Quagga
Jurnal Pendidikan dan Biologi

p-ISSN 1907-3089, e-ISSN 2615-5869
Vol 18, No. 1, 2026, pp 32 - 38
Available online https://journal.fkip.uniku.ac.id/quagga
DOI: 10.25134/quagga.v18i1.470

# Antibacterial Activity of Clove-derived Endophytic Bacteria of *Niallia nealsonii* DCL1 and *Bacillus paranthracis* DCL3

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APA Citation: Quraini, N. N., Priyanto, J. A., Astuti, R. I. (2026). Antibacterial Activity of Clove-derived Endophytic Bacteria of Niallia nealsonii DCL1 and Bacillus paranthracis DCL3. *Quagga: Journal of Education and Biology*, 18(1), 32-38. DOI: 10.25134/quagga.v18i1.470.

Received: 04-06-2025 Accepted: 22-07-2025 Published: 06-01-2026

***Abstract.*** *Pathogenic bacterial infection is still a major health problem that causes death. Endophytic bacteria in medicinal plants have the potential as antibacterial agents. Thus, endophytic bacteria in the medicinal plant, clove (Syzygium aromaticum), allow it to produce the same bioactive compounds as those contained in its host. This study aimed to analyze the antibacterial activity of five endophytic bacterial isolates from clove leaves. Antibacterial activity was evaluated using disc diffusion assay. Based on the results, isolate Niallia nealsonii DCL1 have strong antibacterial activity against Escherichia coli ATCC 8739, while Bacillus paranthracis DCL3 isolate has intermediate antibacterial activity against Pseudomonas aeruginosa ATCC 15442. In addition, extracellular metabolites of these two isolates of DCL1 and DCL3 which harvested at stationary phase showed antibacterial activity against and E. coli ATCC 8739 and P. aeruginosa ATCC 15442. These data indicate that the potential antibacterial compounds from DCL1 and DCL3 are considered extracellular secondary metabolites. Further analysis on the mode of antibacterial action should be carried out for further medical application.*

***Keywords:*** *antibacterial, disc diffusion, endophytic bacteria, pathogenic bacteria*

## 1. INTRODUCTION

Pathogenic bacterial infections remain one of the leading causes of disease and death worldwide (WHO, 2020). Antibiotics are a primary treatment for bacterial infections; however, their extensive use has contributed to the growing issue of antibiotic resistant (Larsson and Flach, 2021). Traditional herbal medicine, based on medicinal plants, is often used as an alternative treatment for bacterial infections in limited settings. These herbal remedies are typically made from plants known for their health-promoting properties and their ability to treat infections (Anand et al., 2019; Rakhman et al., 2024). One such plant that has been traditionally used to treat infections is clove (*S. aromaticum*) (Cortés-Rojas et al., 2014).

Eugenol is one of the bioactive compound derived from clove that exhibits antioxidant, anti-inflammatory, antitumor, and antimicrobial properties (Afrendi et al., 2023; Amini et al., 2022; Barboza et al., 2018; Rahmi et al., 2021). Bioactive compounds from plants can be extracted through various methods, which typically require a large amount of plant biomass. Obtaining bioactive compounds directly from plants demands significant land use, time, and financial resources. Endophytic bacteria, which reside within plant tissues, are known to possess the ability to produce metabolites similar to those of their host plants (Singh et al., 2017).

In the previous study, we successfully isolated five endophytic bacteria, namely *B.*

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*cereus* DCN1 (OP363657.1), *B. cereus* DCT2 (OP363659.1), *N. nealsonii* DCL1 (OP363654.1), *C. kochii* DCL2 (OP363655.1), and *B. paranthracis* DCL3 (OP363656.1) (Utami et al., 2023). Several endophytic bacteria have been reported to produce bioactive compounds with similar or identical characteristics to those produced by their host plants. The antimicrobial potential of bioactive compounds from plant endophytes has been previously reported. For instance, *B. pseudomycoides* strain DJ8 and *Bacillus* sp. isolated from *Archidendron pauciflorum* (jengkol), which were shown to inhibit the growth of pathogenic bacteria such as *Staphylococcus aureus* and antibiotic-resistant *P. aeruginosa* strain M18 (Priyanto et al., 2023a, 2023b).

To date, studies on the antibacterial activity of endophytic bacteria from clove remain limited. Therefore, this study aims to analyze the antibacterial activity of five endophytic bacterial isolates from clove against pathogenic bacteria *S. aureus* ATCC 6538, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 15442. The findings of this study may reveal the potential application of metabolites derived from clove endophytic bacteria as antibacterial agents, which could be incorporated into various health-related products such as antiseptics or disinfectants.

## 2. RESEARCH METHOD

### *Bacterial cultivation*

Each bacterial isolate from stock cultures was inoculated into sterile media appropriate to its original growth medium. *B. cereus* DCN1 was cultured on Nutrient Agar (NA); *N. nealsonii* DCL1, *C. kochii* DCL2, and *B. paranthracis* DCL3 were grown on Luria-Bertani (LB) Agar; and *B. cereus* DCT2 was cultured on Tryptic Soy Agar (TSA). The cultures were incubated at approximately 28°C for 24 hours. The grown endophytic bacterial isolates were then inoculated into liquid media and incubated for 24 or 40 hours at ~28°C in a rotary shaker at 120 rpm. The bacterial cultures were subsequently used for antibacterial activity assays. Following incubation, the bacterial suspensions were centrifuged at 5000 rpm for 15 minutes to obtain the supernatant (Utami et al., 2023).

### *Screening of antibacterial activity*

The screening assay was conducted using the disc diffusion method (Dany et al., 2023). A total of 20 µL of the supernatant from each endophytic bacterial culture was applied onto a 6 mm sterile paper disc until fully absorbed. Each supernantat was obtained from 24 hours bacterial culture. The discs were then placed on Nutrient Agar (NA) plates previously inoculated with the target bacteria at a concentration of $10^8$ CFU/mL. The plates were incubated at approximately 28°C for 24 hours at room temperature. The target bacteria used were *S. aureus* ATCC 6538, *E. coli* ATCC 8739, and *P. aeruginosa* ATCC 15442, each at a concentration of $10^8$ CFU/mL. The inhibition zone was measured based on the diameter of the clear zone formed around the disc. Ciprofloxacin 500 mg (Ciprofloxacin®, Hexpharm Jaya, Indonesia) was used as the positive control, and sterile distilled water served as the negative control. Each test was performed in duplicate. The diameter of the inhibition zones was calculated using the inhibition zone formula (Figure 1), and antibacterial activity was categorized accordingly (Table 1).

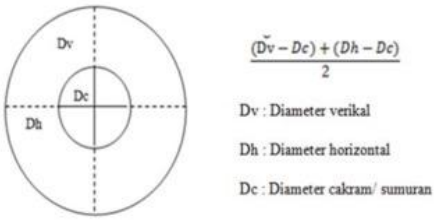


**Figure 1.** Formula for the inhibitory diameter zone (Gerstein et al., 2016)

**Table 1.** Category of inhibitory level based on the diameter of inhibitory zone (Priyanto et al., 2023a)

| Diameter (mm) | Inhibitory level |
|---|---|
| < 5 mm | *resistant* |
| 5-10 mm | *intermediet* |
| 10-20 mm | *strong* |
| > 20 mm | *very strong* |

### *Bacterial growth curve*

Endophytic bacterial isolates were inoculated to Luria Bertani (LB) broth medium, and incubated for 24 hours at ±28°C, as subculture. As much as 2.5 ml (5% v/v) were then transferred to 50 mL LB broth medium and incubated at ±28°C

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hours in a rotary shaker with agitation (120 rpm). Cell turbidity was measured by spectrophotometer at λ600 nm for each four hours for 48 hours (Asfahani et al., 2023).

***Antibacterial activity assay of Supernatant***

Culture of endophytic bacteria in a LB broth medium were incubated in a *rotary shaker* (120 rpm) at ±28°C for 40 hours. Antibacterial activity was measured using disc diffusion methods as described previously (Dany et al., 2023). Inhibition zone was marked by a clear zone around disc, and measured using the formula described previously. Ciprofloxacin was used as positive control (50 ppm). Assays were done in triplicates.

## 3. RESULTS AND DISCUSSION

***Screening of antibacterial activity***

Five isolates were tested for antibacterial activity against pathogenic bacteria, *S. aureus*, *E. coli*, and *P. aeruginosa*. Based on the screening assay, four isolates had antibacterial activities as shown by the development of clear zone (Table 2, Figure 2). *N. nealsonii* DCL1 was capable to strongly inhibit *E. coli,* yet moderately inhibit *S. aureus* (Figure 2A, B). However, this isolate could not inhibit the growth of *P. aeruginosa.* On the other hand, isolate *B. paranthracis* DCL3 showed the strongest inhibitory effect against *P. aeruginosa*, compared to other endophytic bacteria tested (Table 2, Figure 2C). Interestingly, no inhibitory activity was exhibited by *B. cereus* DCN1 against all pathogenic bacteria.

**Table 2**. Antibacterial activity assay by clove-derived endophytic bacterial supernatant of 24-hour culture, using disc diffusion method, against pathogenic bacteria.

| **Bacterial isolate** | **Diameter of inhibition zone (mm) ± SD** | | |
|---|---|---|---|
| | *S.a* [ATCC 6538] | *E.c* [ATCC 8739] | *P.a* [ATCC 15442] |
| *Niallia nealsonii* DCL1 | 4,5 ± 0,7 | 19 ± 1,4 | 0 |
| *Cytobacillus kochii* DCL2 | 3,0 ± 1,4 | 9,5 ± 4,9 | 2,0 ± 0 |
| *Bacillus paranthracis* DCL3 | 1,5 ± 0,7 | 3,5 ± 2,1 | 10,5 ± 2,1 |
| *Bacillus cereus* DCN1 | 0 | 0 | 0 |
| *Bacillus cereus* DCT2 | 0 | 0 | 2,5 ± 2,1 |
| *Ciprofloxacin (positive control)* | 25,3 ± 0,8 | 27 ± 1,0 | 26,6 ± 0,7 |

Note : *S.a: Staphylococcus aureus; E.c: Escherichia coli; P.a: Pseudomonas aeruginosa*

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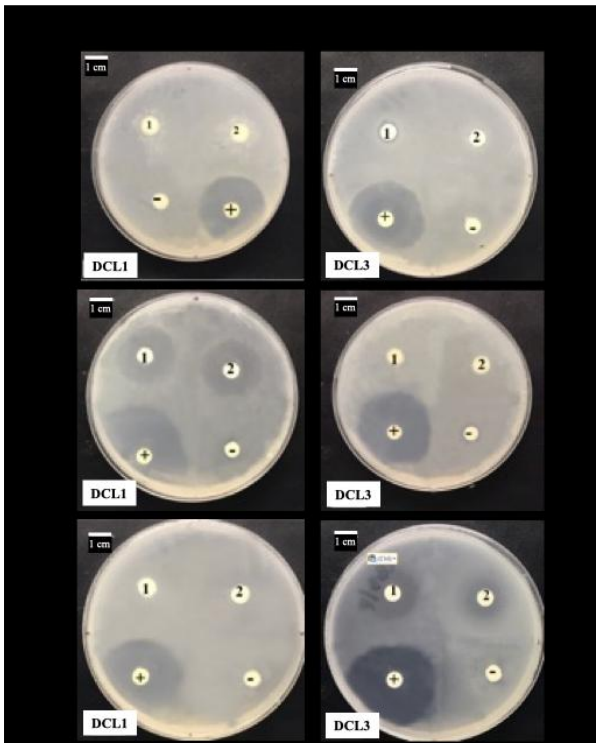


**Figure 2.** Antibacterial activity of endophytic bacteria *Niallia nealsonii* DCL1 and *Bacillus paranthracis* DCL3 against pathogenic bacteria (A) *Staphylococcus aureus* ATCC 6538, (B) *Escherichia coli* ATCC 8739 and (C) *Pseudomonas aeruginosa* ATCC 15442. 24 hours of supernatant from each endophytic bacteria was put on top of the disc and placed on top of agar medium containing pathogenic bacteria. Plates were incubated for 48 hours. 1 and 2 indicate replication, ciprofloxacin and sterile water were assigned as positive (+) and negative (-) control, respectively.

Based on the screening assay, isolate DCL1 was found as the most potential isolate to inhibit the growth of pathogenic bacteria. The distinct antibacterial activity elicited by endophytic bacteria might be occurred due to the different profile of secreted antibacterial metabolites as well as their antibacterial activities (Priyanto et al., 2023a). The development of clear zone shows the activity of secreted metabolites by endophytic bacteria in inhibiting the growth of pathogenic bacteria. Such antibacterial activities can be delivered by various mode of actions including modulation of metabolism activity, inhibition of cell wall formation, disruption of the permeability of membrane cells, and impairment of nucleic acid synthesis (Kapoor et al., 2017; Raheem and Straus, 2019) .

***Bacterial growth curve***

The analysis of the growth of endophytic bacterial isolates is required to determine the growth phases. Most of the antibacterial metabolites produced by microbes are generally secondary metabolites which are produced and potentially secreted during the stationary phase.

The bacterial isolates *N. nealsonii* DCL1 and *B. paranthracis* DCL3 went lag phase for four hours after inoculation (Figures 5A and B). Following the lag phase, each isolates were then enter exponential phase as indicated by an increase in absorbance values. Both of the isolates experienced log phased for 12 hours, which then continued with stationary phase up to 48 hours. The growth curve data indicate that *N. nealsonii* DCL1 and *B. paranthracis* DCL3 share the same late stationary phase at hour 40, which is considered the optimal time for harvesting secondary metabolites from the endophytic bacteria.

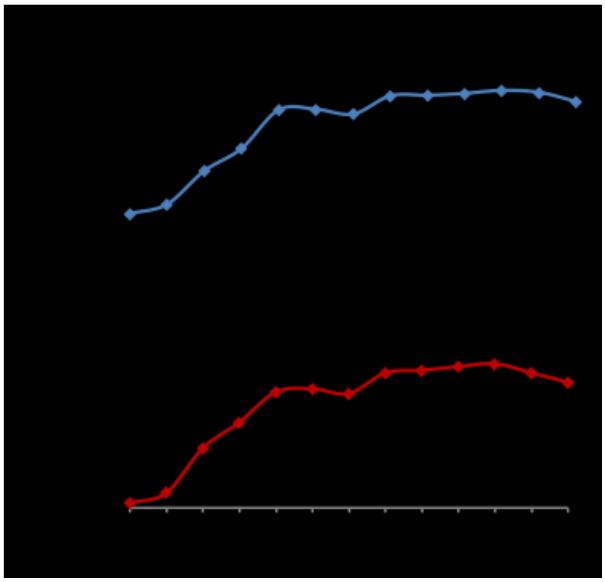

**Figure 3.** The growth curve of clove endophytic bacteria (A) *Niallia nealsonii* DCL1 and (B) *Bacillus paranthracis* DCL3 in Luria Bertani medium, at ±28°C.

***Antibacterial activity assay of Supernatant***

By using supernatant from 40 hours of culture, we found that no significant increase of antibacterial activity of DCL1 towards pathogenic

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bacteria E. *coli* compared to that 24 hour of culture. Similarly, to that activity of DCL3 towards *P. aeruginosa* (Table 3). However, it is worth noting that DCL1 showed strong antibacterial activity against *E. coli*.

**Table 3**. Antibacterial activity assay by clove-derived endophytic bacterial supernatant of 40 hour culture, using disc diffusion method, against pathogenic bacteria.

| Bacterial isolate | Diameter of inhibition zone (mm) ± SD | |
|---|---|---|
| | *E.c* [ATCC 8739] | *P.a* [ATCC 15442] |
| *Niallia nealsonii* DCL1 | 21 ± 1 | 0 |
| *Bacillus paranthracis* DCL3 | 4,3 ± 1,1 | 10 ± 6,2 |
| *Ciprofloxacin* | 32 ± 1 | 33,3 ± 1,5 |

Note: *E.c: Escherichia coli; P.a: Pseudomonas aeruginosa*

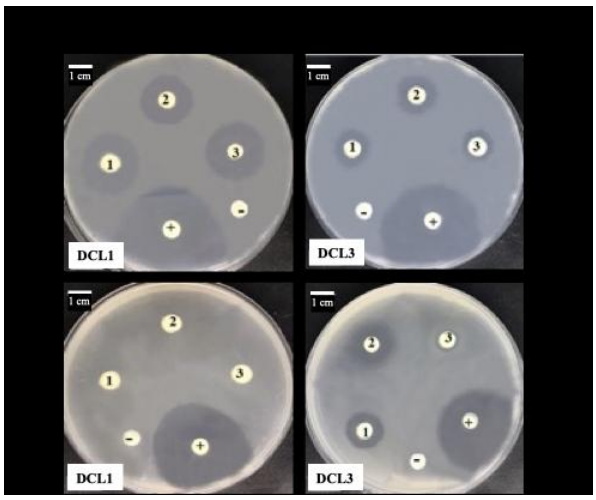


**Figure 4.** Antibacterial activity of endophytic bacteria *N. nealsonii* DCL1 and *B. paranthracis* DCL3 against pathogenic bacteria (A) *Escherichia coli* ATCC 8739 and (B) *Pseudomonas aeruginosa* ATCC 15442. 40 hours of supernatant from each endophytic bacteria was put on top of the disc and placed on top of agar medium containing pathogenic bacteria. Plates were incubated for 48 hours. 1, 2 and 3 indicate replication, ciprofloxacin and sterile water were assigned as positive (+) and negative (-) control, respectively.

Antibacterial activity is categorized wide spectrum due to its ability in inhibiting both Gram negative and positive bacteria (Almulhim and Alotaibi, 2018). In this study, DCL1 and DCL3 are regarded to have narrow spectrum of antibacterial activity as both inhibited Gram negative bacteria only. Gram positive bacteria has thicker cell walls compare to that Gram negative bacteria. Such characters are reported to maintain cell integrity following environmental stress, including antibacterial exposure. Disruption over cell wall in Gram positive bacteria often limit cellular lysis since supported by the thickness of cell wall (Jubeh et al., 2020). It is well-known that Gram positive bacteria such as *S. aureus* is one of the multidrug resistant pathogenic bacteria that generated a great challenge to treat the infections (Nandhini et al., 2022).

## 4. CONCLUSION

Endophytic bacterial isolates from S. aromaticum leaves exhibit antibacterial activity against pathogenic bacteria. Of the five bacterial isolates tested, the endophytic bacteria *N. nealsonii* DCL1 and *B. paranthracis* DCL3 were found to have potential antibacterial activity against the pathogenic bacteria *E. coli* ATCC 8739 and *P. aeruginosa* ATCC 15442. The antibacterial activity of metabolites from *N. nealsonii* DCL1 harvested during the stationary phase (40 hours of incubation) was classified as strong against *E. coli*, while *B. paranthracis* DCL3 showed intermediate activity against *P. aeruginosa* ATCC 15442

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