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Analysis of Bacterial Diversity in Temple Bricks Using Phenetic Numeric Taxonomy Method

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Abstract. *Temple bricks-based constructions often face various challenges, including physical, chemical, and biological weathering. Previous studies identified various biological factors contributing to brick weathering, including exudate produced by microorganisms, such as bacteria. In addition, bacteria often live synergistically and antagonistically with other species, exhibiting diverse morphological and physiological traits (bacterial diversity). Various methods have been developed to explore bacterial diversity, with phenetic numerical taxonomy being the most popular. Therefore, this study aims to determine bacterial diversity on the surfaces of temple bricks using phenetic numerical taxonomy method. Bacterial isolation was carried out aseptically, followed by labeling and transferring the isolate to the laboratory for further tests. The tests included morphological characterization, biochemical assays, physiological reactions, and potential enzymatic activities. Subsequently, dendrogram was constructed using MVSP (Multi-Variate Statistical Package) software with isolated grouping based on the Unweighted Pair Group Method Averages (UPGMA) algorithm. The similarity between isolates was analyzed using the Simple Matching Coefficient (SSM) similarity value. The dendrogram analysis revealed the presence of 3 clusters namely A (4 isolates), B (1 isolate), and C (2 isolates), with a similarity index of 0,543 to 0,857. Clusters A, B, and C had a similarity index of ≤0.700, indicating the occurrence of distinct species in each cluster. Based on the profile-matching results of critical characters, the 7 bacterial isolates were identified as belonging to the genera Bacillus, Corynebacterium, and Mycobacterium.*

Keywords: *diversity, numerical taxonomy, phenotype, temple building*

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INTRODUCTION

Temple is a building with a high historical value, which has become one of the most tourist-attracting places in Indonesia, significantly contributing to the country's foreign exchange earnings (Rahma, 2020). In addition, the majority of temple in East Java region are often constructed with red clay bricks (Poernama & Putra, 2022). These red bricks typically possess a porosity level of approximately 30-38%, leading to the ability to easily absorb water and become damp (Fatmawati, 2018).

Damp-surface bricks have been reported to have the ability to facilitate weathering processes caused by microorganisms, such as bacteria. According to a previous report, bacteria require moist conditions to carry out the cellular metabolism processes (Widarti et al., 2015), with organic acids being a major by-product. However, these organic acids can break down $CaCO_3$ content, a binding agent in rocks, leading to rapid biodeterioration (Obidi & Okekunjo, 2017).

A study by Qi Wang et al. (2011) identified several bacterial genera with the ability to cause rock biodeterioration, including *Bacillus, Massilia, Brevibacillus, Glacialice, Acinetobacter, Brachysporum, and Achromobacter* (Qi-Wang et al., 2011). Recently, there has been an extensive use of 16S rRNA gene-based molecular methods to investigate bacteria responsible for the deterioration of various rock types (Mihajlovski et al., 2017; Zulaika et al., 2021; Meng et al., 2020; Qi-Wang et al., 2011; Lepinay et al., 2018). However, 16S rRNA gene analysis has various drawbacks, including limited resolution as it can only identify closely related species, posing challenges to identifying microbes at the genus or family level. This method also fails to consider the phenotypic properties of the identified strains because 16S rRNA region represents only a small portion of the genome, rendering it insufficiently informative for determining phenotypic traits (Akihary & Kolondam, 2020).

According to several studies, phenotypic traits play an essential role in bacterial identification and taxonomy. These characteristics typically arise from the direct expression of genes in microorganisms, providing readily observable features. In addition, environmental factors have the potential to modulate gene expression, enabling microorganisms to adapt to changing environments over time. Phenotypic traits also offer comprehensive information about various aspects, including the ecological role of bacteria, the potential hazards to humans and animals, and the development of effective control strategies (Rosselló-Móra & Amann, 2015).

In nature, diverse bacteria coexist, forming communities known as bacterial diversity. A previous report revealed that bacterial diversity can be studied using phenetic numerical taxonomy method, offering various advantages. These include the ability to produce a more stable classification of bacteria and the formed clusters can be interpreted as taxa with similar potential (Vane-Wright, 2017). Therefore, this study aims to determine the diversity of bacteria on the surface of temple bricks using phenetic numerical taxonomy method. These results can be used as a reference for conserving temple rocks and other historical buildings.

MATERIALS AND METHODS

Isolation and Screening

The isolation of bacteria-producing organic acids was carried out aseptically using a composite method on the rocks of Brahu Temple, Trowulan Site, Mojokerto, and East Java, with coordinates -7.5431, 112.374538. Details of maps location sampling were

presented in Figure 1, while sampling site locations selected from 4 points on the brick temple were shown in Figure 2.

The isolates used in this study were obtained using the aseptic swapping method. The isolation and screening media used were selected for bacteria-producing organic acids according to Shah (2019) with modificatios. These media contained 1% sucrose (w/v), 1% $CaCO_3$ (w/v), and 28 g/L Nutrient Agar. A total of 1-gram sample of brick was diluted with sterile distilled water from 10-1 to 10-3 (Sari et al., 2021), and inoculation was performed using the pour plate method. The process was carried out by placing 100 uL of inoculum into a sterile petri dish and supplementing with selective media. In addition, incubation was carried out at room temperature for 1-5 days until clear zone was formed (Shah, 2019).

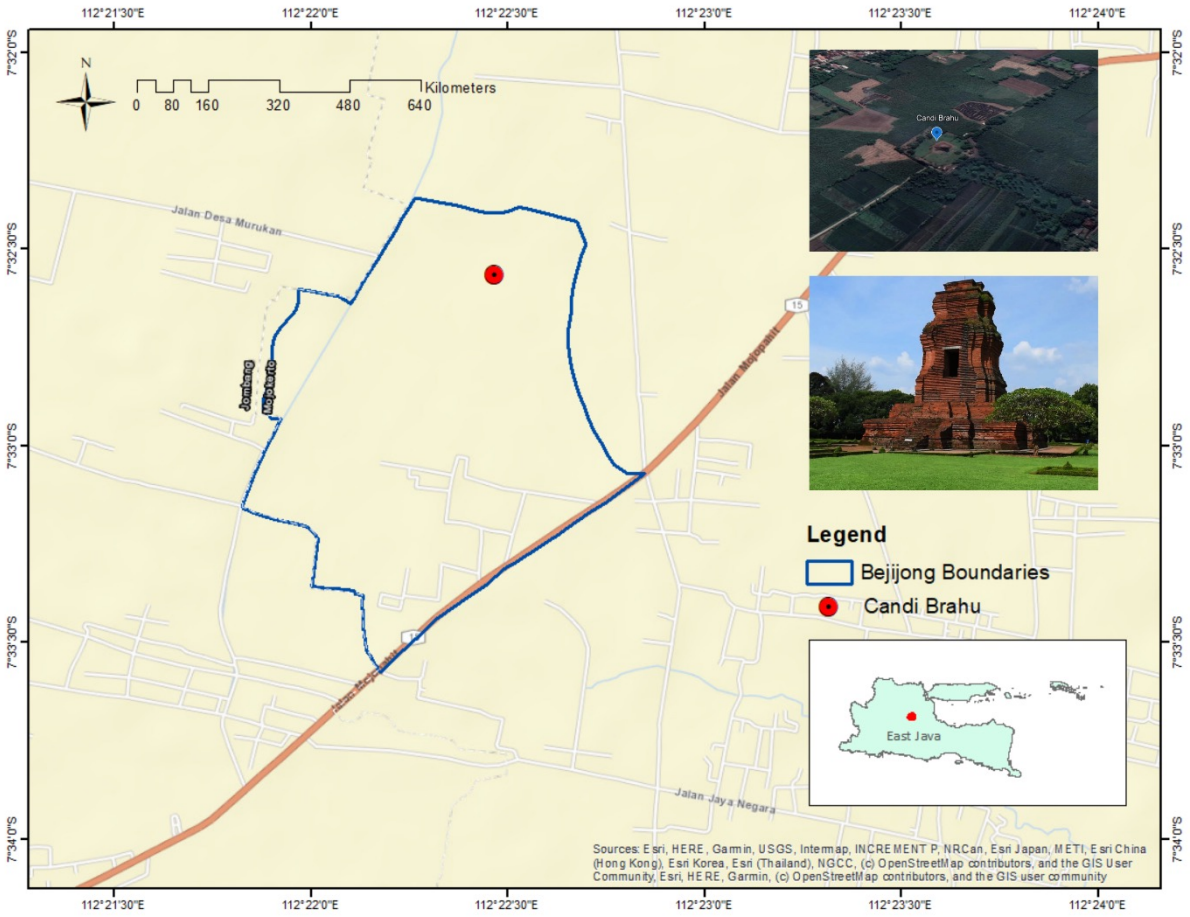

Figure 1. Location of Brahu Temple, Mojokerto, East Java, Indonesia

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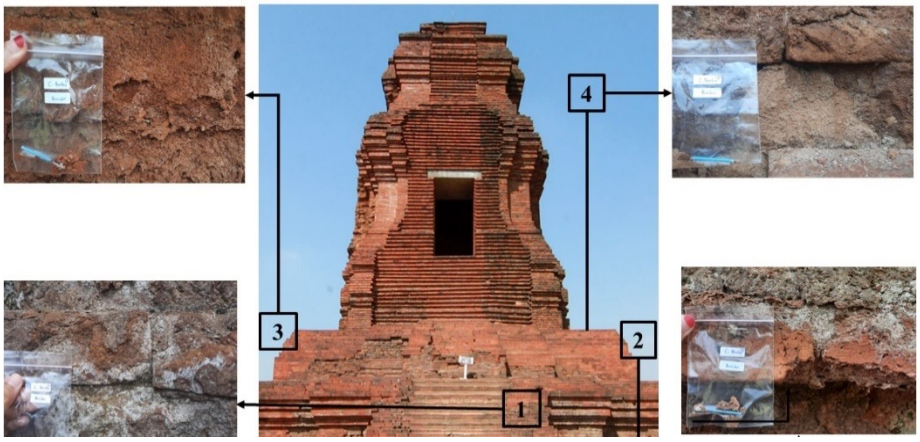

Figure 2 . Bricks sampling point at Brahu Temple (1. Front side, 2. Right side, 3. Left side, 4. Back side).

Clear Zone Test

The clear zones in bacteria were measured to assess the ability to produce organic acids. During the observation of clear zones, a modified selective medium consisting of Nutrient Agar (NA) supplemented with 1% (w/v) $CaCO_3$ and 1% (w/v) sucrose (Shah 2019) was used. In addition, observation was performed using the spot inoculation method on a selective medium, and the media was then incubated at room temperature for 1 – 5 days. Organic acids-producing bacteria were indicated by clear zones (Shah, 2019), which were then measured for the diameter using the clear zone index formula with the following equation.

$$\text{Clear Zone Index for Organic Acid} = \frac{\text{Diameter of clear zone}}{\text{Diameter of colony bacterial}}$$

Phenotypic Characterization

The screened isolates were characterized based on direct observation of bacterial morphology, including colony shape, elevation, and margin. Characterization was continued with biochemical tests and physiological reactions of bacterial strains, which consisted of the oxygen requirement test, OF test (oxidative fermentative test), motility test in SIM (Sulfide Indol Motility) medium, and Ziehl-Neelsen method for acid-fast staining. Bacterial cell characteristics were observed using a light microscope to observe gram characteristics, cell shape, and the presence of endospores. The enzymatic potential of the strains was determined using qualitative tests for amylase, urease, catalase, and laccase enzymes (Mulyawati et al., 2019; Zulaika et al., 2016).

Dendrogram Construction

The characterization data of isolates were converted into binary digits, and tabulated in the next table. The letters "N" and "T" represented the number of organisms and the upper triangular portion of the matrix, and the data were inputted into a text editor or notepad application. The analysis was performed using the Multi-Variate Statistical Package software, and the similarity values between isolates were calculated using the Simple Matching Coefficient. In addition, the values obtained were grouped using the Unweighted Pair Group Method with Averages algorithm. Cophenetic correlation analysis was conducted by comparing unsorted and sorted similarity matrices. A correlation index (R) equal to or greater than

0.7 was considered acceptable, and when the correlation value was ≤0.7, the dendrogram formed did not represent the similarity matrix that served as the foundation (Vane-Wright, 2017).

Generic Assignment

The screened isolates that had been constructed underwent generic assignment based on key characteristics following the guidelines of Bergey's Manual of Determinative Bacteriology up to the genus level (Holt & Smith, 1994).

RESULTS AND DISCUSSION

Isolation and Clear Zone Test

Among the 15 organic acid-producing bacterial isolates obtained through the isolation process, only 7 isolates exhibited the ability to produce organic acids effectively, as evidenced by the formation of clear zones. These 7 isolates were identified as CB.V3.C, CB.V3.D, CB.V3.F, CB.V4.B, CB.V4.C, CB.V6.A, and CB.V6.C. Clear zones and the measurement calculation clear zone indicated for each isolate could be observed in Figure 3 and Table 1

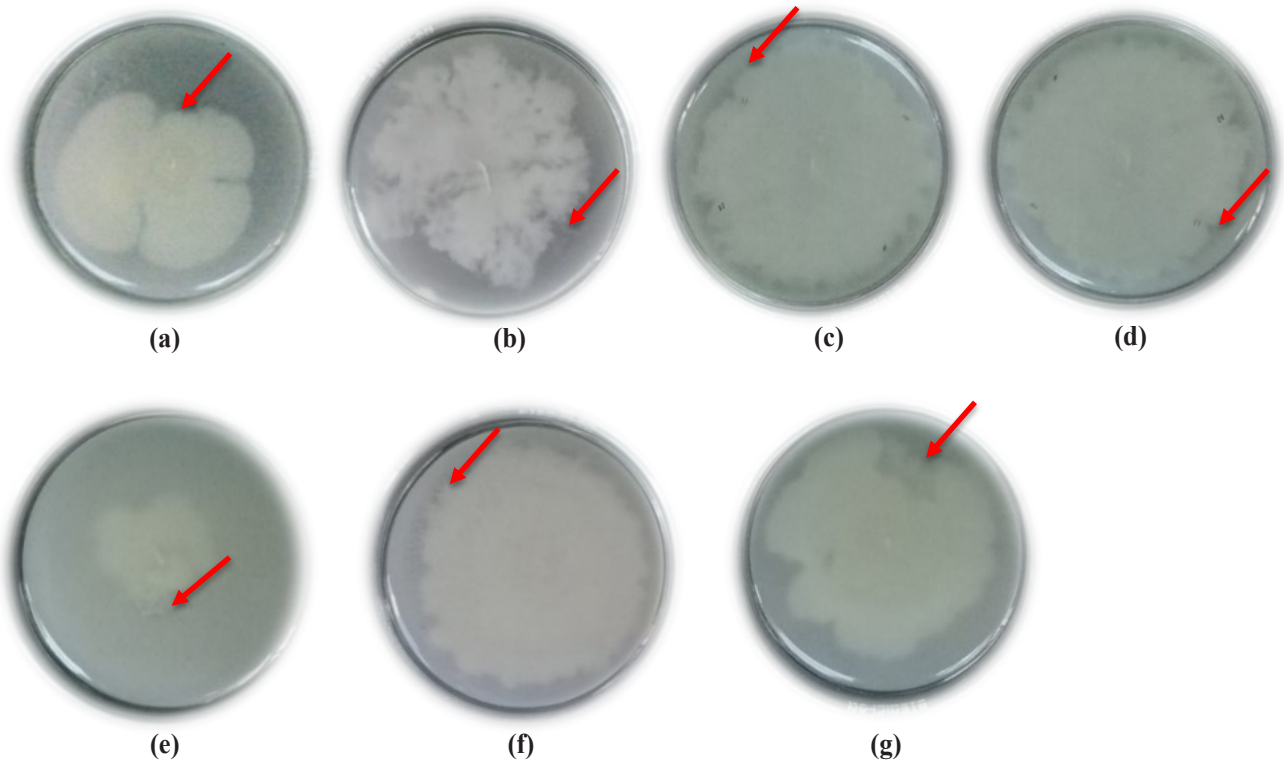

Figure 3. Clear zone formed in isolate a.) CB.V6.C, b.) CB.V3.F, c). CB.V3.C, d.) CB.V3.D, e.)CB.V4.B, f.) CB.V4.C, g.) CB.V6.A.

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Table 1. The calculation results of clear zone index for bacterial isolates producing organic acids.

No.	Isolate	Clear Zone Index (cm)
1.	CB.V3.C	0.137
2.	CB.V3.D	0.111
3.	CB.V3.F	0.129
4.	CB.V4.B	0.088
5.	CB.V4.C	0.060
6.	CB.V6.A	0.028
7.	CB.V6.C	0.059

$CaCO_3$ in the media served as an indicator of organic acids produced by bacteria, and the sucrose in the media served as a carbon source that could be used by bacteria during the fermentation process. According to Shah et al (2019), $CaCO_3$ could be precipitated in the presence of organic acids resulting from bacterial metabolism, and these reduced the pH value of the media, subsequently, breaking down $CaCO_3$ by forming calcium hydrogen carbonate compounds $(Ca(HCO_3)^+$ through the interaction between hydrogen ions (H^+) and calcium ions (Ca^+). Additionally, other carbonate ions (CO_3^{2-}) could precipitate and form clear zone around bacterial colonies (Natasia et al., 2020). Organic acids produced by bacteria, such as oxalic acid, lactic acid, and gluconic acid, could act as chelating agents and demineralize rock substrates including calcium, iron, magnesium, and manganese (Munawati et al., 2017). The width of the clear zone can serve as an indicator of increased bacterial metabolic activity in enzyme production during the $CaCO_3$ degradation process, which is also associated with a decrease in the pH of the medium. According to Kigawa et al. (2013), a wider clear zone formed during $CaCO_3$ degradation corresponds to a more acidic pH in the medium (Kigawa et al., 2013).

Phenotypic Characteristic

Phenotypic characterization comprised the assessment of morphological colony, biochemistry, and physiology traits. Gram staining results, all isolates showed gram-positive bacilli cells presented in Figure 4, and the following were the results of gram staining of bacterial isolates.

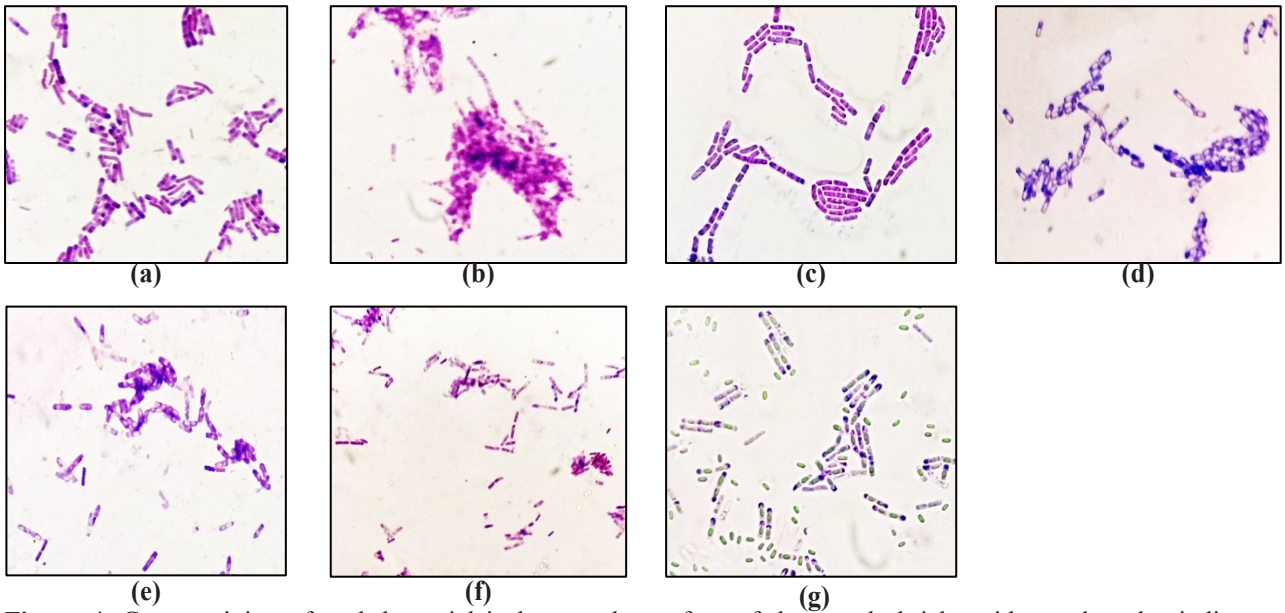

Figure 4. Gram staining of each bacterial isolate on the surface of the temple bricks with purple color indicates gram-positive bacteria and all of shape bacterial are bacilli. a.) CB.V3.C, b.) CB.V3.D, c.) CB.V3.F, d.) CB.V4.B, e.) CB.V4.C, f.) CB.V4.A, g.) CB.V6.C.

Regarding oxygen demand testing, there were isolates categorized as aerobic and facultative anaerobic isolates, and in the Oxidative-Fermentative test, fermentation was observed in all isolates. Fermentation was the process of decomposing organic compounds by microbes under limited or no oxygen (Hanson, 2016), and 5 isolates were found to synthesize the amylase enzyme. This enzyme was responsible for hydrolyzing organic molecules, like amylum, into simple compounds such as dextrin (Prescott, 2002). All isolates tested positive for the catalase enzyme, 4 isolates were positive for the urease enzyme, and 1 isolate was positive for the laccase enzyme, and this was an oxidoreductase enzyme that contributed to lignin degradation, alongside lignin peroxidase and manganese peroxidase enzymes that oxidize various compounds such as ortho- and para-diphenols, aminophenols, polyphenols, polyamines, aryl diamines. Additionally, some inorganic ions were involved in reducing 2 molecules of O_2 to H_2O (Efiyanti & Hidayat, 2017). The phenotypic characteristics of the selected isolates are summarized in Table 2.

Dendrogram Construction and Generic Assignment

The selected and phenotypically characterized isolates were then analyzed using phenetic numerical taxonomy method. The cluster analysis result employing the Simple Matching Coefficient (SSM) method based on phenotypic characters revealed that the test strains were divided into 3 clusters, and this included clusters A, B, and C with a similarity index of 0.543-0.857 (Figure 5). he phenetic correlation coefficient index value calculation in dendrogram construction was 0.811, and the acceptable correlation coefficient index was ≥ 0.700 (Sokal, 1986). Therefore, the dendrogram represented the similarity matrix, and that was the basis for the construction. The lower the cophenetic correlation value, the lower the reliability of the obtained dendrogram, thereby this did not represent the similarity matrix on which it was based (Afrelia et al., 2021).

The genus identification results showed that cluster A (CB.V6.C, CB.V4.B, CB.V4.C, and CBV3.F) belonged to the genus Bacillus characterized by bacilli (rod)-shaped cell, Gram-positive, positive motility, endospore positive, facultative anaerobic or aerobic, chemoorganotroph, and positive catalase. According to Holt & Smith (1994), members of the genus Bacillus typically exhibited rod-shaped cells, occasionally arranged in chains, and possessed the capability to produce endospores. Members of the genus Bacillus exhibited motility with peritrichous flagella, although some were non-motile. Additionally, the genus Bacillus was Gram-positive, capable of fermenting carbohydrates with gas production, positive for catalase, either aerobic or facultatively anaerobic, and saprophytic (Holt & Smith, 1994).

Cluster B (CB.V3.D) was identified as the genus Corynebacterium, characterized by rod-shaped cells, Gram-positive staining, motility, non-acid resistant, facultative anaerobic, chemoorganotroph, and positive catalase activity, and this was widely distributed in soil. According to Holt & Smith (1994), members of the genus Corynebacterium had straight to slightly curved rod-shaped cells with irregularly stained segments, and these members were non-motile, Gram-positive, catalase-positive, and mostly aerobic, although some were microaerophilic or even anaerobic. These species could or could not ferment sugars, but many species of the genus Corynebacterium oxidized glucose to CO_2 and H_2O without producing gas (Holt & Smith, 1994).

The genus Mycobacterium, characterized by rod-shaped cells, Gram-positive staining, motility (either positive or negative), acid

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Table 2. Phenotypic characteristics of bacteria isolates producing organic acids.

Character	Bacteria Isolate						
	CB.V3.C	CB.V3.D	CB.V3.F	CB.V4.B	CB.V4.C	CB.V6.A	CB.V6.C
Dry Colony	+	+	-	-	-	+	-
Moist Colony	-	-	+	+	+	-	+
Form Colony							
Irregular	-	-	-	+	-	+	-
Circular	+	-	-	-	-	-	+
Filamentous	-	+	+	-	+	-	-
Margin Colony							
Undulate	-	+	-	+	-	-	+
Entire margin	+	-	-	-	-	-	-
Erose/Creanate	-	-	-	-	+	+	-
Filamentous	-	+	+	-	-	-	-
Elevation Colony							
Flat colony	-	+	-	+	-	+	-
Convex colony	-	-	-	-	-	-	+
Raised colony	+	-	+	-	+	-	-
Cell form							
Diplobacillus	-	-	+	+	+	-	+
Streptobacillus	+	+	-	-	-	+	-
Gram Staining							
Gram-positive	+	+	+	+	+	+	+
Gram-negative	-	-	-	-	-	-	-
Endospore							
Endospore	-	-	+	+	+	-	+
Biochemical test and physiological response							
Aerobic	+	-	-	-	-	-	-
Facultative anaerobe	-	+	+	+	+	+	+
Oxidative Fermentative test	+	+	+	+	+	+	+
Motility test	+	-	+	+	+	+	+
Acid-fast staining	+	-	-	-	-	+	-
Enzyme							
Amylase	+	+	-	+	+	+	+
Urease	+	-	+	-	-	+	+
Catalase	+	+	+	+	+	+	+
Laccase	+	-	+	-	-	+	-

Note : (+) positive test result and (-) negative test result

resistance, endospore, aerobic, and positive catalase activity was identified as Cluster C (CB.V6.A and CB.V3.C). This aligned with the characteristics of the genus Mycobacterium as described by Holt and Smith (1994), which stated that members of this genus were characterized by rod-shaped cells that were straight or slightly curved, non-motile, aerobic, and fluorescent or red when stained with acid-fast stains (Holt & Smith, 1994).

According to Qi Wang et al (2011), the Firmicutes phylum, specifically from the genus Bacillus, was majorly found in weathering bricks of old buildings, and over 50% of the 7 selected isolates belonged to the genus Bacillus. This observation was supported by the ability of Bacillus to form endospores, enabling bacteria to spread widely from a prolonged resting stage and withstand various nutrient limitations, drought, salinity, temperature fluctuations, radiation, and redox changes (Qi-Wang et al., 2011).

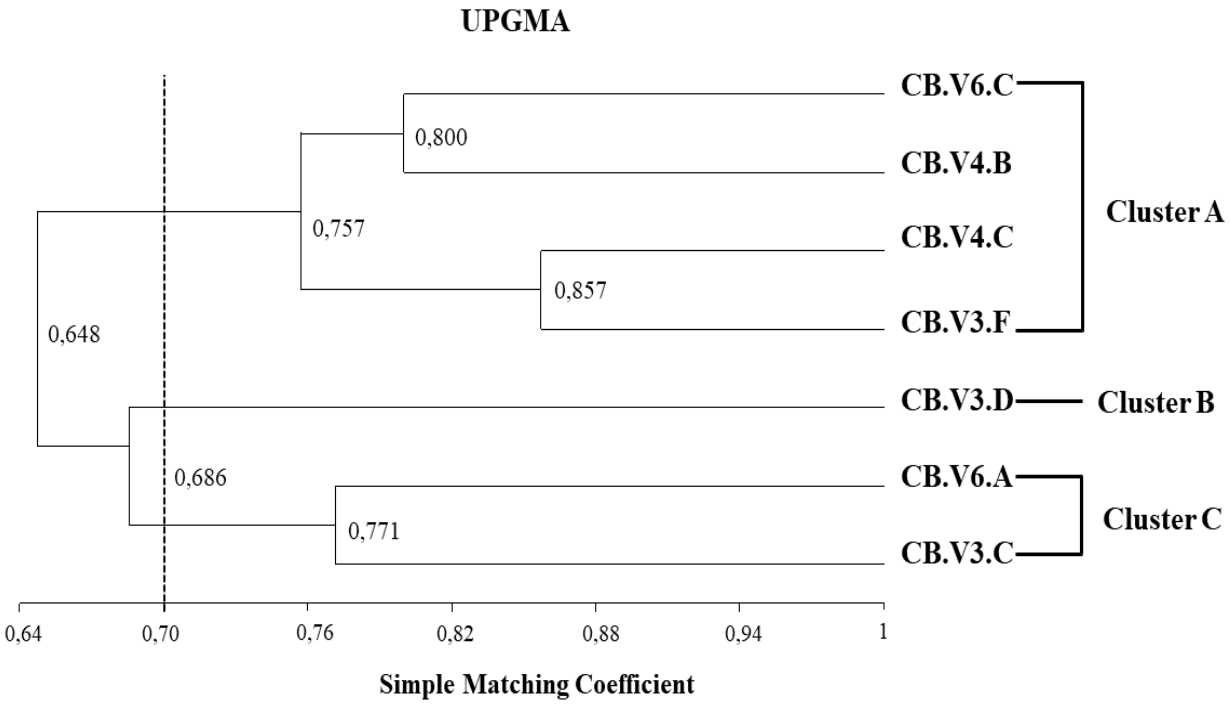

Figure 5. Dendrogram of Organic Acid Producing Bacteria (UPGMA, Unweighted Pair Group Methods Averages. The numbers on nodes indicated the similarity values among isolates, and arrows indicated the minimum accepted similarity matrix values).

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CONCLUSION

In conclusion, the dendrogram of bacteria isolated from temple bricks showed the presence of 3 clusters, namely A, B, and C with a range of similarity index values of 0.543 - 0.857. However, profile matching categorized bacterial isolates into 3 genera namely *Bacillus* (Cluster A), *Mycobacterium* (Cluster C), and *Corynebacterium* (Cluster B). The results showed that Cluster A constituted 50% of bacterial isolates from surface temple bricks.

AUTHOR CONTRIBUTION

L.A: Contribution to collected data, conducted experiment, analyzed, an interpreted the data. Drafted the manuscript and critically revised it for important intellectual content. Approved the final version to be published. **E.Z** and **S.S** : Concept and design research. Contributed to the drafting and revision of the manuscript. Provided critical feedback on intellectual content. Approved the final manuscript for submission.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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