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FULL LENGTH ARTICLE

Evidence for the microbial degradation of imidacloprid in soils of Cameron Highlands

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Abstract Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine), with a novel mode of action is a recent systemic and contact insecticide with high activity against a wide range of pests. Continuous dispersion of this pesticide in the environment and its stability in soil results in environmental pollution which demands remediation. The present research was attempted to isolate and characterize imidacloprid degrading bacteria from vegetable farms of Cameron Highlands in Malaysia. The degradation ability of the isolates was tested in minimal salt medium (MSM) for a duration of 25 days and the selected strains were characterized based on their biochemical and molecular characteristics. Levels of imidacloprid in MSM medium were analyzed by high performance liquid chromatography (HPLC). Among 50 soil bacterial isolates *Bacillus* sp., *Brevibacterium* sp., *Pseudomonas putida* F1, *Bacillus subtilis* and *Rhizobium* sp. were able to degrade 25.36–45.48% of the initial amount of imidacloprid at the concentration of 25 mg L^{-1} in C limited media. *Brevibacterium* sp. was isolated from organic farms that had never been exposed to imidacloprid while the other farms had previously been exposed to different levels of imidacloprid. All bacteria introduced in this study were among the first reports of imidacloprid degrading isolates in C limited media from tropical soil. Therefore, the results of this study demonstrate the effectiveness of using soil bacteria for microbial degradation of imidacloprid. These findings suggest that these strains may be promising candidates for bioremediation of imidacloprid-contaminated soils.

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1. Introduction

Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine) is a neonicotinoid insecticide that is commonly applied as a systemic pesticide for controlling many species of sucking insects, termites and other chewing pests (Nauen and Denholm, 2005; Matsuda et al., 2001; CCME (Canadian Council of Ministers of the Environment), 2007; Moriya et al., 1992; Sheets, 2001). Imidacloprid is a polar compound

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with high solubility in water, relatively non-volatile and persistent in soil with a half life of about 156 days (Jeschke and Nauen, 2005). Owing to its physical properties and high insecticidal activity at low application rates, imidacloprid has drawn attention as an efficient pesticide and because imidacloprid is routinely sprayed over fields to protect crops, it is directly released into the environment. The fate of imidacloprid and its products in the environment and in drinking water is of concern to the public. While many in the industry consider imidacloprid to be a pesticide of relatively low toxicity it has been found to be enormously toxic to non-target insects such as bees, and recently has led to resistance in the Colorado potato beetle (Sheets, 2001; Sladan et al., 2012). Studies have shown that imidacloprid has a similar function to that of nicotine, which acts as an agonist on the postsynaptic nicotinic acetylcholine receptors (nAChR) causing paralysis and death in insects (Hassal, 1987; Matsuda, 2007; Matsuda et al., 2001).

In general, degradation of pesticides in soil is facilitated by both biotic and abiotic factors including chemical, sunlight and microbial agents and among these factors biodegradation is the most commonly used method for converting synthetic chemicals into inorganic products (Alexander, 1999; Bassey and Grigson, 2011). Degradation caused by light or water and metabolic process in plants, give rise to several products such as imidacloprid urea, 6-chloronicotinic aldehyde, 6-chloro-N-methylnicotinacidamide and 6-chloronicotinic acid (Krohn and Hellpointner, 2002). Soil studies on the biodegradation of imidacloprid have demonstrated that imidacloprid urea, imidacloprid guanidine and 6-chloronicotinic acid are all possible metabolites for this purpose (Jeschke and Nauen, 2005; Krohn and Hellpointner, 2002). Previous investigations on the microbial degradation of imidacloprid have revealed that relatively few species of bacteria are actually able to degrade this compound. Degradation of imidacloprid by Leifsonia strain PC-21 is the first report on imidacloprid degradation by an isolated microorganism (Anhalt et al., 2007). In this study six metabolites were characterized by high performance liquid chromatography (HPLC) using ^{14}C-methylene-imidacloprid and liquid chromatograph electrospray–mass spectrometer (LC–MS). Two of the metabolites were identified as imidacloprid-guanidine and imidacloprid-urea by HPLC standards and LC–MS.

The vast majority of investigations of pesticide degradation in soil have been conducted under temperate conditions, predominately in Europe and North America. Nevertheless, approximately one-half of earth's population, and roughly one-third of its land mass are located in the tropics. Countries in the tropical zone use pesticides for controlling agricultural and other pests (Racke et al., 1997). The objective of the present study was to isolate and identify bacteria able to degrade imidacloprid from vegetable farms of the Cameron Highlands in Malaysia.

2. Materials and methods

2.1. Chemicals and reagents

Imidacloprid standard was supplied by Sigma Aldrich as a colorless powder with 99.9% purity and 6-chloronicotinic acid ($C_6H_4ClNO_2$) with 99.5% purity, gray-yellow crystalline solid was obtained from Dr. Ehrenstorfer GmbH Co. All the media including Tryptic Soy Broth (TSB) and Nutrient Agar (NA) were obtained from Sigma Aldrich. All solvents were of HPLC grade and purchased from Fisher Scientific. HPLC grade water was produced by a Milli-Q water purification system (Millipore, Milli-Q Advantage A10). HPLC grade water and acetonitrile were passed through a 0.45 μm filter prior to application. Commercial imidacloprid (Confidor®) was provided by the Mardi Research Center (Cameron Highlands, Malaysia). All other chemicals were of HPLC grade. Analytical standards for HPLC calibration in the range of 0.1–100 mg L^{-1} were prepared from aliquots of the working standard solution.

2.2. Media preparation

TSB and NA media were prepared using distilled water according to the manufacturer's instructions (Sigma–Aldrich, USA) and were autoclaved at 121 °C for 15 min to ensure that the solution was sterilized. After cooling, an appropriate amount of imidacloprid was added using a syringe filter and dispensed into petri dishes. Mineral salt medium (MSM) was prepared according to the Kaufman and Kearney recipe with some modifications (Kaufman and Kearney, 1965). The basal mineral salt medium used in this study contained 2.5 g L^{-1} KH_2PO_4, 2 g L^{-1} K_2HPO_4, 1.0 g L^{-1} NaCl, 0.25 g L^{-1} $MgSO_4$, and 1.5 g L^{-1} $(NH_4)_2HPO_4$ and some trace elements such as 0.001 g L^{-1} $FeSO_4{\cdot}7H_2O$, 0.07 g L^{-1} $CoCl_2{\cdot}6H_2O$, 0.04 g L^{-1} $Na_2MoO_4{\cdot}2H_2O$ and 0.1 g L^{-1} $CaCl_2{\cdot}2H_2O$ were added. The medium pH was adjusted to 6.5 using HCl. Finally using the syringe filter (0.45 μm), 1 g glucose and sucrose were also added as a sole source of carbon.

2.3. Instruments

HPLC analysis for imidacloprid was performed using an Agilent 1200 series system equipped with a diode array detector (DAD) and ChemStation software was used for data acquisition and processing. All samples were separated using a reversed-phase Zorbax Eclipse® C_{18} analytical column (150 × 4.6 mm i.d.; 5 μm particle size) from IT Tech (Malaysia). The column was operated isocratically at room temperature at a flow rate of 1 ml min^{-1} using 60:40 (v/v) Milli-Q water/acetonitrile. A UV–VIS detector operating at 270 nm was also used. Calibration was done using external standards any time a sample was analyzed and a linear regression analysis was used for quantification. Identification of imidacloprid and 6-chloronicotinic acid was based on their retention time. Optical density (OD) of the bacteria was measured by a Spectrophotometer (Cary 50Bio) at 600 nm wave length.

2.4. Soil sample collection

Soil samples were collected from vegetable farming areas of the Cameron Highlands in Malaysia, from locations with a history of applying neonicotinoid pesticide. The majority of the farms selected from this area had been under cultivation of cabbage, cauliflower and other vegetables for several years. Soil with no background of any pesticide concentration was also collected from an organic farm as a sample. The samples were randomly collected from the top 15–25 cm depth following the standard procedure and stored in plastic bags at ambient temperature (Carter and Gregorich, 2006). A part of each sieved soil sample

(through a 2 mm sieve) was taken for residual analysis using the liquid extraction method (Sabourmoghaddam et al., 2012). The remaining soil samples were used for bacterial isolation. Soil samples used in the experiments were sandy loam with an average pH of 6.23 in water. Properties of the soil samples are listed in Table 1.

2.5. Isolation of bacteria for biodegradation studies

Bacteria were isolated from the soil samples using serial dilution on different media plates including NA, MSM and TSB and incubated for two days at 28 °C. After two days, the plates were screened for colonies that appeared visually different from others. The colonies were randomly selected and re-cultured. The plates were incubated for 24–48 h at 28 °C (Cappuccino and Sherman, 2008). After multiplication, biodegradation ability of each bacterium was tested.

All biodegradation assays were carried out using sterilized 50 mL falcon tubes. The tubes were filled with 30 mL of MSM and spiked with imidacloprid (at the concentration of 25 mg L^{-1}) to serve as the sole source of carbon in a carbon-limited medium and the sole source of nitrogen in a nitrogen-limited medium. One positive control containing full MSM and two negative controls without any carbon and nitrogen sources were prepared to follow the bacterial growth. Control tubes containing media and pesticide (without any bacteria) were prepared to eliminate environmental effects such as photodegradation. The falcon tubes containing 10^5 bacterial cells per ml were incubated in the dark at 28 °C on a rotary shaker at 120 rpm and then monitored for four weeks. The sub-samples from each treatment were removed on days 15 and 25 for pesticide residual analysis and optical density (OD) was measured using a spectrophotometer (Cary 50Bio). In order to determine the concentration of each treatment, 0.5 mL of each sub-sample was removed and mixed with 0.5 mL of acetonitrile in 2 mL Eppendorf tubes followed by centrifuging at 12,000 rpm for 5 min. The supernatant was transferred to the amber HPLC vials using Pasteur pipettes and kept in a refrigerator. Twenty-five microliter of each sample was injected into the HPLC. Concentrations of bacteria in suspension were estimated by light absorbance values at OD_{600}.

2.6. Statistical analysis

Data obtained from HPLC for different concentrations of imidacloprid for each treatment were subjected to a one-way analysis of variance (ANOVA). Determinations of significant difference between means were made by Duncan's test ($p < 0.05$) using the Statistical Package for Social Sciences (SPSS) program version 17. Controls were used only to monitor the growth of isolates and were not included in data analysis.

2.7. Molecular characterization

The bacterial isolates which were capable of utilizing imidacloprid were identified using 16S ribosomal DNA. DNA extractions were performed by applying the boiling method cited in Medici et al. (2003) with some amendments. The 16S rRNA gene was amplified by using the following primers (Baker et al., 2003). Forward: 5′-AGA GTT TGA TCC TGG CTC AG-3′ Reverse: 5′-GGT TAC CTT GTT ACG ACT T-3′.

The PCR reaction was performed in a final volume of 50 μL containing 25 μL Taq Master Mix (produced by Vivantis, Malaysia), 1 μL of each primer and 2 μL of bacterial DNA template. The final volume was adjusted to 50 μL using nuclease-free water. DNA amplification was performed in a thermo cycler (BioRad, USA) with the following thermal profile: an initial denaturation step of 94 °C for 3 min (1 cycle), followed by 35 cycles of 94 °C for 1 min, 41 °C for 1 min, and 72 °C for 2 min with the final extension step of 75 °C for 5 min (Yeates et al., 1998). The amplified DNA was analyzed by electrophoresis on 1.5% agarose (5 μL aliquot of each PCR product) and stained with ethidium bromide. PCR products were sent to the NHK Company (NHK Bio Science Solutions SDN. BHD) for purification and DNA sequencing.

3. Results and discussion

3.1. HPLC calibration

Retention times for 6-chloronicotinic acid and imidacloprid under experimental conditions were 1.3 and 2.7 min, respectively (Fig. 1). HPLC was calibrated by working standard solutions of imidacloprid and 6-chloronicotinic acid, prior to sample analyses, at levels of 1–100 mg L^{-1}. A linear relationship between the injected amounts of standard solutions and the resulting peak area was observed with a correlation coefficient of 0.999 for both imidacloprid and 6-chloronicotinic acid.

3.2. Imidacloprid degradation

Biodegradation of imidacloprid using different isolates of bacteria was studied over 25 days of incubation. A one-way ANOVA was used to test differences between 50 independent isolates of Cameron Highlands for C limited treatments. The means of concentrations for both days (days 15 and 25) were determined as being significantly different at the confidence

Table 1 Selected characteristics of the soil samples used in this study.

Soils	pH in Water	% Organic carbon	Moisture content	% Clay	% Sand	% Silt	Residue of imidacloprid (mg L^{-1})
1	6.4	3.17	20.32	13.68	62.77	23.52	2.83
2	5.8	2.82	20.48	12.22	65.19	22.48	2.07
3	6.3	3.14	21.06	14.20	62.03	23.74	1.73
4	5.8	2.9	20.14	12.14	64.87	22.84	0.35
5	6.3	3.16	21.5	17.99	64.76	17.02	2.21
Organic farm	6.8	3.2	22.73	17.06	60.26	22.52	ND

ND: not detected.

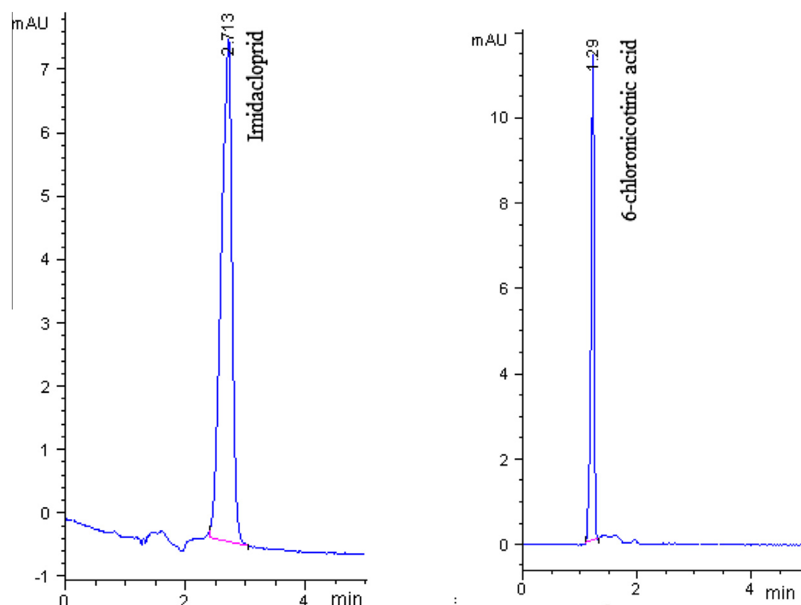

Figure 1 Separation of the 1 mg L-1 imidacloprid (2.7 min) and 6-chloronicotinic acid (1.29 min) on a C18 column, at a flow rate of 1 mL min^{-1}.

level of 95%. The null hypothesis (means are equally the same) was rejected due to low *p* value and high *F* ratio (both *p* values were less than 0.05). According to this analysis, between group and within group variance was less on day 15 and increased on day 25. A post hoc test was used in conjunction with ANOVA to determine which specific group of pair(s) was statistically different from the others.

According to the Duncan test for day 15, the isolates were placed in 8 different groups while 5 independent groups were classified for day 25. Seven different isolates of bacteria (Nos. 45, 3, 25, 32, 8, 17 and 47) were placed in the first three groups on day 15 and five bacterial isolates (Nos. 45, 3, 32, 25 and 17) were among the first three groups for day 25. Final selections of biodegradative isolates were made according to the Duncan test for day 25 due to low concentrations of treatments.

The results showed that five isolates were able to degrade imidacloprid at the concentration of 25 mg L^{-1} in C limited media. Biodegradation of imidacloprid in spiked media varied substantially among the strains of bacteria (Table 2). During the first two weeks of incubation, biodegradation of imidacloprid was slow; it was between 9.8 and 22.88% on day 15; however, it accelerated later, so that on day 25 of incubation it reached between 25.36% and 45.48% of the spiked amount.

A significant correlation was confirmed between growth of isolate and day, post inoculation (day 15 and day 25) by Pearson Correlation, SPSS. The confidence level for the correlation between growth of bacteria and concentration of imidacloprid on day 15 was 95% while this value was 99% for day 25. SPSS analysis confirmed a strong relationship between populations of bacteria with degradation ability.

Growth rates for the five different biodegradative isolates during 25 days of incubation are presented in Fig. 2. The results demonstrate that the maximum growth was achieved on day 10 for all isolates with a higher growth recorded for isolate number 32. The measured OD for isolate 32 was higher than that of other isolates during the incubation period, but its efficiency in biodegradation of the pesticide was less than that of other two isolates. It could only degrade 32.4% of the initial amount of imidacloprid. The performance of isolates 45 and 3 in terms of imidacloprid biodegradation was better than that of other isolates while the growth rate of these isolates was slower than that of others (Fig. 3).

Concentration of 6-cloronicotinic acid for strain 45 on day 15 was 2.2 mg L^{-1} while at the final record on day 25 it increased to 5.8 mg L^{-1}. During the experiment some peaks become well separated but the existing peaks could not be exposed due to lack of middle metabolite standards. On day 15, a peak with considerable area with retention time of 3.2 was detected; however, it did not remain the same in the final reading. Concentration of 6-chloronicotinic acid for isolates 3, 32, 25 and 17 on the final record was 3.5, 2.6, 4.3 and 3 mg L^{-1}, respectively.

Table 2 Ability of different isolates in biodegradation of imidacloprid (25 mg L^{-1}).

Bacterial code	Concentration of imidacloprid in selected soils (mg kg-1)	Av. concentration (day 15)	Av. concentration (day 25)	Biodegradation ability (day 25) (%)
45	2.83	19.28	13.63	45.48
3	0.35	19.92	15.70	37.20
32	1.73	21.39	16.90	32.40
25	Organic farm	20.32	17.53	29.88
17	2.83	22.55	18.66	25.36

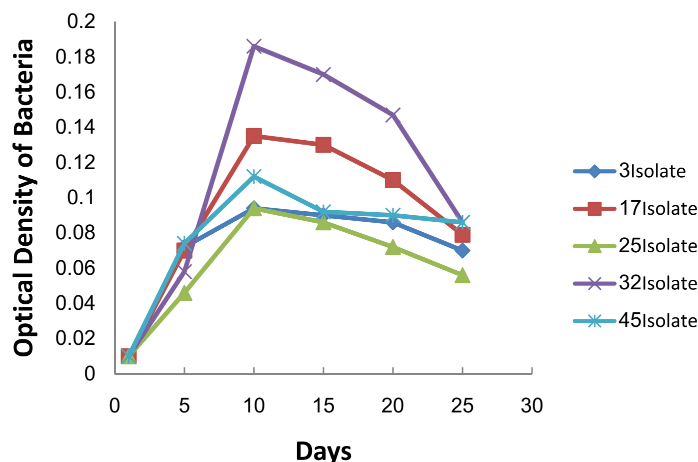

Figure 2 Growth of different biodegradative isolates during 25 days.

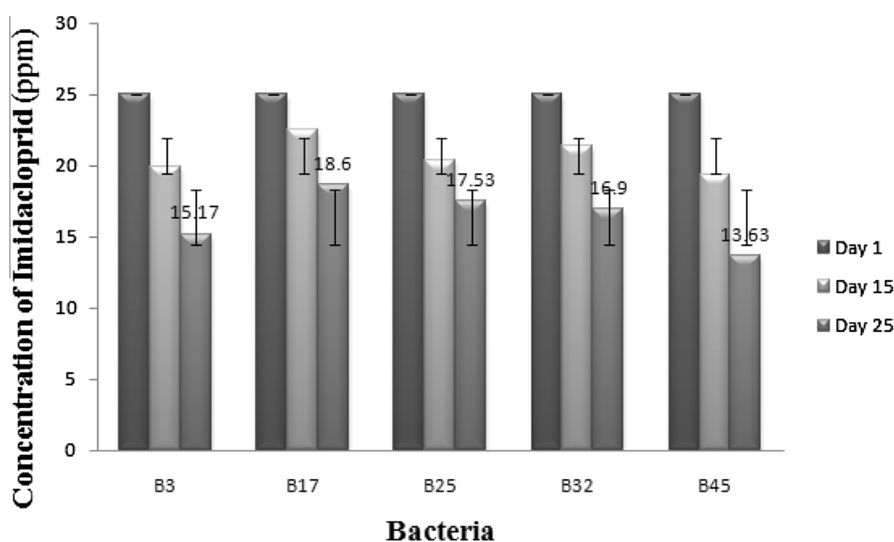

Figure 3 Effect of different bacteria on imidacloprid concentration (C Limited Media).

According to Anhalt et al. (2007), some metabolites of imidacloprid such as imidacloprid-guanidine and imidacloprid-urea and some unknown metabolites during biodegradation period of imidacloprid by *Leifsonia* sp. could be detected using HPLC-MS, but unfortunately it was not possible to detect the metabolites using HPLC-DAD without appropriate standards of imidacloprid metabolites.

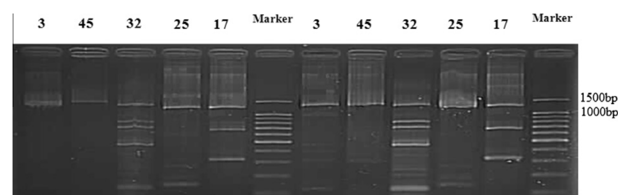

Figure 4 Detection of 1450 bp amplicon in isolates with replication.

3.3. Identification of imidacloprid degrading isolates

Molecular identification of the selected isolates was performed using 16S rRNA gene sequence analysis. The 16S rRNA sequence of the isolates was compared with the related sequences of other microorganisms obtained from GenBank (http://www.nbci.nlm.nih.gov) as a result of ntBlast analysis.

Results from PCR indicated replicatively the presence of a 1450 bp band for all the isolates (Fig. 4). This band was used for sequencing 16S rRNA gene (Table 3) (Shen et al., 2008). The near complete 16S rRNA region of strain 45 exhibited 87–88% identity to the 16S rRNA of two strains of *Rhizobium* sp. in Genbank (accessions GU591754.1 and GQ483459.1). It was, therefore, classified as *Rhizobium* sp. species.

Analysis of 16S rRNA sequence for isolate No. 3 indicated that it was related to *Bacillus subtilis* with 98% similarity (accession GU391355.1). Also, strain No. 17 was identified as *Bacillus* sp. (GQ406788.1) with 97% similarity.

The coverage of nucleotides between strain 32 and *Pseudomonas putida* F1 (accession CP000712.1) was 99%, and isolate No. 25 was referred to as *Brevibacterium* sp. (accession

Table 3 Results of blast.

Bacterial code	Name of isolates using blast	Query coverage (%)	Max ident (%)
45	*Rhizobium* sp.	100	88
3	*Bacillus subtilis*	89	98
32	*Pseudomonas putida* F1	99	97
25	*Brevibacterium* sp.	97	99
17	*Bacillus* sp.	94	97

Table 4 Results of biochemical tests for selected strains.

Bacteria code	Gram staining	Oxidation fermentation	
		Tube without Vaseline	Tube with vaseline
45	Negative	+	–
3	Positive	+	+
32	Negative	+	–
25	Positive	+	–
17	Positive	+	–

GQ199716.1) with 99% similarity. Morphological observation of strains and biochemical tests all supported the results from the 16S rRNA sequences (Table 4).

Biodegradation of imidacloprid by various species of bacteria was reported earlier (Anhalt et al., 2007; Pandey et al., 2009). Phugare et al. (2013) isolated the bacterium *Klebsiella pneumonia* strain BHC1 from the pesticide contaminated agricultural soil. The bacterium was capable of degrading imidacloprid up to 78% within 7 days at 30 °C. The metabolites were identified as nitrosoguanidine, imidacloprid-guanidine and 6-chloronicotinic acid by gas chromatography and mass spectroscopy. Similar to the present work, Madhuban et al. (2011) have studied the biodegradation of imidacloprid, and an aerobic bacterium capable of degrading imidacloprid was isolated from an agricultural field soil by enrichment culture. *Burkholderia cepacia* (strain CH9) was able to degrade imidacloprid and in a mineral-salt medium supplemented with 50 $\mu g\,mL^{-1}$ of imidacloprid resulted in 69% degradation of imidacloprid within 20 days.

Our results also are in agreement with Sundaram et al. (2013) who reported the degradation of cypermethrin with *Bacillus* sp. on soil samples. Despite our results their study indicated complete mineralization of cypermethrin by *Bacillus* sp. within 30 days. Zhang et al. (2014) reported the degradation of triazine herbicide metribuzin by enrichment culturing of soil contaminated with metribuzin. With an initial concentration of 20 $mg\,L^{-1}$, the degradation rate by *Bacillus* sp. was 73.5% in 120 h. They reported that in higher concentrations, the biodegradation rates decreased. Their results indicate that this strain of *Bacillus* sp. can significantly increase the degradation rate of metribuzin in contaminated soil.

4. Conclusions

This study showed that some species of bacteria had the potential for degradation of imidacloprid. Over 25–45% of imidacloprid was lost on day 25 by the selected soil bacterial isolates. Although *Leifsonia* sp. was previously introduced by Anhalt et al. (2007) as a biodegradation agent for imidacloprid in N limited media all the bacteria introduced in this study were the first reports of imidacloprid degrading isolates in C limited media from tropical soil samples. These transformations of imidacloprid by bacteria present new possibilities for chemical degradation in soil. Further studies are required for the evaluation of exact mechanism of imidacloprid biodegradation by these isolates. Additional experiments should be designed to determine optimal pH, temperature and concentration to increase biodegradation ability of selected bacteria.

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