evaporation and dissolved in HPLC grade acetonitrile for analytical studies. Imidacloprid concentration was determined by following linear graph line equation Y= m X + C

Isolate VG5 showed 58% degradation for the sample collected on 3rd day, 76% on 5th day and 87% degradation on 7th day. Whereas after 20 days residual remained was 8.4 ppm with degradation up to 91% (Fig.1 and 2 a, b, c). After 5 days, a new peak at 224 nm to 230 nm was noticed in the broth of VG5 (Fig. 2b). The standard peak of 6-chloronicotinic acid and the newly observed peak were matched (Fig. 2a). The findings of study are in parallel with the findings of Phugare *et al.,* 2013 where they found the appearance of new peak of imidacloprid product 6-chloronicotic acid on 7th day by *Klebsiella* sp. The depletion in peak height and appearance of new peak is an indication of removal of imidacloprid from the broth with the formation of product (Fig.2.(b). The findings in the present study were more effective than the findings of Ferreira *et al.,* 2016 where a strain of *Bacillus thuringiensis* isolated from contaminated marine sediments was able to degrade 71% of imidacloprid in 11 days. Phugare *et al*., 2013 reported that the *K. pneumoniae* strain BCH1 was able to degrade 78% of 50 mg l^{-1} imidacloprid in 7 days under static conditions. *Enterobacter* sp. strain ATA1 identified by Sharma *et al*. 2014 could degrade 30-40% of imidacloprid in 72 hrs till 15 days of incubation in MMG medium. But the degradation rate of imidacloprid by our isolates was more effective than the earlier reported strains. The *Aspergillus terreus* YESM3 strain of Mohammed and Badawy, 2017 could degrade 85 % of 25 mg l^{-1} of imidacloprid more rapidly and efficiently in 6 days. Our strains could degrade >80% of 100 mg l^{-1} of imidacloprid in 7 days. Guo *et al*., 2021 reported 64.4 % 100 mgl^{-1} of

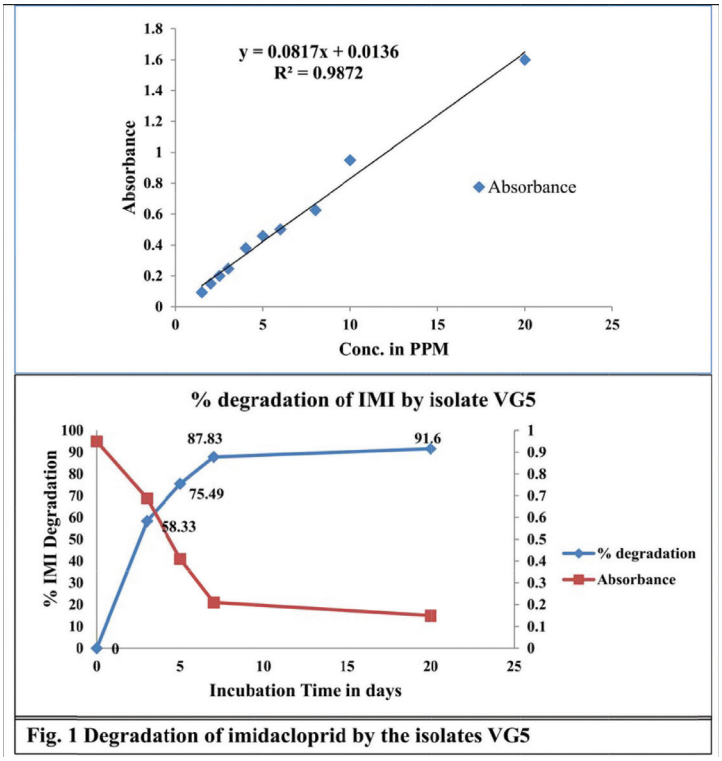


Fig. 1 Degradation of imidacloprid by the isolates VG5

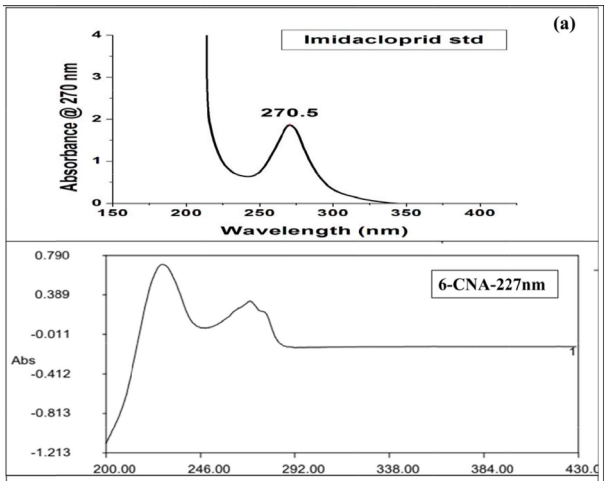


Fig. 2.(a). UV spectra of standards: Imidacloprid and its metabolite

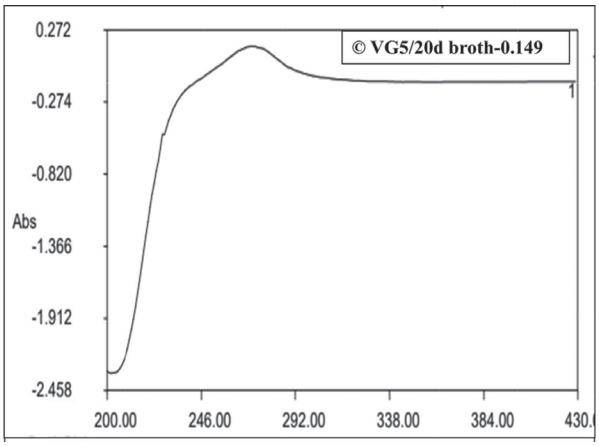


Fig. 2(c). UV spectra of isolate VG5 after 20 days of incubation

imidacloprid degradation in 6 days by the resting cells of *Hymenobacter latericoloratus* GCMCC 16346 co-metabolically in water environment. Similarly Ma *et al*., 2014 noticed that *Pseudoxanthomonas indica* CGMCC 6648 could degrade imidacloprid to 5-hydroxy imidacloprid and olefin in presence of glucose in 96 hours. We could not detect these metabolites by UV spectrophotometry because of absence of standard metabolite solutions. While *Mycobacterium* sp. MK6 strain of Kandil *et al*., 2015 and *Rhodopseudomonas* 6hysic strain of Wu *et al*., 2020 were noticed to be more effective than our strain. However, compared to majority earlier available literature VG5 and VG10 could degrade imidacloprid faster and show more percentage degradation ability.

Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) analysis

For the determination of metabolites, processed VG5 sample was initially spotted on TLC sheets. Three bands with Rf values 0.19, 0.28 and 0.6 were observed under UV chamber confirms the formation of products in the test sample (Farouk *et al.,* 2013). Bands with Rf value 0.19 was matched with the Rf value of control (standard imidacloprid -Rf 0.20). An appearance of band of imidacloprid in test

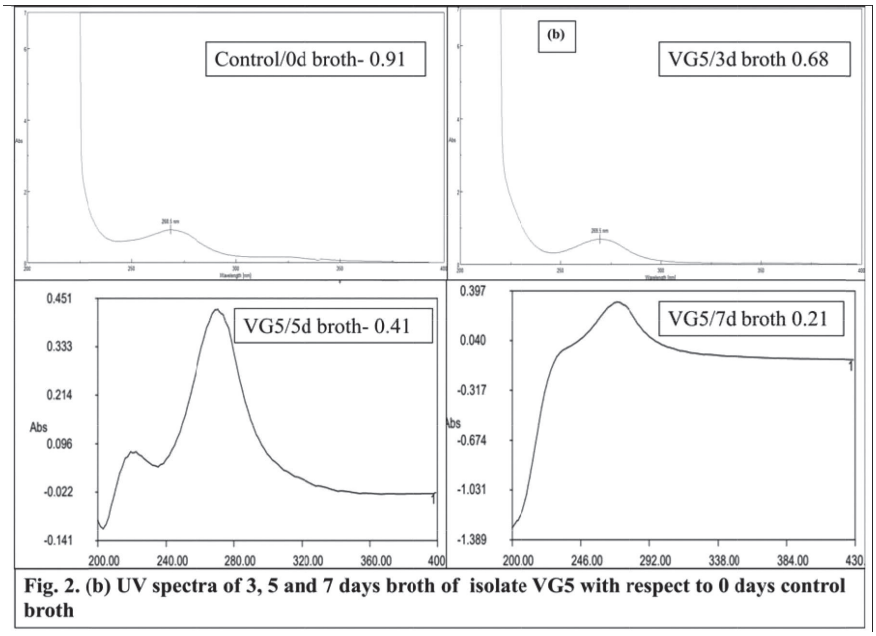
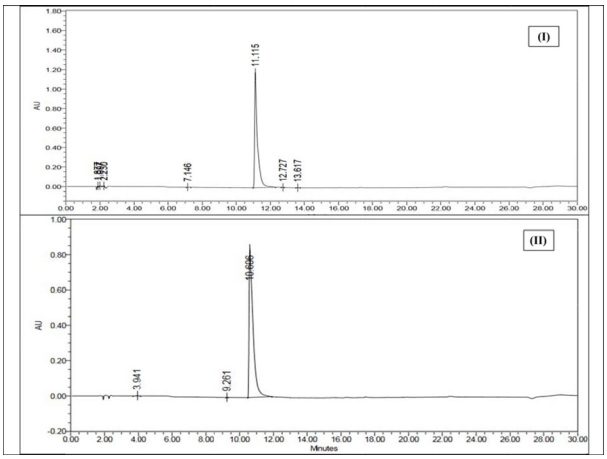


Fig. 2. (b) UV spectra of 3, 5 and 7 days broth of isolate VG5 with respect to 0 days control broth

sample indicates the presence of residual imidacloprid. Chandegaonkar *et al*., 2009 observed imidacloprid spot at RF 0.55 by using chloroform: acetone 7:3 as a mobile phase. In another mobile phase hexane–acetone–ethanol 8:1:1, he observed distinct spot at RF 0.42. Kavrakovski and Rafajlovska, 2015 also worked on the development and validation of TLC method for the determination of chlorophenoxy and benzoic acid herbicides in water. Hamada *et al*., 2012 performed densitometric method by using TLC for the determination and quantification of nine pesticides in water. Rezic *et al*., 2005 also used TLC for the separation of pesticides in honey. The earlier reported work and the results of current study ended with the conclusion that TLC can be used successfully for the separation of pesticides residue and their metabolites.

The developed TLC bands were scratched, collected separately in 2 ml Eppendorf tubes and redissolved in small quantity of HPLC grade methanol (Phugare *et al*., 2013) for the further identification of metabolites by HPLC. The mixtures were centrifuged at 6000 g for 10 min to remove silica gel from the scratched bands and the supernatant was used for HPLC analysis.

HPLC data of scratched TLC band with Rf values 0.28 showed peak at retention time (R.T.) 10.605 min. This was matched with the metabolite standard 6-chloronicotinic acid (6CNA) with R.T- 10.606 min. The peak of band with Rf values 0.19 was comparable with the imidacloprid standard which retention time was 11.11 min. While the Rf values 0.60 showed totally different peak pattern as compared to the other bands (Fig. 3. (V). The current study findings are in sync with the findings of Phugare *et al*., 2013 where two TLC scratched bands were com-



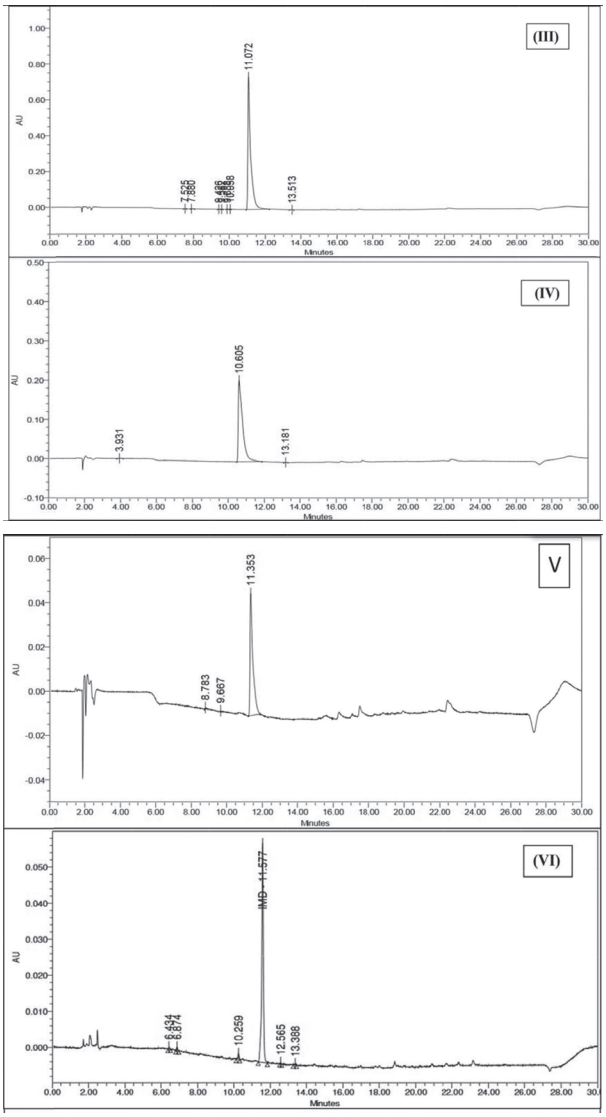


Fig. 3. HPLC analysis of (I) Standard imidacloprid, (II) Standard 6-chloronocotinic acid and bands with different Rf values (III) 0.19 (IV) Rf 0.28 and (V) Rf 0.60 separated through TLC analysis on 7th day (VI) 20 days sample of VG5 strain in Fig.3.

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parable to the Rf values of standards imidacloprid and 6-chloronicotinic acid. The HPLC chromatogram of test sample VG5 showed new peaks with R.T 6.4 min, 6.8 min, 10.25 min and 12.56 min. The new peaks detected in test broths were below the detection limit. This may be that they were produced in a trace amount or may be removed from the treated broth with incubation time. In absence of reference metabolite standards we could not identified such metabolites in our study. None of the peaks matched with reference metabolite 6-

chloronicotinic acid by 20 days treatment. This showed that 6-chloronicotinic acid which was produced in first 7 days of treated broth (Fig. 3) must have further degraded to CO_2 and H_2O and hence not detected in 20 days treated treatments. The findings are matched with the earlier findings of Mohammed and Badawy, 2017 where the fungal strain *A. Terreus* YESM3 was able to produce 6-chloronicotinic acid as one of the major metabolites after 2 days and disappeared after 10 days of incubation. They concluded that this may be due to further biodegradation of 6-chloronicotinic acid by *A. Terreus* YESM3 strain. Hu *et al*., 2013 reported the formation of nitroguanidine, imidacloprid guanidine as IMI metabolites. *Pseudomonas* sp. 1G strain is capable of converting imidacloprid into urea metabolites and denitrification products (Pandey *et al*., 2009). In contrast to our results, Anhalt *et al*., 2007 were not found 6-chloronicotinic acid production during the experiments by PC-21 strain. They observed production of trace amount of NO_3^- / NO_2^- and characterized six metabolites by HPLC and LC-MS, the two metabolites identified were imidacloprid guanidine and imidacloprid urea. Whereas Kandil *et al*., 2015 reported the formation of 6-chloronicotinic acid by *Mycobacterium* sp strain MK6 in less than 2 weeks along with the production of small amount of desnitro- olefin and desnitro degradates of imidacloprid. The earlier reported work and the results of current study ended with the conclusion that TLC can be used successfully for the separation of pesticides residue and their metabolites.

Conclusion

The residues identified by HPLC demonstrated 6CNA as a significant metabolite during imidacloprid's biodegradation. As a result, *Cytobacillus firmus* strain VG5 can be utilised to bioremediate imidacloprid in contaminated water or other environmental samples, thereby minimizing environmental pollution. There is currently no literature available that reports this bacterium for imidacloprid bioremediation.

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Conflict of Interest: Nil

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