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Imidacloprid degradation by potential soil bacteria isolated from rice fields in Grobogan, Central Java, Indonesia

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Abstract. *Hermawan MA, Pangastuti A, Setyaningsih R. 2024. Imidacloprid degradation by potential soil bacteria isolated from rice fields in Grobogan, Central Java, Indonesia. Nusantara Bioscience 16: 284-291.* Imidacloprid a widely used pesticide is known for its polar nature, resistance to evaporation, and persistence in soil. When concentrations exceed environmental thresholds, imidacloprid can act as a pollutant, disrupting ecosystems, altering soil pH, and decreasing soil fertility. This study aimed to isolate and identify soil bacteria from rice fields capable of degrading imidacloprid and to highlight their potential role in bioremediation. Isolated bacteria are identified based on morphological characteristics, their ability to degrade imidacloprid and through molecular tests using 16S rRNA. Four bacterial colonies were obtained from the isolation results with different morphological variations. The degradation test results showed that the isolates were able to grow in media containing imidacloprid and were able to reduce imidacloprid by 26.66-31.75%. Based on 16S rRNA gene analysis, isolate IT1 was identified as *Enterobacterales*, IT2 was identified as the Enterobacteriaceae, IT3 as *Pectobacterium aroidearum* strain CCRMPA670, and IT4 was identified as *Bacillus thuringiensis* strain FDAARGOS_791.

Keywords: *Bacillus thuringiensis*, Bacteria, biodegradation, imidacloprid, *Pectobacterium aroidearum*, rice field

INTRODUCTION

The effective use of pesticides in controlling pests is a short-term solution. Pesticides create a dependency among farmers to consistently use them as a determinant factor for high yields and quality agricultural products (Putri et al. 2021). Imidacloprid is a type of pesticide that is widely used and its effect lasts about 156 days (Zamule et al. 2021). Previous studies have reported varying half-life values for imidacloprid in different soil types, namely 455-518 days on sandy clay soils in Australia and 233-366 days on muddy clay soil in India (Bhattacherjee et al. 2020).

Imidacloprid exceeding the environmental threshold becomes a pollutant and can disrupt the natural balance. Its uncontrolled use of imidacloprid can lead to various problems (Erguven and Demirci 2021). Approximately 20% of imidacloprid pesticides hit the target, while the remaining 80% falls into the soil, causing soil acidification and reducing soil fertility (Sabourmoghaddam et al. 2015). Continuous use of imidacloprid leads to environmental accumulation, resulting in soil and water pollution, potentially accumulating in the food chain (Bhattacherjee et al. 2020). Its residues in the soil affect the decline in the diversity of soil fauna (Bandeira et al. 2020). This occurs because imidacloprid can influence the growth and reproduction of soil fauna, reducing the quantity and variety of existing fauna. Additionally, imidacloprid can disrupt interactions among soil fauna, disturbing the balance of the soil ecosystem. The low quantity of soil fauna will reduce their contribution to soil quality and productivity.

Grobogan District has a rice harvest area of 179,124 hectares, with the highest rice production in Central Java, Indonesia, with a production of 787,275 tons-GKG in 2022. Grobogan District is indeed the largest among other districts in Central Java, so it has become an essential factor in Indonesia's rice production. Considering that it is the largest rice harvest area in Central Java, it is essential to maintain the soil condition of the area so as not to experience an increase in soil acidity due to the residue of the imidacloprid pesticide which in terms affects the rice crop productivity level. Imidacloprid can cause the accumulation of pesticide residues in the soil, potentially killing the diversity of soil fauna, increasing plant pest resistance and reducing soil fertility (Bandeira et al. 2020).

Imidacloprid can undergo natural environmental processes, including hydrolysis, photodegradation and biodegradation. Biodegradation is a promising process for reducing residues due to its relatively easy, selective, effective, safe, and cost-efficient operation (Hu et al. 2013). Cycoń and Seget (2015) demonstrated that various bacterial isolates can degrade imidacloprid residues as the sole carbon or nitrogen source or through metabolic transformation. Bhattacherjee et al. (2020) reported that *Burkholderia cepacia* from agricultural land can degrade 50 µg/mL of imidacloprid by 69% within 20 days. Gupta et al. (2016) used *Pseudomonas* sp. RPT 52 with a 0.5 mM imidacloprid solution, achieving approximately 46.5% degradation within 40 hours.

This study aimed to isolate and identify soil bacteria from rice fields capable of degrading imidacloprid and to highlight their potential role in bioremediation. The urgency of research is getting bacterial isolates that have

a high ability to grow in rice fields that are applied by imidacloprid and the ability to degrade the residue of the imidacloprid pesticide and know the level of efficiency of the isolates selected to re-feminist the imidacloprid residue in the soil.

MATERIALS AND METHODS

Study area

The sampling was conducted in the Godong, Wirosari, and Ngaringan Sub-districts of Grobogan, Central Java, Indonesia (Figure 1). Grobogan is located at an altitude of 100-500 meters above sea level with coordinates 7° 1' 18.188" S 110° 57' 45.306" E. The land in Grobogan is mostly used for the agricultural sector, such as rice fields and plantations.

Sample collection

The samples used were collected from the Grobogan region, consisting of soil exposed to imidacloprid pesticides based on a long history of using the pest-repellent pesticide for brown planthopper, namely Avidor 25 WP brand (imidacloprid 25%). The sampling locations were at three points in the Sub-districts of Godong, Wirosari, and Ngaringan, with soil samples taken from the central area due to the likelihood of containing a significant amount of pesticide residues and being the main rice cultivation area. Using a scoop, 500 grams of soil samples were taken from the top layer of soil (depth of 0–15 cm) (Gautam and Dubey 2022). The collected soil was then placed in an ice box (filled with ice bags to maintain a temperature of ±4°C) to preserve the soil conditions (Alwi et al. 2023).

Isolation and purification of potential pesticide imidacloprid degrading bacterial isolates

The soil was dried and ground with a mortar and then sieved through a 0.2 mM mesh to remove physical impurities. Each soil sample was weighed and 5 grams were taken using an analytical balance. Vortex was used to homogenize the soil samples after they were put in bottles with 45 mL of distilled water. Subsequently, centrifugation was carried out at 10,000 x g for 20 minutes (Irfan et al. 2021), referred to as a 10^{-1} dilution. One millilitre of the liquid was pipetted from the 10^{-1} dilution and added to a reaction tube holding nine millilitres of distilled water to make a 10^{-2} dilution. This process was repeated sequentially up to a 10^{-7} dilution using the serial dilution technique. Serial dilution was performed to reduce the density of microorganisms in the soil samples, facilitating the isolation of purer bacterial colonies on culture media (Bhattacherjee et al. 2020).

All the dilutions (10^{-1} to 10^{-7}) were spread using the pour plate method on 15 mL of MSM agar media supplemented with 2 ppm of imidacloprid as the sole carbon source in petri dishes. The minimal salts medium (MSM, g/L) consisted of K_2HPO_4 2.27 g, KH_2PO_4 0.95 g, and $(NH_4)_2SO_4$ 0.67 g per 1 L of deionized water, adjusted to pH 7.0 (Coleman 2002). The inoculated plates were then incubated for 48 hours at 28°C (Yadav et al. 2021). Bacteria obtained from the mixed culture were purified using the quadrant streaking method with four streaks until no other bacterial mixtures were present. Pure isolates were also inoculated into glycerol stocks and stored in the freezer.

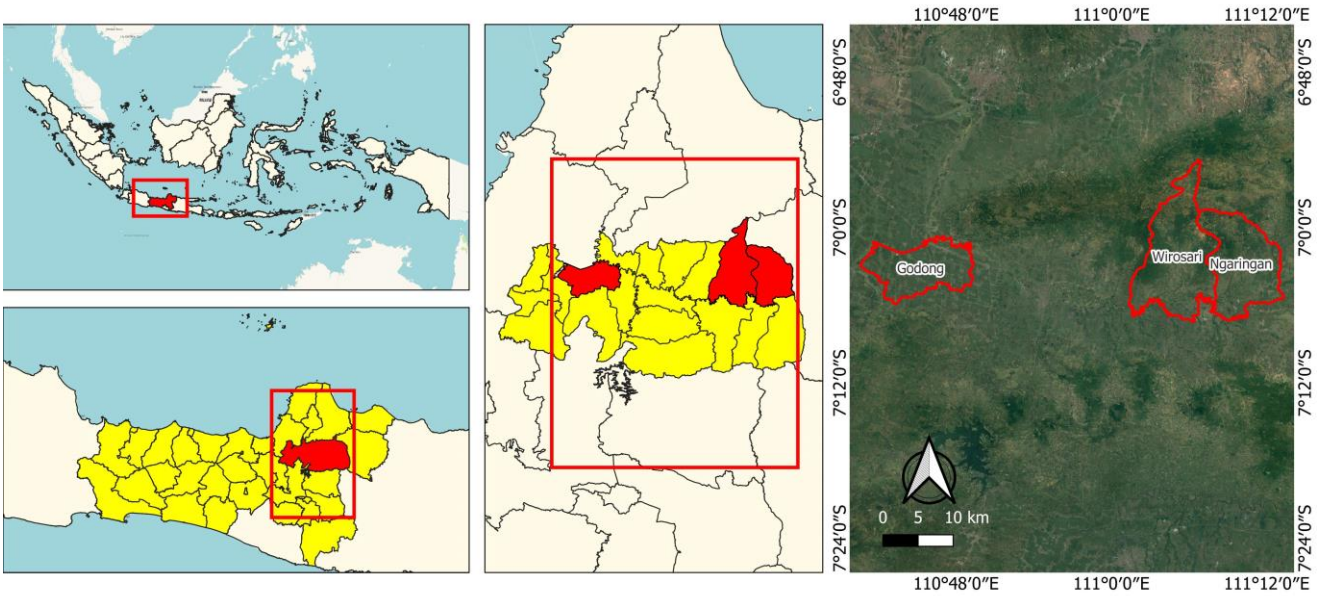

Figure 1. Locations of soil sampling sites: Wirosari, Godong, and Ngaringan Sub-districts of Grobogan, Central Java, Indonesia (7° 1' 18.188" S 110° 57'45.306" E)

Growth of bacterial isolates

Bacterial isolates were cultured in erlenmeyer flasks containing 250 mL of Minimal Salts Medium (MSM) supplemented with two ppm imidacloprid. Subsequently, the cultures were placed on a shaker incubator at 28°C and 150 rpm for 24 hours, allowing them to reach the exponential growth phase. A 10 mL sample was extracted and centrifuged at 8000 x g for 15 minutes, the supernatant was then discarded and replaced with 10 mL of sterile distilled water, followed by vortex. The Optical Density (OD) values were measured using a UV-Vis spectrophotometer at a wavelength of 600 nm (Mishra et al. 2014). Absorbance values were recorded every three hours until the bacterial culture entered the stationary phase.

Imidacloprid pesticide degradation test

The isolates cultured were transferred in a volume of 10 mL into a reaction tube containing 250 mL of liquid MSM with two ppm imidacloprid. The reaction tube was incubated at 170 rpm and 28°C. Samples were detected and measured on days 0, 3, 5, 7, 10, 12, 14, 17, 19, and 21. Pesticide imidacloprid degradation was assessed using High-Performance Liquid Chromatography (HPLC) (Hu et al. 2013).

Identification of selected bacterial isolates

The bacterial DNA genome from the isolate was extracted using the Quick-DNA™ Kit from Zymo Research, following the manufacturer's protocol. DNA obtained was then used as a template for the Amplification of DNA 16S rRNA. Amplification using a pair of 67F (5′-CCTACGGGNGGCWGCAG-3′) and Primer 1387R (5′-ACTACHV GGGTATCTAATCC-3′) and for sequencing using a primary 785F (5′-GGATTAGATACCCTGGTA-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) to target the partial region of the 16S rRNA gene. PCR reactions were performed in a thermocycler with 7.5 µL My Taq Red Mix buffer (2x), 0.5 µL forward primer, 0.5 µL reverse primer, 0.5 µL DNA template, and 19.8 µL ddH_2O. The denaturation, amplification, and annealing processes each had 30 cycles. The cycle parameters were as follows: initial primer denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 45 seconds, annealing at 62°C for 45 seconds, extension at 72°C for 2 minutes, and a final extension at 72 °C for 72 minutes (McCabe et al. 1999). The PCR products were then kept at 4°C for subsequent analysis by electrophoresis. The amplified products were electrophoresed on a 1% agarose gel. Electrophoresis was carried out for 45 minutes at an electric voltage of 84 V in 1x TAE buffer. The DNA amplicons in the agarose gel were stained with gel red dye. DNA visualization was performed using a UV transilluminator (Gao et al. 2021). The amplified DNA was then subjected to DNA sequencing. The 16S rDNA amplicon was subsequently sent to a third party for sequencing processing.

Data analysis

The growth curve of imidacloprid degrading bacteria was constructed, and the findings were obtained by incubating the bacteria with a UV-Vis spectrophotometer to measure Optical Density (OD) at 600 nm. The bacterial growth curve was generated based on the OD values obtained over time. Following the construction of the bacterial growth curve, a test for bacterial resistance to imidacloprid was conducted by observing the OD values at 600 nm under various concentrations of imidacloprid. Subsequently, a test of imidacloprid pesticide degradation by bacteria was performed using High-Performance Liquid Chromatography (HPLC). The data obtained consisted of residual imidacloprid concentrations after incubation with bacteria. The acquired data were then analyzed descriptively and qualitatively. The sequences obtained from the sequencing process underwent a similarity test using BLASTn features on NCBI, utilizing the 'nr/nt' or 'Bacteria' database.

RESULTS AND DISCUSSION

Imidacloprid residue

The soil sample from Wirosari exhibited the highest residue levels compared to the soil samples from Godong and Ngaringan (Table 1). According to the Indonesian Minister of Health and Minister of Agriculture Decree No. 881/MENKES/SKB/VIII/1996 and No. 711/Kpts/TP.270/8/1996 Regarding the Maximum Residue Limits of Pesticides in Agricultural Products, the maximum residue limit for imidacloprid in soil should be 0.5 ppm. The soil in Ngaringan and Godong has imidacloprid residues approaching the maximum allowable limit, while the Wirosari soil exceeds the imidacloprid residue limit permitted by the Department of Agriculture.

Isolation of indigenous bacteria

The isolates capable of growing on MSM + imidacloprid media were coded as IT1, IT2, IT3, and IT4. Color dissimilarity was the most noticeable aspect of isolates characterization. According to Table 2 and Figure 2, The color of isolates IT1, IT2, IT3, and IT4 were light pink color, brick-red color, whitish-yellow color, and white color, respectively.

Bacterial growth

All bacterial isolates experienced an exponential growth phase from hour 3 to 51, except for isolate IT1, which that it ended its exponential phase at hour 54 (Figure 3). The bacterial growth results from the unique capabilities of each bacterial isolate in utilizing nutrients present in the media, ultimately leading to variations in metabolic efficiency.

Table 1. The content of imidacloprid residue Grobogan, Central Java, Indonesia, soil sample

Soil samples	Residue (ppm)
Ngaringan	0.47
Godong	0.45
Wirosari	0.50

Imidacloprid pesticide degradation test

Bacterial isolates can grow in MSM + imidacloprid media. Isolates IT1, IT2, IT3, and IT4 can reduce the concentration of imidacloprid in MSM+imidacloprid 2 ppm media. The highest (31.75%) percentage of decreased imidacloprid was recorded in the IT1 isolate (Figure 4), and the percentage of decreased imidacloprid IT3 isolates was lower than the other three isolates. The percentage of reduced imidacloprid was consistently increased from day 0 to the 21st day; this shows the potential for the isolate's degradation or reduction of imidacloprid.

Identification of bacterial isolates

The results of BLAST-n analysis (Table 3) showed that identified bacterial isolates had a similarity of 16S rRNA gene sequences with bacteria from the genus *Pectobacterium* and *Bacillus*. IT 1 and IT 2 had a percentage of similarity <95% with a low category after comparing species data in Genbank. Based on the 16S rRNA enclosure gene, the results revealed that isolates IT1 and IT2 were not identified because the similarity was only 86.83% and 89.51% with *Serratia nevei* and *S. marcescens*, respectively. Isolates IT3 and IT4 showed 99.83% and 98.57% similarities with *Pectobacterium aroidearum* strain CCRMPA670 and *Bacillus thuringiensis* strain FDAARGOS_791. With this percentage, IT1 and IT2 were identified into Enterobacterales and Enterobacteriaceae, respectively.

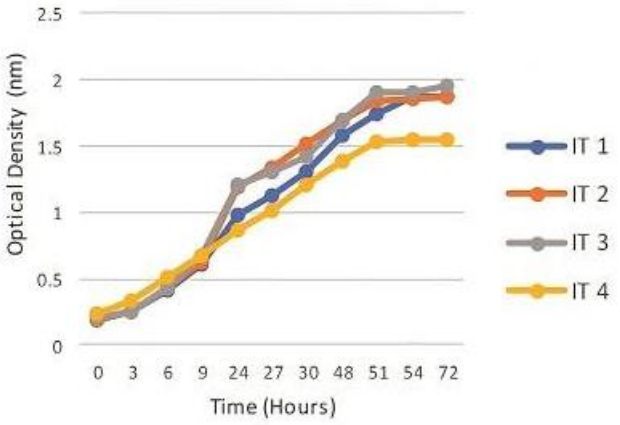

Figure 3. Growth of bacterial isolates on MSM with imidacloprid media

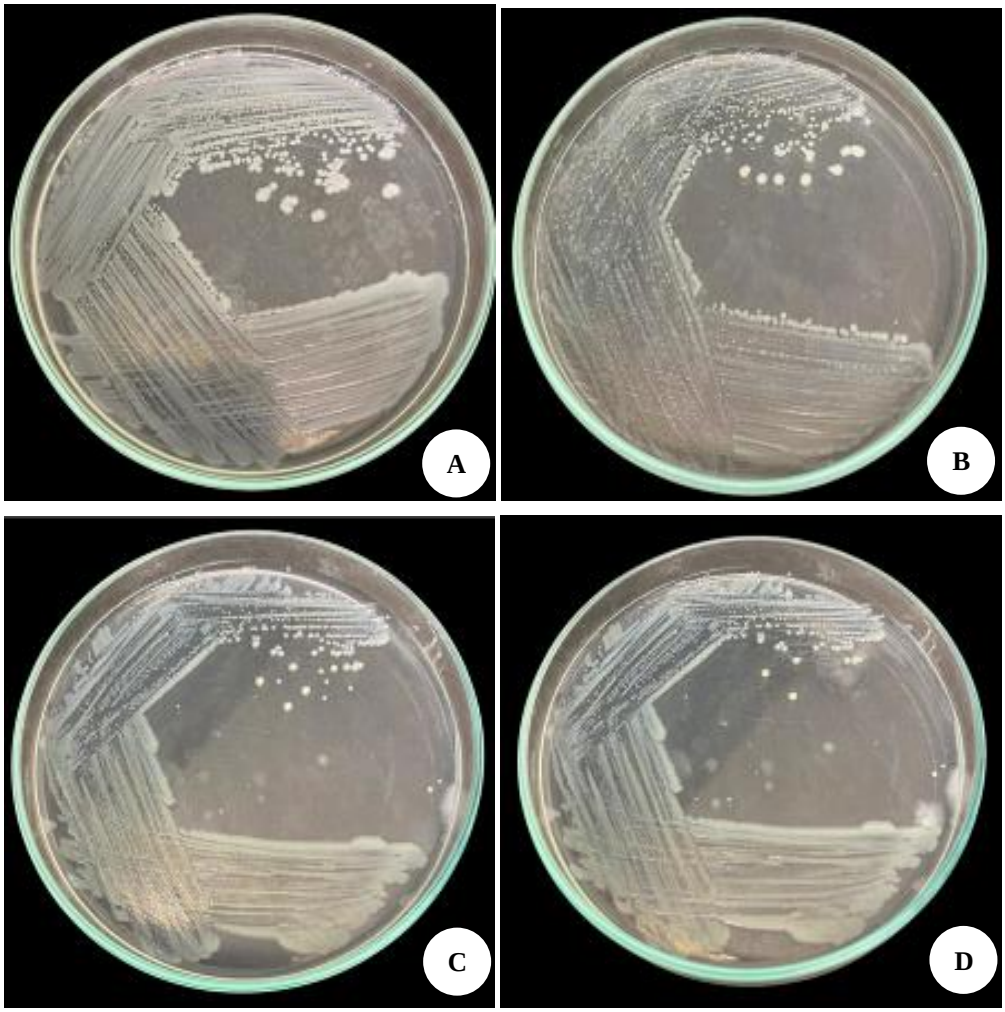

Figure 2. Bacterial colonies. A. IT1, B. IT2, C. IT3, D. IT4

Table 2. The characteristics of bacterial isolates from the rice fields in Grobogan, Central Java, Indonesia

Isolates code	Elevation	Margin	Colony color	Shape of colony	Shape of cell
IT1	Convex	Entire	Light pink	Circular	Bacilli
IT2	Convex	Entire	Brick red	Circular	Bacilli
IT3	Convex	Entire	Whitish yellow	Circular	Bacilli
IT4	Convex	Entire	White	Circular	Bacilli

Table 3. Similarity of 16S rRNA gene sequences of bacterial isolates using the BLAST-n program

Isolates code	Related species	Query cover (%)	Similarity (%)	ACC Numbers
IT1	*Serratia nevei* strain 2017-45-174	76	86.83	CP109739.1
IT2	*Serratia marcescens* strain JW-CZ2	100	89.51	CP055161.1
IT3	*Pectobacterium aroidearum* strain CCRMPA670	90	99.83	MN883868.1
IT4	*Bacillus thuringiensis* strain FDAARGOS_791	100	98.57	CP054568.1

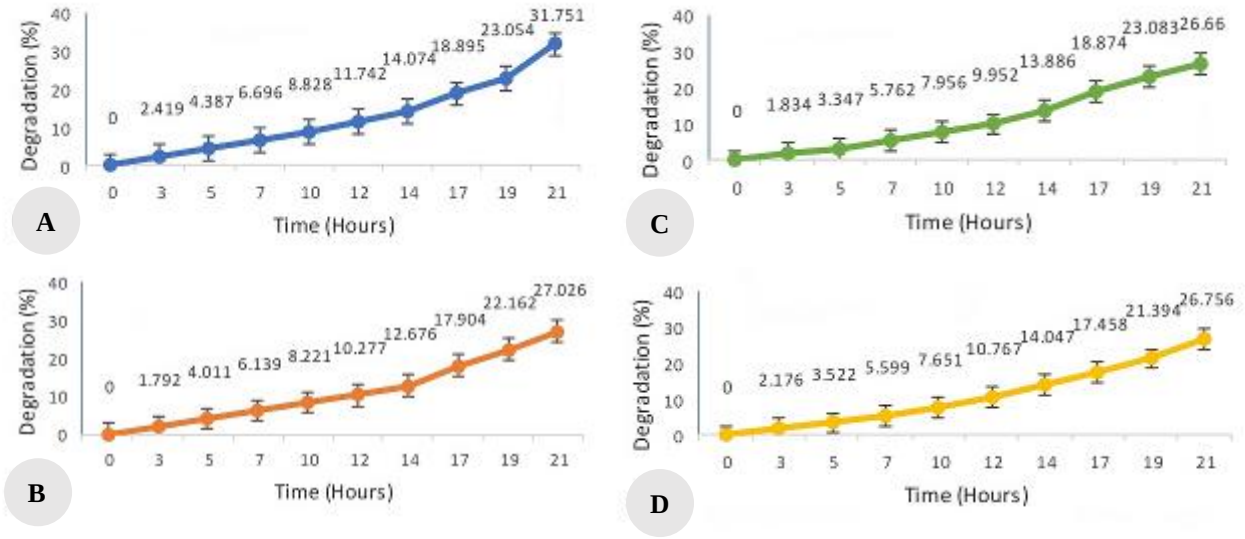

Figure 4. Growth of bacterial isolates on MSM + imidacloprid media: A. IT1, B. IT2, C. IT3, D. IT4

Discussion

Imidacloprid residue can affect the presence of soil bacteria through various mechanisms, both directly and indirectly. The impact of imidacloprid on soil bacteria depends on several factors such as bacterial types, imidacloprid concentrations, environmental conditions, and degradation of microorganisms. Excessive use of imidacloprid can interfere with the balance of soil microbes and decrease soil ecological function. Several studies have shown that imidacloprid residues can affect the composition of bacterial communities in the soil. Akter et al. (2023) reported that high imidacloprid concentrations could reduce the abundance of *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* in the soil. Astaykina et al. (2020) noted that imidacloprid changed the relative abundance of several eukaryotic and prokaryotic genera, such as *Apiotrichum*, *Gamicola*, *Humicola*, *Kitasatospora*, *Solicoccozyma*, *Sphingomonas*, *Streptomyces* and *Terrabacter*, respectively. He also stated that imidacloprid also reduces the relative abundance of Methylophilaceae, Coribacteraceae, Coxiellaceae, and Rhodospirillaceae in the soil, but imidacloprid increases the abundance of Nitrospirae bacteria in soil. Nitrospirae bacteria play a role in the nitrogen cycle, which is the conversion of ammonia into nitrate, a source of nitrogen that is important for plants (Yu et al. 2020).

All growth of bacterial isolates undergoes an exponential phase at a vulnerable time of 3 to 51 hours, except IT1 isolates, which ended the exponential phase at 54 hours. The growth of these bacteria arises due to the unique capabilities of each bacterial isolate in taking advantage of the nutrients contained in the media, which ultimately impacts variations in metabolic efficiency. The death of bacteria sensitive to imidacloprid can provide an opportunity for bacteria that are resistant to breed. Mohammed and Badawy (2017) reported that soil bacteria can quickly degrade imidacloprid through various metabolic pathways. Gonzalez and Aranda (2023) reported that growth in the exponential phase is influenced by the nature and shape of microbes in the environment, using

nutrients in the growth medium, temperature conditions, and media pH. Then, enter the stationary phase until 72 hours. The stationary phase occurs if the number of bacterial cells stops increases (Jõers et al. 2020). Although there is no growth in the stationary phase, cells can still grow and divide themselves. In this phase, the number of growing bacteria is balanced with the number of dead bacteria (Risna et al. 2022). Bacterial isolates demonstrate the ability to thrive in MSM+imidaclopridmedia due to their utilization of carbon and nitrogen sources in the media. This aligns with the findings of Zamule et al. (2021), who reported that various bacterial strains, including *Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas aeruginosa, Alcaligenes faecalis, Escherichia coli* and *Streptococcus lactis* can flourish in imidacloprid-containing media.

The highest percentage of decreased imidacloprid in test was 31.75%, in IT1 isolates, while the percentage of reduction in IT3 isolates was lower than in the other three isolates. The percentage of decreased imidacloprid was increased from day 0 to 21st day, this shows the potential for degradation or reduction of imidacloprid by the isolates. Bacteria that can grow in MSM+imidacloprid media are bacteria that have enzymes that can break the chemical structure of imidacloprid into simpler molecules, which bacteria can then use as a source of carbon and nitrogen. Other bacteria that are unable to grow in MSM+imidacloprid media do not have the enzymes needed to utilize the carbon imidacloprid content. These bacteria have enzymes that can break imidacloprid, but these enzymes are not efficient enough to produce enough energy for bacterial growth. Cycoń and Seget (2015) reported that the activity of the enzyme dehydrogenase, hexaphosphatase, and urease in soil bacteria given imidacloprid has decreased performance. Every pesticide application that affects the microbial community and its biochemical activity in the soil can be estimated to produce changes in the level of soil enzyme activity. Akoijam and Singh (2015) observed that *Bacillus aerophilus* and *B. alkalinitrilicus* are capable of degrading over 90% of imidacloprid in clay loam within 56 days. The degradation produces metabolites, such as 6-chloronicotinic acid, nitrosimine, and imidacloprid-NTG, which remain unaffected by sterilization. In another study, *B. cepacia* strain CH 9 was able to degrade 69% of the 50 ppm imidacloprid in 20 days after inoculation in MSM media (Bhattacherjee et al. 2020). *Ochrobacterium* sp. strain BCL-1 can degrade 67.67% from 50 ppm imidacloprid in 48 hours after the application as mentioned in the literature (Hu et al. 2013). Akoijam and Singh (2015) have observed that the loss of imidacloprid follows the first pseudo-order kinetics when applied at levels of 50, 100, and 150 ppm in sandy clay enriched with *B. aerophilus* with a part-time value of 14.33, 15, 15, 05, and 18.81 days. A strain of *B. thuringiensis* isolated from polluted marine sediments has been shown to degrade 71% of imidacloprid within 11 days (Obayori et al. 2024). *Trichoderma*, one of the most promising biological control agents, is found across various agricultural climates and is prevalent in soil and root ecosystems, it has the ability to serve as both a biological control and a plant growth promoter. In another study, *Tepidibacillus decaturensis* strain ST1 was able to degrade imidacloprid effectively in liquid media, slurry, and soil microcosms (Tiwari et al. 2023).

The results of molecular analysis revealed that IT1 was identified at the level of the order Enterobacterales and IT2 isolates identified at the level of the Enterobacteriaceae family. Isolates IT3 and IT4 were identified as *Pectobacterium proidearum* strain CCRMPA670 and *B. thuringiensis* strain FDAARGOS_791. The similarity of the 16S rRNA gene is one of the characteristics of a close-related bacterium. The 16S rRNA gene is very conservative, so changes that occur in this gene usually occur slowly and gradually. This causes closely related bacteria to have a similar similarity to the 16S rRNA gene (Sharma et al. 2014). Determination of potential bacterial identity is based on the criteria for the percentage of similarities ≥99% shows the similarity of species, the percentage of similarity ≥95%-<99% shows the similarity of the genus, and the percentage of similarity <95% shows the similarity of the family (Collins et al. 1994). Church et al. (2020) reported that comparing sequences of the 16S rRNA gene can help distinguish organisms at the genus level across key bacterial phyla and classify strains at various levels. *Pectobacterium* is included in the gram-negative bacteria Enterobacteriaceae found in rice fields' soil (Rossmann et al. 2018). *Bacillus* is generally used as a plant growth booster agent found in plantations and rice fields (Akinrinlola et al. 2018). Pang et al. (2020) state that *Pectobacterium* can grow and survive under high levels of imidacloprid. According to Vu et al. (2022), *Pectobacterium* can resist imidacloprid pesticides, and the bacteria have developed mechanisms to protect themselves. Ferreira et al. (2016) research states that *B. thuringiensis* is able to degradate imidacloprid. Other members of the genus *Bacillus* who also showed the ability to degrade imidacloprid compounds such as *Bacillus cereus* (Talpur et al. 2023), and *Bacillus wehenstephanensis* (Shetti et al. 2021). *Bacillus striatum*, which contains CYP353D1v2 genes exhibits strong resistance to imidacloprid (Pang et al. 2020). Soil-dwelling bacteria from the genus *Bacillus* have the ability to break down pesticides into simpler residues. *B. cereus* was identified as an efficient catalyst for degrading imidacloprid, metabolizing 92% of it within 11 days at a neutral pH. Through optimization using the Box-Behnken design, the bacteria transformed imidacloprid into 6-CNA via the intermediate's guanidine and 5-hydroxy imidacloprid (Talpur et al. 2023). The *B. cereus* is considered a promising tool for removing imidacloprid from contaminated water and soil (Gangola et al. 2021). Isolating the enzyme responsible for this degradation could provide a pathway for commercial use of purified enzymes.

In conclusion, 4 colonies obtained with different morphological variations namely IT1, IT2, IT3, and IT4. Analysis of degradation activity using the HPLC method showed that all isolates have the ability to grow in MSM, which contains imidacloprid and succeed in reducing the imidacloprid content by 26.66-31.75%. Based on 16S rRNA gene analysis, isolate IT1 was identified as *Enterobacterales*, IT2 was identified into the

Enterobacteriaceae, IT3 as *P. aroidearum* strain CCRMPA670 and IT4 were identified as *B. thuringiensis* strain FDAARGOS_791.

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