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Molecular Identification of Endophytic Bacteria Isolated from *Allium cepa* L. Waste as Antibiofilm Agent Against *S. mutans*

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Abstract. *Streptococcus mutans is a key contributor to dental caries due to its ability to form resilient biofilms. Endophytic bacteria are a promising source of natural antibiofilm agents. This study aimed to isolate and characterize endophytic bacteria from red onion (Allium cepa L) waste with potential antibiofilm activity against S. mutans. This research employed an exploratory laboratory design with descriptive and quantitative approaches. Morphological and Gram-staining analyses were conducted alongside molecular identification through 16S rRNA sequencing. Antibacterial and antibiofilm assays were performed to evaluate the biological activity of isolate EA1. Absorbance values from biofilm inhibition and degradation tests were analyzed using descriptive statistics to calculate inhibition percentages. Isolate EA1 exhibited strong biofilm degradation (77% inhibition), moderate inhibition of biofilm formation (53%), and relatively lower antibacterial activity (30% growth inhibition). Molecular analysis confirmed its identity as a member of the Enterobacter genus, closely related to E. tabaci and E. asburiae. EA1 holds significant potential as a source of novel antibiofilm compounds for controlling S. mutans. Further investigation is needed to characterize its active metabolites and evaluate its clinical applicability.*

Keywords*: antibiofilm, caries, endophyte, red onion, S. mutans*

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INTRODUCTION

Red onion (*Allium cepa* L.) is a major horticultural commodity widely cultivated in Indonesia, particularly in Enrekang Regency, South Sulawesi. According to the Central Statistics Agency (BPS) of Enrekang Regency (2024), red onion production reached 1,759,330 quintals in 2023, with a harvested area of 13,669 hectares in 2022—a 38% increase from the previous year. This rise in cultivation area was accompanied by a 20% increase in production, reflecting its growing agricultural significance.

Endophytic bacteria are microorganisms that inhabit plant tissues without harming to their host. These bacteria have been identified in various crops, including eggplant (Achari & Ramesh, 2018), peanuts (Wang & Liang, 2014) and pistachio (Etminani & Harighi, 2018). Studies have also reported the presence of endophytic bacteria in red onion tissues (Sudewi et al., 2023), indicating their potential as sources of bioactive compounds with diverse biological activities (Sogandi et al., 2019), including antibiofilm properties.

Dental caries remains one of the most prevalent oral diseases globally, *Streptococcus mutans* plays a central role in the development of dental caries, a condition that, if left untreated, can lead to chronic oral infections, tooth loss, and systemic complications such as cardiovascular disease and diabetes. These health outcomes underscore the importance of early intervention and effective biofilm-targeted strategies to mitigate the broader impacts on human health (Gao Z et al., 2023; Pu X et al., 2024). Its development is influenced by several factors, including dietary habits, oral hygiene, and the presence of cariogenic bacteria such as *Streptococcus mutans*, which are capable of forming biofilms (Tong et al., 2020). Biofilms are microbial communities embedded in self-produced extracellular polymeric substances that confer resistance to antimicrobial agents and host immune responses (Ciofu et al., 2022; Jo et al., 2022; Mahamud et al., 2022).

Given the potential of endophytic bacteria to produce novel antibiofilm agents, investigating isolates from red onion may provide promising alternatives for preventing or controlling biofilm-related infections caused by *S. mutans*.

MATERIALS AND METHODS

Research Design and Sample Collection

This study employed an exploratory laboratory design to isolate and identify endophytic bacteria from the red onion (*Allium cepa* L.) cultivated in Enrekang Regency, South Sulawesi, Indonesia. The research involved isolating and characterizing endophytic bacteria from red onion (*Allium cepa* L.) waste. Initial isolation, purification, and preliminary screening were conducted at the Molecular Biology Laboratory, Universitas Negeri Makassar. Further molecular identification through 16S rRNA gene sequencing and phylogenetic analysis was performed at PT. Genetika Science Indonesia. The study was carried out over a period from June to September 2024.

Isolation of Endophytic Bacteria and Preparation of Pure Cultures

Plant samples (Root, Skin, and leaf) were collected from both healthy and diseased onion farms. Samples were washed thoroughly under running tap water to remove adhered soil particles. Surface sterilization was performed by sequential immersion in 70% ethanol for 1 minute, 2% sodium hypochlorite solution for 5 minutes, and sterile distilled water for 1 minute (three times). Using the spread plate method, the sterilized root tissues were aseptically macerated and inoculated on Nutrient Agar (NA) isolation media containing nystatin (0.01% b/v). Plates were incubated at 30°C for 48 hours, and morphologically distinct bacterial colonies were selected for further purification (Leonita et al., 2016).

Screening of Antibacterial Activity of Endophytic Bacteria against S. mutans

The antibacterial activity assay was performed by incorporating 0.4 mL of each pathogenic bacterial suspension into 80 mL of nutrient agar (NA) medium, then by pouring 20 mL into sterile dishes and solidifying them. The endophytic bacterial isolates were then inoculated using an inoculating loop onto the surface of the agar containing the test pathogen. Plates were incubated at 37 °C for 24–48 hours. The antibacterial activity was evaluated by measuring the diameter of the clear zone (inhibition zone) formed around the colonies. Isolates that produced distinct inhibition zones against the test pathogens were considered to possess promising antibacterial potential (Sogandi et al., 2022)

Biofilm Inhibition Assay

The biofilm inhibition assay was performed to evaluate the ability of endophytic bacterial isolates to inhibit the initial adhesion of *Streptococcus mutans* on the well surface of a 96-well microplate. The test began with preparing an endophytic bacterial suspension in broth medium, adjusted to a final concentration of 10^8 CFU/mL (OD_{600} = 0.1). A volume of 100 µL of the suspension from the most potent endophytic isolate and the positive control (0.1% chlorhexidine) and negative control (broth medium without bacteria) were added to each well. The plate was incubated at 37°C for 24 h. After incubation, the wells were gently washed three times with sterile physiological saline to remove non-adherent cells. Subsequently, 100 µL of *S. mutans* suspension was added to each well, followed by incubation at 37°C for 48 h to allow biofilm formation. Post-incubation, the wells were washed three times with sterile saline to eliminate planktonic cells. The remaining adhered biofilm was stained using 0.1% crystal violet for 15 minutes. The excess stain was removed by rinsing with sterile distilled water. To quantify the biofilm, 200 µL of 96% ethanol was added to dissolve the bound dye and incubated at room temperature for 15 minutes (Kining et al., 2022). The ethanol-crystal violet solution was then transferred to a cuvette and the absorbance was measured at 600 nm using a UV-Vis spectrophotometer. The degree of biofilm inhibition was determined based on the reduction in absorbance compared to the control groups.

% Inhibition of Attachment =

$$\frac{\text{OD negative control - OD experimental sample}}{\text{OD negative control}} \times 100\%$$

Biofilm Degradation Assay

This assay evaluated the capacity of endophytic bacterial extracts to degrade pre-formed *Streptococcus mutans* biofilms. Initially, *S. mutans* suspension was prepared and distributed into the wells of a sterile 96-well microplate, similar to the biofilm prevention assay. The plate was then incubated at 37°C for 48 h to allow biofilm formation. Following incubation, the culture medium was carefully removed, and 100 µL of the endophytic bacterial suspension (10^8 CFU/mL, OD_{600} = 0.1), as well as the positive control (0.1% chlorhexidine) and negative control (broth medium only), was added to each well. The plate was incubated again at 37°C for 24 h. After the treatment, the wells were washed three times with sterile saline to remove non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet for 15 minutes and rinsed with sterile distilled water. Subsequently, 200 µL of 96% ethanol was added to dissolve the dye retained by the biofilm, and the plate was incubated for 15 minutes at room temperature (Kining et al., 2022). The ethanol solution was then transferred into a cuvette, and the absorbance was measured at 600 nm using a UV-Vis spectrophotometer to determine the extent of biofilm degradation.

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% Inhibition of Cell Degradation =

$$\frac{\text{OD negative control - OD experimental sample}}{\text{OD negative control}} \times 100\%$$

Biofilm Growth Test

This assay was conducted to evaluate the effect of endophytic bacterial extracts on the growth and maturation of *Streptococcus mutans* biofilms during extended incubation. The procedure followed the same setup as the biofilm prevention assay. A total of 100 µL of *S. mutans* suspension and 100 µL of endophytic bacterial suspension (10^8 CFU/mL, OD_{600} = 0.1) were co-inoculated into each well of a sterile 96-well microplate. The plate was incubated at 37°C for 48 h to allow simultaneous biofilm development in the presence of endophytic bacteria. After incubation, the wells were gently washed three times with sterile saline to remove non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet for 15 minutes and then washed with sterile distilled water to remove excess dye. Subsequently, 200 µL of 96% ethanol was added to each well to solubilize the retained crystal violet, and the plate was incubated for 15 minutes at room temperature (Kining et al., 2017). The ethanol solution was then transferred to a cuvette, and absorbance was measured at 600 nm using a UV-Vis spectrophotometer to assess the level of biofilm formation.

% Cell Growth Inhibition =

$$\frac{\text{OD negative control - OD experimental sample}}{\text{OD negative control}} \times 100\%$$

Morphological and Presumptive Identification of Isolate Endophytic Bacterial

The characterization of endophytic bacterial isolates was conducted through macroscopic and microscopic observations. Macroscopic characterization included the assessment of colony morphology such as shape, margin, elevation, and pigmentation on solid media. Microscopic characterization was performed using the Gram staining technique. The bacterial smears were observed under a light microscope at 1000× magnification to determine the cell morphology and Gram reaction of each isolate (Leonita et al., 2016)

16S rRNA Molecular Identification and Phylogenetic Analysis

The endophytic bacterial isolate exhibiting the highest antibacterial activity was subjected to species-level molecular identification. Genomic DNA was extracted using the Quick-DNA Magbead Plus Kit (Zymo Research, D4082) following the standard protocol (B/7.2.1/IKP/009). Amplification of the 16S rRNA gene was conducted using the MyTaq HS Red Mix, 2X kit (Bioline, BIO-25048) (B/7.2.1/IKP/002), and the PCR products were analyzed by electrophoresis (B/7.2.1/IKP/005). The amplified 16S rRNA gene products were subjected to bidirectional sequencing using the Sanger sequencing method based on Capillary Electrophoresis (conducted by 1st BASE Subcontract Lab Testing). The resulting sequence data were analyzed through bioinformatics tools for species identification (B/7.2.1/IKP/006).

RESULTS AND DISCUSSION

This research began by collecting red onion (*Allium cepa*) agricultural waste from Cakke Village, Anggeraja District, Enrekang Regency one of the largest red onion-producing regions in the area. Samples were collected immediately after harvest and consisted of unutilized parts of the red onion plant, including leaves, skins, and roots.

Isolation of Endophytic Bacteria

Endophytic bacteria were isolated from each plant part using standard surface sterilization and plating techniques. Isolates

were coded based on tissue origin: ED (leaves), EK (skin), and EA (roots). Six morphologically distinct isolates (ED1, ED2, EK1, EK2, EA1, EA2) (Figure 1) were selected for further analysis. The diversity of isolates reflects host-related factors such as plant age, tissue type, soil conditions, and geography, which influence bacterial colonization (Hardoim et al., 2015; Compant et al., 2019; Triandriani et al., 2020).

Antibacterial Activity Screening of Endophytic Bacteria

Of the six tested isolates, three (ED1, EK2, and EA1) (Figure 2) demonstrated inhibitory activity against *Streptococcus mutans*, shown by the formation of inhibition zones **(Table 1)**. This confirms the potential of endophytic bacteria from red onion waste as natural antibacterial agents, consistent with previous findings (Sogandi & Nilasari, 2019). Endophytic bacteria can synthesiz pharmacologically active compounds due to the close relationship and interaction between endophytes and their hosts (Yang et al., 2016). The isolate EA1, derived from root tissue, exhibited the highest antibacterial activity with an inhibition zone of 10.19 mm, classified as moderate (Davis & Stout, 1971). This activity is likely due to the production of bioactive metabolites such as antimicrobial enzymes or secondary metabolites (Suhendar et al., 2021).

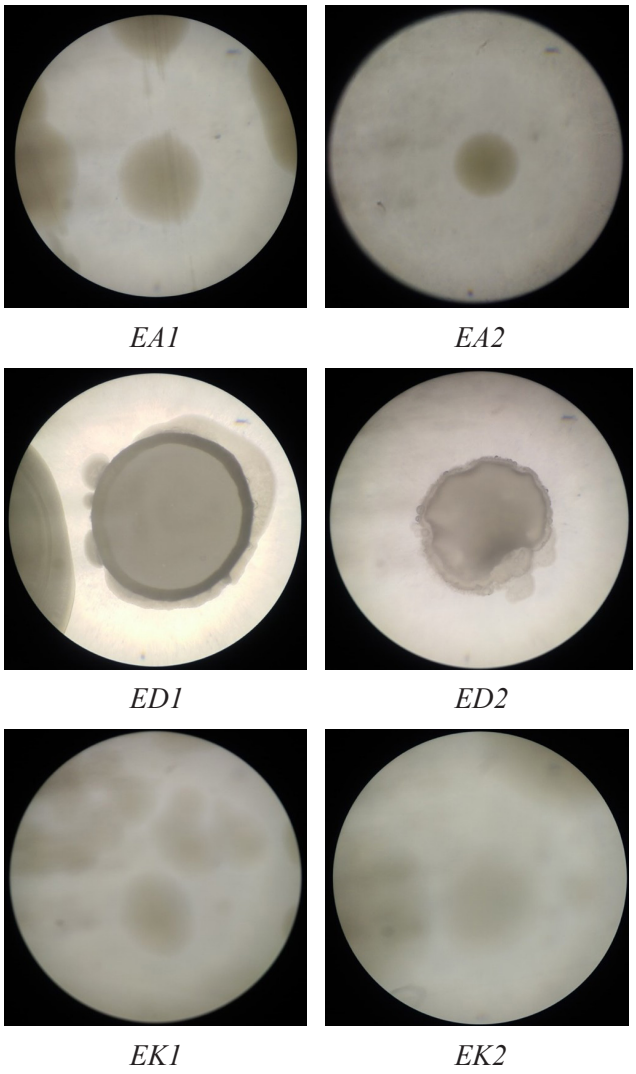

Figure 1. Endophytic bacteria were isolated from each plant part

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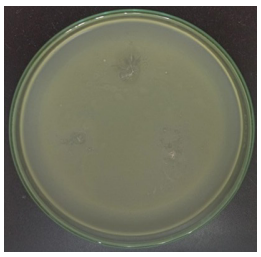
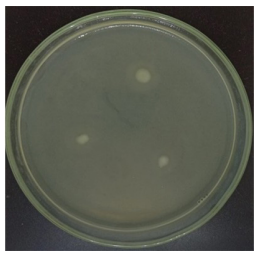
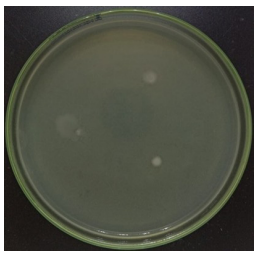
Sample	Source Tissue	Inhibition Zone
ED1	Leaf	
EA1	Root	
EK2	Skin	

Figure 2. Antibacterial activity of endophytic bacteria against *S. mutans*

Table 1. Antibacterial activity of endophytic bacteria against *S. mutans*

Sample	Source Tissue	N	Inhibition Zone (mm)	Activity Classification	95% CI	Grouping
EA 1	Root	3	10,190 ± 0,98	Moderate	(9,317; 11,063)	A
ED 1	Leaf	3	7,517 ± 0,44	Moderate	(6,644; 8,389)	B
EK 2	Skin	3	4,9433 ± 0,06	Moderate	(4,0706; 5,8161)	C

Pooled StDev = 0,617783
Means that do not share a letter are significantly different. Grouping Information Using the Tukey Method and 95% Confidence

Antibiofilm Activity of Isolate EA1

Isolate EA1 showed strong antibiofilm properties across three activity types (Figure 3). The inhibition of biofilm attachment reached 53%, indicating a significant preventive effect. This aligns with findings by Caruso et al. (2023), who reported inhibition values of 49–57% by endophytic bacteria from Eremophila longifolia. EA1 also outperformed the 41% inhibition observed in papaya leaf extract (Kining et al., 2017), the fungal extract isolated from Moringa oleifera was able to inhibit the formation of S. aureus, K. pneumoniae, and C. albicans biofilms by 69.2%, 57.66%, and 55%, respectively (Kaur et al., 2020), highlighting red onion root endophytes as promising biofilm-preventing agents. In terms of biofilm degradation, EA1

achieved an inhibition rate of 77%, surpassing previous studies which reported 49.029% degradation using water leaf extract of papaya (*Carica papaya* L.) against *Pseudomonas aeruginosa* (Kining et al. 2017). For biofilm growth inhibition, EA1 demonstrated moderate activity with 30% inhibition. This is consistent with Ramírez-Puebla et al. (2024), who observed 20–40% inhibition among root-derived endophytes and Elkhouly et al (2021) studied the metabolism of the endophytic fungus *Aspergillus Tubenginses* ASH4 isolated from *Hyoscyamus muticus*, the endophytic extract was able to suppress the formation of the *P. aeruginosa,* and *E. coli* biofilms by 28.44% and 37.68%, respectively.

The inhibition of *S. mutans* biofilm formation by plant-derived agents is a multifactorial process that involves the interaction of phytochemicals with bacterial molecular targets and cellular pathways (Cho et al., 2022). The inhibition of *S. mutans* biofilm formation by isolate EA1 is likely associated with multiple mechanisms. These include interference with bacterial adhesion, where plant-derived phenolics (e.g., catechins, citrus extracts) block surface receptors or compete for binding sites. Additionally, flavonoids and tannins may inhibit the activity of glucosyltransferases (Gtf), reducing the synthesis of extracellular polysaccharides (EPS) essential for biofilm structure. EA1 may also modulate quorum sensing (QS) by downregulating genes involved in EPS production and biofilm regulation. Furthermore, the disruption of cell membrane integrity by phenolic compounds or essential oils can increase membrane permeability, weakening bacterial cell stability and inhibiting biofilm maturation (Atazhanova et al., 2024). Overall, EA1 exhibited triple-action antibiofilm activity preventing attachment, degrading biofilms, and inhibiting growth supporting the development of multi-target antibiofilm agents. Additional molecular and biochemical assays are necessary to confirm the proposed mechanisms underlying the antibiofilm activity of isolate EA1. These results strengthen the position of endophytic bacteria, particularly from red onion roots, as a valuable source of natural anti-biofilm compounds.

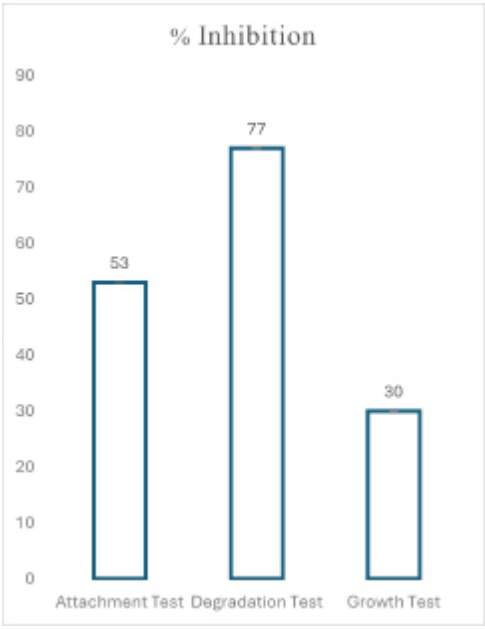


Figure 3. The inhibition of *S. mutans* antibiofilm of EA1

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Morphological and Presumptive Identification of Isolate EA1

Morphological observations (Figure 4) revealed that isolated EA1 forms round colonies with irregular edges, convex elevation, and non-sporulating characteristics. Microscopic analysis following Gram staining showed that EA1 appears pink under safranin staining and does not retain crystal violet, indicating it is a Gram-negative rod.

Based on these characteristics and reference to Bergey's Manual of Determinative Bacteriology, EA1 is presumed to belong to the genus *Enterobacter*, a member of the *Enterobacteriaceae* family commonly found as plant endophytes (Davin-Regli et al., 2019).

Certain *Enterobacter* strains exhibit antimicrobial activity and function as biocontrol agents (Ananda et al., 2019). They produce protease, amylase, and cellulase, enzymes that contribute to the inhibition of pathogenic bacteria (Ahmad et al., 2008). The genus has been widely reported in association with various plants, including *Aloe vera* (Akinsanya et al., 2015), and has shown antimicrobial activity against multiple pathogens, with inhibition zones reported between 6.0–57 mm (Khalifa et al., 2016).

Molecular Identification of Isolate EA1

The molecular analysis of isolate EA1 revealed a DNA concentration of 12.2 ng/µL, with an A260/280 ratio of 1.90 and an A260/230 ratio of 1.01, indicating high DNA purity with minimal protein contamination. Electrophoresis results (Figure 5) showed a clear DNA band at ~1400 bp, matching the target region, and no bands in the negative control, confirming the absence of contamination.

Sequence assembly generated a 1409 bp 16S rRNA gene, which was analyzed using BLAST. The results indicated a high similarity to sequences from the genus *Enterobacter* (Table 2), supporting morphological identification. The top matches included *Enterobacter tabaci*, *E. ludwigii*, *E. asburiae*, and *E. cloacae*. Interestingly, the closest hit was an unclassified *Bacterium strain ZYM9001*, suggesting that EA1 may represent a novel strain.

A phylogenetic tree constructed using the Neighbor-Joining method (Figure 6) demonstrated EA1's taxonomic placement within the Enterobacter genus, showing close evolutionary relationships with multiple *Enterobacter* species. This analysis supports EA1's potential novelty and reinforces its relevance for further study.

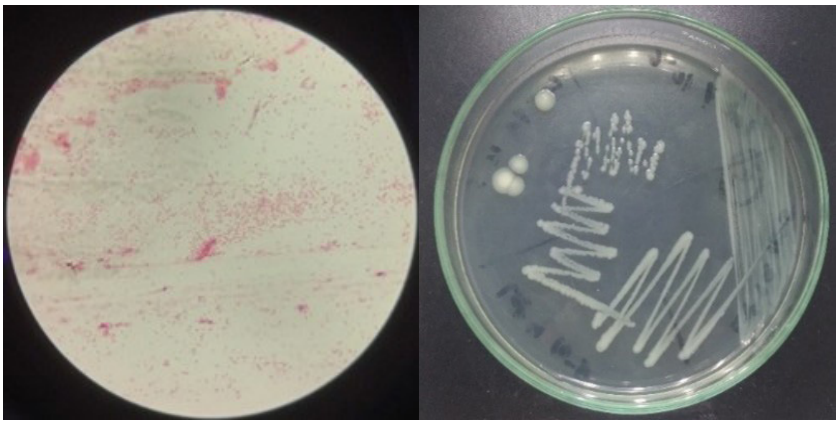

Figure 4. Gram staining results of EA1

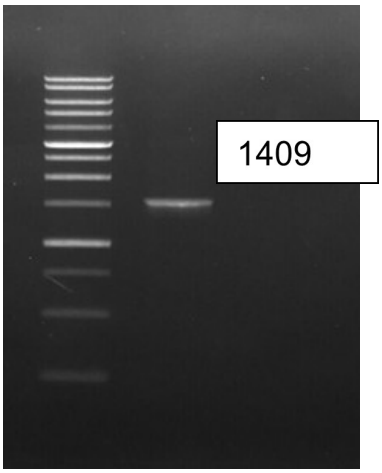

Figure 5. Electrophoresis results of 16S rRNA gene amplification products

Table 2. BLAST Analysis Results of EA1

No	Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
1	*Enterobacter* sp. FF16 16S ribosomal RNA gene, partial sequence	2558	2558	99%	0.0	99.50%	KR778808.1
2	*Enterobacter* sp. SR19 16S ribosomal RNA gene, partial sequence	2556	2556	99%	0.0	99.43%	KF896099.1
3	*Enterobacter cloacae* strain Os_Ep_VPA_27 16S ribosomal RNA gene, partial sequence	2553	2553	99%	0.0	99.43%	MN932286.1
4	*Bacterium* strain ZYM9001 16S ribosomal RNA gene, partial sequence	2553	2553	99%	0.0	99.36%	PP990591.1
5	*Enterobacter* sp. strain Md1-53 16S ribosomal RNA gene, partial sequence	2553	2553	99%	0.0	99.43%	MF581459.1
6	*Enterobacter* sp. strain ICMP 663 16S ribosomal RNA gene, partial sequence	2553	2553	99%	0.0	99.36%	PP103237.1
7	*Bacterium* strain CH9 16S ribosomal RNA gene, partial sequence	2553	2553	99%	0.0	99.43%	KY069038.1
8	*Enterobacter asburiae* strain MSSRF QS66 16S ribosomal RNA gene, partial sequence	2553	2553	99%	0.0	99.36%	KJ877656.1
9	*Enterobacter mori* strain P3 16S ribosomal RNA gene, partial sequence	2551	2551	99%	0.0	99.36%	MK774801.1
10	*Enterobacter ludwigii* strain AA1 16S ribosomal RNA gene, partial sequence	2549	2549	99%	0.0	99.29%	MT613360.1

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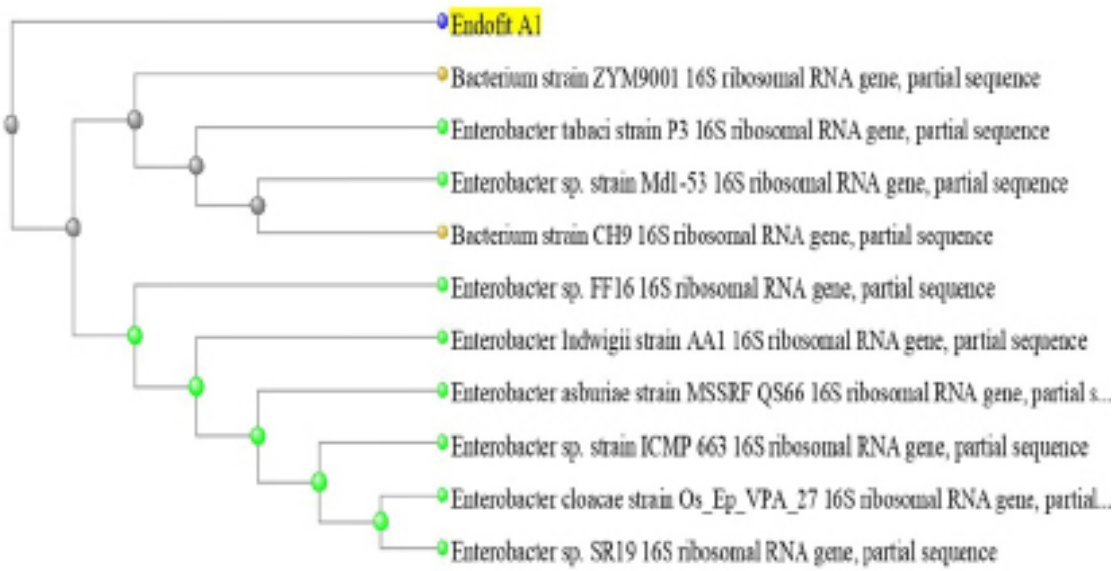

Figure 6. Phylogenetic tree of EA1

CONCLUSION

The endophytic bacterium EA1, isolated from red onion (*Allium cepa*) waste, demonstrates promising antibacterial and antibiofilm activity against *Streptococcus mutans*. Morphological and molecular analyses identified EA1 as a member of the *Enterobacter* genus, closely related to *E. tabaci* and *E. asburiae*. The isolate showed strong biofilm degradation (77%) and moderate inhibition of biofilm formation (53%), indicating its potential as a multi-target antibiofilm agent. These findings support the potential application of EA1 or its metabolites in preventing and treating biofilm-related infections caused by *S. mutans*. However, further studies are necessary to isolate and characterize its bioactive compounds, explore genomic features, and evaluate its safety and effectiveness in vivo.

AUTHOR CONTRIBUTION

1. Ekajayanti Kining: Coordinate all research activities, prepare proposals, develop antibiofilm test methods, make research reports, and prepare publication manuscripts.
2. Sogandi: Collaborated with the chairman to develop the proposal and conducted molecular identification of potential endophytic bacteria.
3. Dian Firdiani: Identified bacteria macroscopically and microscopically, together with the chairman analyzed the data and drafted the publication manuscript.
4. Muh. Achyar Ardat: Assisting in preparing tools and materials, site surveys, sampling, accompanying testing activities, and documentation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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