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Research Article

# Exploration of Acetic Acid-Producing Bacteria from *Heterotrigona itama* Honey in Sukabumi, Indonesia

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## ABSTRACT

Honey produced by stingless bees is generally characterized by higher acidity, which is associated with organic acid–producing microorganisms, such as lactic acid and acetic acid–producing. These bacteria can survive in this environment due to their tolerance to high sugar concentrations and low pH. Therefore, this study aimed to explore the presence of lactic acid– and acetic acid–producing bacteria and to analyze their relationship with honey sugar, with a particular focus on the characterization of acetic acid–producing bacteria isolated from *Heterotrigona itama* honey. This study involved characterizing honey for physicochemical properties and sugar content, and isolating bacteria. Additionally, isolates of acetic acid-producing bacteria were selected on selective media, and molecular identification was performed using 16S rRNA gene sequences. The results show that the proportion of lactic acid– and acetic acid–producing bacteria may influence the sugar content of honey. Honey Hi3 exhibited higher electrical conductivity (EC), total dissolved solids (TDS), moisture content, and acidity compared to the other honey samples. A total of 11 selected isolates showed diverse characteristics and were non-hemolytic. Isolate Hi11 produced 0.3% acetic acid over 48 hours and has optimal acetic acid production in glucose, yeast extract, peptone, and ethanol (GYPE1) medium. Based on 16S rRNA gene sequence analysis, this isolate was identified as the genus Enterobacter. These findings indicate the presence of acetic acid–producing bacteria and their potential contribution to the sour sensory characteristics of *H. itama* honey in Indonesia.

## 1. Introduction

Bacteria present in honey may originate from the digestive tract of bees and the hive environment, whereas secondary sources involve contamination during post-harvest handling, processing, and the use of equipment (Olaitan *et al.* 2007). Furthermore, bacterial communities have been identified in various types of honey, including monofloral, wildflower, manuka, and feral honey, in which the genus *Lactococcus* was found to be dominant (Xiong *et al.* 2023). Therefore, these bacteria may come from the guts (Tola *et al.* 2021), nests (Karyawati *et al.* 2020), nectar (Pitiwittayakul *et al.* 2015), pollen, and honey (Salomón *et al.* 2024; Nurjanah *et al.* 2026).

Lactic acid-producing bacteria constitute the core microbiota associated with both bees and honey. Lactic acid-producing bacteria, such as *Lactiplantibacillus plantarum* and *Pediococcus acidilactici*, were detected in honey produced by *H. itama* and *A. cerana* from Bogor, Indonesia (Fatma *et al.* 2022). Lactic acid-producing bacteria are a group of fermentative bacteria capable of utilizing sugars, particularly glucose and fructose, as their primary substrate (Wang *et al.* 2021). Lactic acid-producing bacteria have been reported to possess various beneficial properties, including probiotic potential (Meradji *et al.* 2023), antifungal (Bulgasem *et al.* 2016), and antimicrobial activities (De Simone

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*et al.* 2023). Aside from lactic acid-producing bacteria, the functional roles of other bacterial groups potentially involved in the formation of acidic taste in honey remain poorly characterized, as most existing studies have focused primarily on lactic acid-producing bacteria, while information on other bacteria in honey remains limited.

One group of bacteria with the potential to contribute to the development of sour taste is acetic acid–producing bacteria. These bacteria are known to accumulate during oxidative fermentation and to produce acetic acid, which contributes to the characteristic sour flavors of fermented products (Hao *et al.* 2025). Acetic acid–producing bacteria are aerobic bacteria that oxidize carbohydrates and ethanol to produce acetic acid (De Vuyst *et al.* 2023). These bacteria generally belong to the genera *Acetobacter, Komagataeibacter,* or *Gluconobacter*, which are microorganisms capable of oxidizing alcohol to acetic acid under aerobic conditions (Lynch *et al.* 2019). The presence of these bacteria may contribute to the formation of organic acids associated with the sour sensory characteristics of honey (Hungerford *et al.* 2023). However, the presence of acetic acid-producing bacteria in stingless bee honey from Indonesia has not yet been reported. These bacteria also have antibacterial and antioxidant properties and block α-glucosidase (Kim *et al.* 2023). In addition, other bacteria, such as *Enterobacter* sp. T4384 has also been reported to produce acetic acid (Kim *et al.* 2013). Therefore, lactic acid– and acetic acid–producing bacteria need to be further investigated regarding their presence and relative abundance in Indonesian honey, particularly in *Heterotrigona itama* honey.

*H. itama* is a species of stingless bee and one of the successfully domesticated species in Indonesia (Buchori *et al.* 2022; Sayusti *et al.* 2026). Stingless bees produce honey with a distinctive sour taste and unique physicochemical characteristics (Vit 2022; Dos Santos *et al.* 2024), and it also contains bacteria and yeasts capable of producing organic acids (Hungeford *et al.* 2021; Vit 2022). Organic acids, such as acetic acid, in *H. itama* honey were reported at 0.27% (Wong *et al.* 2019). In addition, the quantity and composition of sugars and organic acids in stingless bee honey are also thought to play an important role in shaping its characteristic taste and aroma (Hungeford *et al.* 2023). Therefore, the sour taste of honey can be investigated through the presence of acetic acid–producing bacteria in order to better understand the fundamental characteristics of *H. itama* honey. To date, no studies have reported the contribution of acetic acid–producing bacteria to the characteristics of *H. itama* honey in Indonesia. Therefore, this study was conducted to explore the presence of lactic acid– and acetic acid–producing bacteria and to analyze their relationship with honey sugar, with a particular focus on the characterization of acetic acid–producing bacteria isolated from *H. itama* honey.

## 2. Materials and Methods

### 2.1. Honey Sampling

Honey samples of *H. itama* were obtained from beekeepers in Cijangkar Village, Nyalindung, Sukabumi, Indonesia. *H. itama* honey was collected from three colony sites, labeled Hi1, Hi2, and Hi3. *H. itama* honey was aseptically collected from sealed honey pots using sterile syringes and transferred into sterile 15 mL tubes. All honey was stored in an ice box containing ice gel during transport. In this study, each honey sample was used for 35 mL for sugar content analysis and bacterial isolation. An inventory was also conducted to identify potential nectar sources of the bees, and the plant species found around the study site were identified using the iNaturalist application.

### 2.2. Physicochemical Characteristics of Honey

The physicochemical characteristics of honey, including pH, temperature, total dissolved solids (TDS), and electrical conductivity (EC), were assessed during the honey sampling process. These measurements were conducted based on El Sohaimy *et al.* (2015), with certain modifications. Each 5 mL of *H. itama* honey was diluted with 45 mL of distilled water (pH 7.0). The pH of the honey was measured using an HI-98127 meter, while temperature, TDS, and EC were assessed with an HI-98311 meter. The moisture content was determined by applying a drop of honey onto the glass prism of a digital refractometer.

### 2.3. Sugar Content

The quantification of reducing sugar content was conducted using the 3,5-dinitrosalicylic acid (DNSA) method, as described by Miller (1959). The honey solution (1 mL)was diluted with distilled water. A 1 mL aliquot of this diluted solution was mixed with 1 mL of the DNS solution, vortexed, and incubated in a boiling water bath for 15 min at 90-100°C. The mixture was allowed to cool to room temperature for ten minutes. Absorbance at 540 nm was used to assess reducing sugars, which convert DNSA to 3-amino-5-nitrosalicylic acid, producing a reddish-orange solution.

Total sugar was determined by the phenol-sulfuric method based on Dubois *et al.* (1956). Honey (1 mL) was diluted with distilled water. A 1 mL aliquot of this diluted solution was mixed with 0.5 mL of phenol, vortexed, and then diluted with 2.5 mL of sulfuric acid. The mixture is incubated at room temperature for 20 minutes, then vortexed. Absorbance was measured photometrically at 490 nm.

## 2.4. Isolation of Bacteria Capable of Producing Acetic Acid and Lactic Acid

The GYPEC medium with ethanol and a color indicator provides specific nutrients and facilitates the differentiation of acetic acid–producing bacteria through color changes resulting from acid oxidation (Al-Kharousi *et al.* 2024). The honey samples (1 mL) were serially diluted with physiological saline (0.85% NaCl) and plated on the suitable microbiological medium. Acetic acid-producing bacteria are isolated on agar plates containing glucose, yeast extract, peptone, ethanol, and calcium carbonate (GYPEC) with bromocresol green. An aliquot of 100 µL was spread onto a GYPEC agar plate consisting of 8 g of yeast extract, 5 g of peptone, 20 g of D-glucose, 3 g of $CaCO_3$, 15 g of bacteriological agar, 0.022 g of bromocresol green, and 5 mL of absolute ethanol per Liter of distilled water (Al-Kharousi *et al.* 2024). The bacterial isolates were incubated at 30°C for 48 hours. The yellow zone formed surrounding the colony showed the bacteria's capacity to lower the pH and produce acetic acid. The bacterial colony was measured using the total plate count method. Colonies of various forms were chosen and studied.

Honey (1 mL) was mixed with sterile water (0.85% NaCl) and diluted in a series to isolate lactic acid bacteria. 100 µL of the sample was spread onto MRS agar plates with 1% $CaCo_3$ (Yu *et al.* 2023). The plates were incubated at 37°C for 48 hours, and bacterial colonies showing clear zones were quantified using the total plate count method.

## 2.5. Screening of Acetic Acid–Producing Bacterial Isolates

Pure bacterial colonies were examined macroscopically by assessing their shape, margin, color, and elevation. Microscopic examination involved observing cell morphology and performing Gram staining. The isolates were inoculated into YPGE medium and incubated at 30°C for 24 hours. Bacterial isolates are also selected based on their capacity to produce hemolysin by cultivating them on blood agar. This process results in the formation of a distinct, clear zone (hemolysis) surrounding the colony after 24-48 hours of incubation, typically at 30°C. The types of hemolysis include β-hemolysis (completely clear zone), β-hemolysis (partial greenish zone), and α-hemolysis (no change) (Bamba *et al.* 2024).

The isolated acetic acid-producing bacteria were assessed for their capacity to produce acetic acid on GYPEC medium containing bromocresol green. The yellow zone in the medium indicated the production of acetic acid, and the size of the yellow zone diameter revealed the potency of each strain. The potency index (PI) was used as the evaluation parameter, with incubation conducted at 30°C for 48 hours. The potential index was calculated using a formula based on El-Askri *et al.* (2022):

$$\text{Potential Index} = \frac{\text{Diameter of the yellow zone formed (cm)}}{\text{Diameter of the bacterial colony (cm)}}$$

Isolate selection was based on the acetic acid production using the titration method (Kim *et al.* 2023). Each bacterium was incubated at 30°C at 20 rpm in GYPE medium. Each preculture ($OD_{600}$ nm = 0.8) was transferred to GYPE production medium and incubated for 24 hours. The acetic acid content was quantified by titration with 0.05 N NaOH, using phenolphthalein as the indicator. The volume of acetic acid was calculated using the following formula:

$$\text{Titratable acidity (\%)} = \frac{(0.05\text{ N NaOH (mL)} \times 0.003 \times 100)}{\text{Sample volume (mL)}}$$

Note: 0.003 = represents the acetic acid equivalent.

## 2.6. Medium Optimization for Acetic Acid Production

Optimization of the producing media was performed for a selected bacterial isolate. Three types of media were utilized in this optimization process: nutrient broth (NB), GYPE1, and GYPE2. The GYPE1 medium composition is based on Al-Kharousi *et al.* (2024). GYPE2 medium was prepared according to Saelee *et al.* (2023), comprising 3 g yeast extract, 5 g peptone, 25 g D-glucose, and 5 mL of absolute ethanol in 1 L of distilled water. Bacteria used for inoculum preparation were cultured for 24 hours on GYPE agar plates at 30°C. The preculture was inoculated in each liquid medium NB, GYPE1, GYPE2, until reaching an optical density ($OD_{600}$) of 0.6-0.8. All cultures were incubated for 48 hours at 20 rpm, and acetic acid production was quantified by titration.

### 2.7. Growth Curve Determination

The Hi11 isolate growth curve was determined based on the method of Marwah *et al.* (2023), with modifications. Observations were conducted every 5 hours for 35 hours to measure total plate count (TPC), pH, and acetic acid production. A total of 1 mL of inoculum with an initial cell density of 108 CFU/mL was cultured in 150 mL of GYPE1 medium. The fermented sample (1 mL) was serially diluted with 9 mL of 0.85% NaCl solution (101-107). Aliquots of 100 µL from appropriate dilutions were spread onto GYPE1 agar plates. Colony enumeration was performed after incubation using a colony counter. In parallel, 4 mL of the culture sample was collected every 5 hours for titratable acidity analysis, while 1 mL was used for pH measurement.

### 2.8. 16S rRNA Sequencing and Phylogenetic Analysis

The bacterial isolate was cultured in GYPE medium at 20 rpm for 24 hours. A total of 1.5 mL of the culture was harvested by centrifugation at 12,000 × g for 2 minutes, and the resulting pellet was washed with 1 mL of sterile distilled water. DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 27F and 1429R (Yalçın *et al.* 2025). The PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 52°C for 1 minute, extension at 72°C for 1.5 minutes, and a final extension at 72°C for 10 minutes. The resulting PCR products were sequenced using the same primers employed for amplification. Sequence data were edited using Geneious Prime and compared with reference sequences in the NCBI database using BLAST for preliminary identification. Reference sequences for the phylogenetic tree were obtained from GenBank and aligned with the sequences from this study. Phylogenetic analysis was conducted in MEGA X using the Maximum Likelihood method based on the Kimura two-parameter model with a gamma distribution and 1,000 bootstrap replicates (Kumar *et al.* 2018). The partial 16S rRNA gene sequence of isolate Hi11 (~1000 bp) was deposited in the NCBI GenBank database under accession number PZ076326.

## 3. Results

### 3.1. Exploring the Link Between Sugars and Bacteria in Honey

The physicochemical characteristics of *H. itama* honey differed significantly in EC, TDS, moisture content, and temperature (Table 1). All honey samples were acidic, with pH values ranging from 3.3 to 4.2.

Sugar analysis was conducted to determine whether significant differences existed in the reducing and total sugar contents of honey and to evaluate the potential impact of lactic acid- and acetic acid-producing bacteria. The findings indicated that the reducing sugar and total sugar contents of Hi1 were significantly lower than those of other honeys, at 24.31±0.44 and 32.76±0.59 g/100 g, respectively. Furthermore, Hi1 exhibited the highest proportions of lactic acid- and acetic acid-producing bacteria, at 39.7 and 6.1 × $10^1$ CFU/mL, respectively (Figure 1).

Various nectar-producing plant species surrounding the *H. itama* nests were observed and identified using the iNaturalist application. The presence of plants around the hives of *H. itama* bees was observed to be a potential nectar source utilized by bee species. The area surrounding the bee habitat has a variety of nectar-providing plants, including *Ageratum* sp., *Alternanthera* sp., *Averrhoa* sp., *Calliandra* sp., *Eupatorium* sp., *Gliricidia* sp., *Heptacodium* sp., *Hippobroma* sp., *Indigofera* sp., *Ipomoea* sp., *Mimosa* sp., *Musa* sp., *Ocimum* sp., *Sechium* sp., *Sphagneticola* sp., *Syzygium* sp., and *Tithonia* sp.

### 3.2. Selection of Bacterial Isolates Capable of Producing Acetic Acid

The analysis of isolate selection based on acetic acid production activity was conducted by evaluating the potential index and titratable acidity values (Table 2). All isolates produced varying amounts of acetic acid. Notably, isolates Hi11 and Hi113 exhibited the highest potential index values, measuring 0.803 cm and 0.783 cm, respectively. These two isolates also recorded the highest acetic acid values, which were significantly higher than those of the other isolates at 0.300% and 0.295%, respectively. In contrast, isolates Hi2 and Hi3

Table 1. Physicochemical profiles of *H. itama* honey collected from three different colonies within each colony using a calibrated multiparameter meter

| Honey code | EC (mS/cm) | TDS (ppm) | pH | Moisture content (%) | Temperature (°C) |
|---|---|---|---|---|---|
| Hi1 | 0.99±1.86$^{a}$ | 494.3±2.33$^{a}$ | 3.9±0.00 | 25.6±0.13$^{a}$ | 29.1±0.32$^{a}$ |
| Hi2 | 1.21±2.85$^{b}$ | 605.3±2.85$^{b}$ | 3.5±0.00 | 29.7±0.47$^{b}$ | 29.6±0.12$^{a}$ |
| Hi3 | 1.66±4.81$^{c}$ | 831.3±2.85$^{c}$ | 3.3±0.00 | 30.2±0.33$^{b}$ | 29.5±0.12$^{a}$ |

*H.itama* (Hi1, Hi2, and Hi3), electrical conductivity (EC), total dissolved solids (TDS). Different letters within the same column indicate significant differences according to Tukey's test (α = 0.05). Data are expressed as mean ± standard error (SE) of triplicate measurements

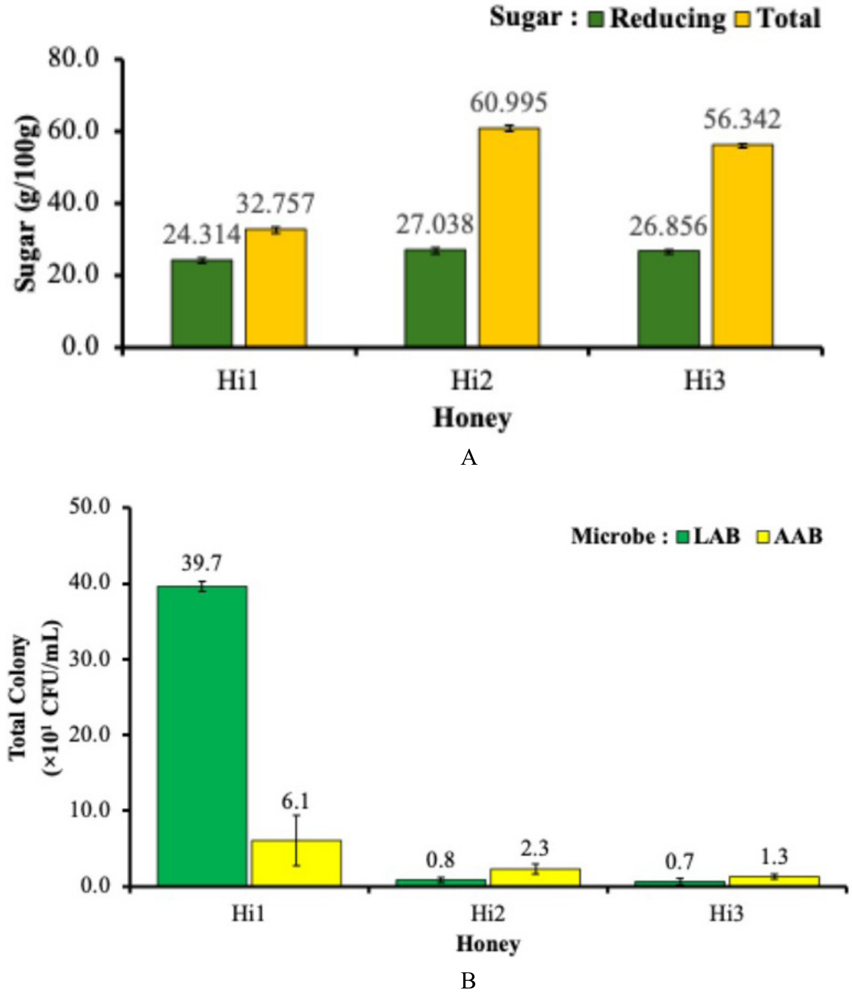

Figure 1. Analysis of sugar content and bacterial populations in various honey types. (A) Concentrations of reducing sugars and total sugars (g/100 g) in honey samples from *H. itama* (Hi1–Hi3), (B) population densities of lactic acid-producing bacteria (LAB) and acetic acid–producing bacteria (AAB) (×10$^{1}$ CFU/mL)

had the lowest potential index and total titratable acid values.

### 3.3. Optimization of Growth Media for Hi11 Isolate in Acetic Acid Production

To evaluate the optimal culture medium for acetic acid production, isolate Hi11 was cultivated in three different media (NB, GYPE1, and GYPE2), and the resulting titratable acidity and acetic acid concentrations were measured. Experimental results showed the isolate Hi11 did not produce acetic acid on NB medium. In contrast, acetic acid was produced on GYPE1 at 0.295%. These findings indicate that variations in the composition of the GYPE medium significantly influenced the acetic acid production capacity of isolate Hi11 (Table 3).

Table 2. Acetic acid production activity of isolates based on yellow zone formation and acetic acid content, calculated using titratable acidity with acetic acid equivalent

| Isolate code | Potential index (cm) | Titratable acidity(%) | Acetic acid (Meq/100 mL) |
|---|---|---|---|
| Hi11 | 0.803±0.016$^{d}$ | 0.300±0.000$^{e}$ | 1.500 |
| Hi112 | 0.533±0.017$^{ab}$ | 0.100±0.005$^{abc}$ | 0.167 |
| Hi113 | 0.783±0.009$^{cd}$ | 0.295±0.005$^{e}$ | 1.450 |
| Hi12 | 0.556±0.060$^{ab}$ | 0.120±0.000$^{c}$ | 0.240 |
| Hi122 | 0.533±0.017$^{ab}$ | 0.105±0.000$^{bc}$ | 0.184 |
| Hi131 | 0.644±0.012$^{b}$ | 0.160±0.005$^{d}$ | 0.527 |
| Hi132 | 0.525±0.014$^{ab}$ | 0.095±0.005$^{ab}$ | 0.150 |
| Hi133 | 0.546±0.012$^{ab}$ | 0.110±0.005$^{bc}$ | 0.202 |
| Hi134 | 0.656±0.012$^{bc}$ | 0.175±0.005$^{d}$ | 0.510 |
| Hi2 | 0.443±0.021$^{a}$ | 0.080±0.005$^{a}$ | 0.107 |
| Hi3 | 0.450±0.050$^{a}$ | 0.080±0.005$^{a}$ | 0.107 |

Different letters within the same column indicate significant differences according to Tukey's test (α = 0.05). Data are expressed as mean ± standard error (SE) of triplicate measurements

Table 3. Acetic acid production by isolate Hi11 in three different media to determine the optimal medium

| Medium | Total titratable acidity(%) | Acetic acid (Meq/100 mL) |
|---|---|---|
| NB | 0.000±0.000$^{a}$ | 0.000 |
| GYPE1 | 0.295±0.017$^{c}$ | 1.450 |
| GYPE2 | 0.240±0.000$^{b}$ | 0.960 |

Different letters within the same column indicate significant differences according to Tukey's test (α = 0.05). Data are expressed as mean ± standard error (SE) of triplicate measurements.

### 3.4. Growth Profile and Acetic Acid Production of Hi11

Observations of the growth dynamics of isolate Hi11 for 35 hours of incubation revealed concurrent changes in cell density, pH, and titratable acetic acid levels (Figure 2). The cell population increased during the early incubation phase and reached a maximum between the 10th and 15th hours, with values ranging from 6.4 to 6.5 log CFU/mL, before declining in the subsequent phase. As the isolate Hi11 grew, the medium pH gradually decreased from approximately 6 to 3, indicating increasing acidity throughout the incubation period. This reduction in pH was accompanied by an increase in acetic acid concentration, which reached 0.27% at the end of incubation. These results suggest that the metabolic activity of isolate Hi11 directly contributes to acetic acid production, therefore driving the observed changes in medium acidity during incubation.

### 3.5. Molecular Identification and Phylogenetic Analysis

Based on the selection results, isolates were chosen for molecular identification. BLAST analysis of the partial 16S rRNA gene sequence showed that isolate Hi11 had 98.69% similarity to *Enterobacter ludwigii*. Furthermore, phylogenetic analysis showed that isolate HI11 clustered with multiple *E. ludwigii* strains. This clustering formed a monophyletic clade supported by a bootstrap value of 79, indicating a relatively strong phylogenetic relationship (Figure 3).

## 4. Discussion

Hi3 honey contains greater TDS levels than other honeys (Table 1). The high EC, acidity levels, and TDS of *H. itama* honey indicate a high amount of ionized ions and organic acids, particularly gluconic and acetic acids (Nweze *et al.* 2017; Majewska *et al.* 2019). On the other hand, *H. itama* honey has more moisture than the maximum limits defined by Codex Alimentarius (2001) and Standar Nasional Indonesia (2018). This water content is influenced by external temperature and humidity (Buba *et al.* 2013). Humid climatic circumstances generate thinner nectar with a bigger volume, whereas high temperatures and low humidity accelerate the rate of nectar evaporation. Variations in nectar water content are crucial for determining the maturation efficiency and final quality of honey (Adgaba *et al.* 2017).

The presence of high water content and available sugars as substrates increases the potential for fermentation by bacteria and yeasts. In contrast, low water content imposes osmotic stress, inhibiting microbial activity (Xiong *et al.* 2023). In addition, temperature parameters affect microbial viability, which in turn influences the physicochemical characteristics of honey during storage (Comi *et al.* 2000). Overall, variations in honey's physicochemical properties can be associated with microbial metabolic activity (Xiong *et al.* 2023). Aside from physicochemical characteristics, the presence of microbes in *H. itama* honey is related to sugar levels.

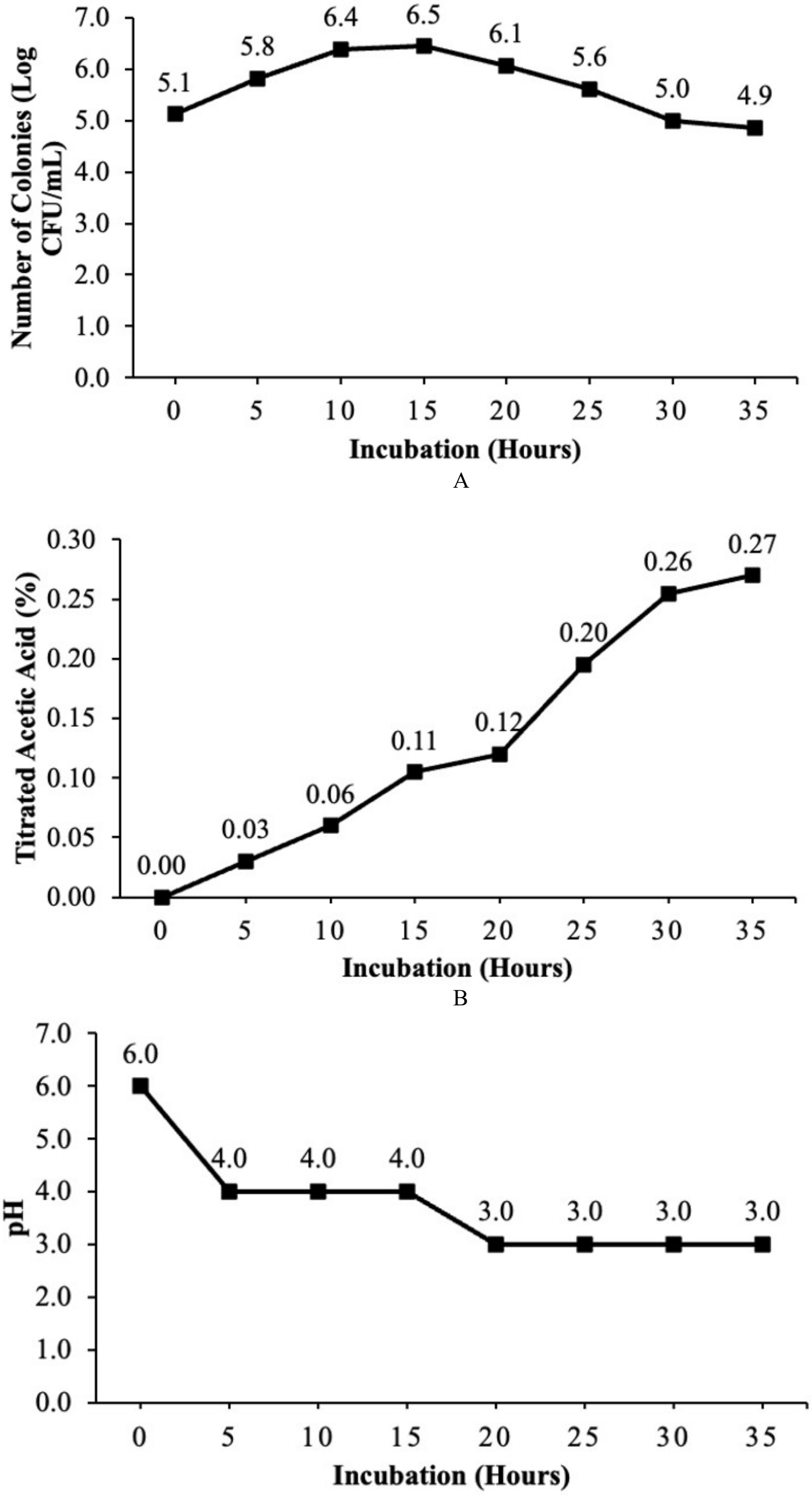

Figure 2. Growth curve of the Hi11 isolate during 35 h of incubation based on changes in (A) cell count (log CFU/mL), (B) titratable acetic acid (%), and (C) pH

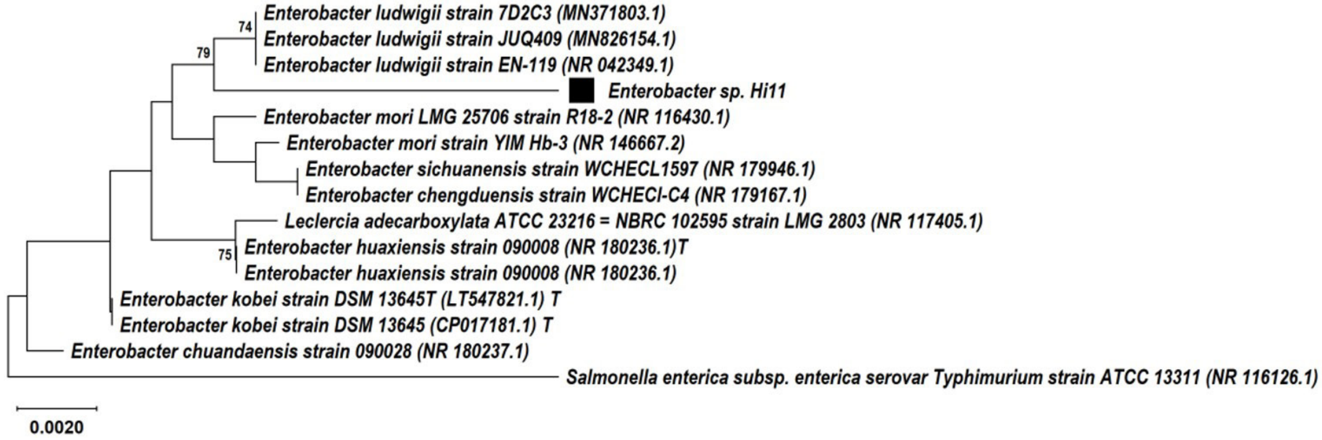

Figure 3. Phylogenetic tree based on 16S rRNA region gene sequences between *Enterobacter* sp. HI11 strain and related species. PCR and re-PCR amplification of the 16S rRNA gene were performed using the universal primers 27F and 1429R

Sugar content is significantly influenced by the nectar source and the environmental conditions in which the bees forage (Rao *et al.* 2016). Furthermore, there is a reciprocal interaction in which microbial activity can alter the sugar composition by utilizing glucose and fructose as metabolic substrates (Callegari *et al.* 2021). In the present study, Hi1 honey had a lower sugar content than other honeys. These disparities in sugar characteristics and environmental conditions ultimately determine the proportions of distinct bacterial populations in each honey.

The bacteria isolated from *H. itama* honey comprised both lactic acid and acetic acid-producing bacteria. The population of lactic acid–producing bacteria tends to be higher, as these bacteria can directly ferment the glucose and fructose present in honey, serving as primary energy sources for rapid growth. In contrast, acetic acid–producing bacteria generally occur at lower populations because, despite their capacity to use carbohydrates, their optimal growth largely depends on the availability of ethanol in honey. Furthermore, lactic acid-producing bacterial populations were quantified to provide an ecological context for the acid-producing microbial community in honey, as acetic acid-producing bacteria are known to coexist and interact with lactic acid producers in natural fermentation ecosystems (Han *et al.* 2024). These two bacterial groups play functional roles in carbohydrate metabolism and the formation of organic acids, directly influencing the physicochemical and sensory characteristics of honey (Mamlouk and Gullo 2013; Wang *et al.* 2021). Subsequently, the acetic acid-producing bacterial isolates from *H. itama* honey were subjected to further analysis.

Eleven bacterial isolates were identified as non-hemolytic and thus considered safe, as no hemolysin activity was observed (Mogrovejo *et al.* 2020). They also exhibited diverse characteristics and varied capacities to produce acetic acid. This variation in production is hypothesized to be associated with differences in the efficiency of each isolate in oxidizing ethanol to acetic acid (El-Askri *et al.* 2022). This capability is linked to the activities of the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), which are integral to ethanol oxidation. ADH catalyzes the conversion of ethanol to acetaldehyde, which is subsequently oxidized to acetic acid by ALDH (Zheng *et al.* 2017). Consequently, the isolates obtained were selected for further analysis concerning the optimal media for growth and acetic acid production.

Isolate Hi11 was identified as the strain producing the highest levels of acetic acid. The isolate was cultured in several media to find the optimal growth medium for acetic acid production. The GYPE1 medium is attributed to the higher yeast extract concentration (0.8%), which provides vitamins and growth factors to bacteria. The availability of appropriate nutrients can facilitate the formation of secondary metabolites and enhance the efficiency of the ethanol oxidation process (Tao *et al.* 2023; Suárez *et al.* 1998). In contrast, the absence of ethanol in the NB medium leads to the deactivation of the oxidative metabolic pathway, resulting in suboptimal acetic acid production (Djenar and Mulyono 2017).

The growth curve of the Hi11 isolate is delineated into four principal phases. The lag phase occurred during the first 5 hours as a period of adaptation to the GYPE1 medium (Rolfe *et al.* 2012). In the logarithmic phase, the isolate produces acetic acid via membrane-associated enzymatic activity, as evidenced by an increase in acetic acid concentration to 0.06% and a decrease in pH to 4 (Hua *et al.* 2024). Subsequently, the culture entered the stationary phase, and acetic acid concentration continued to increase. This suggests that essential enzymatic activities, such as ADH and ALDH, were maintained during ethanol oxidation despite declining cell viability (Hua *et al.* 2024).

Phylogenetic tree reconstruction revealed that isolate Hi11 formed a monophyletic clade with *E. ludwigii*, with robust statistical support (>70%). This isolate was classified at the genus level as *Enterobacter* sp. Hi11 (Janda and Abbott 2007). Although *Enterobacter* sp. Hi11 is not considered a classical acetic acid bacterium; several Enterobacter species have been reported to produce acetic acid as a metabolic by-product during carbohydrate fermentation. Furthermore, this finding aligns with previous studies reporting *Enterobacter* sp. as part of the core microbiota of the digestive tract of stingless bees (Trigona). These bacteria contribute to maintaining digestive tract stability through fermentation, aiding feed degradation and inhibiting colonization of pathogenic microbes (Ramírez-Ahuja *et al.* 2025).

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