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Antibacterial Efficacy of Endophytic Bacterial Fractions from Basil Leaves (*Ocimum sanctum*) against *Streptococcus mutans* and *Pseudomonas aeruginosa*

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Abstract: Endophytic bacteria in *Ocimum sanctum* are promising antimicrobial sources, yet the localized efficacy of their intracellular versus extracellular metabolites against oral pathogens remains underexplored. This study compared the cell mass (CM) and cell-free supernatant (CFS) fractions of six *Bacillus* sp. isolates (EO1–EO6) against *Streptococcus mutans* and *Pseudomonas aeruginosa*. Statistical analysis (Two-Way ANOVA, Tukey's HSD, $p < 0.05$) revealed that the antibacterial efficacy is highly strain-dependent and target-specific. Against the Gram-positive *S. mutans*, the CFS of isolates EO5 and EO6 demonstrated significantly superior inhibition (5.69 ± 0.83 mm and 5.54 ± 0.55 mm, respectively) compared to their CM fractions. Conversely, all fractions exhibited uniformly weak and non-significant inhibition against the Gram-negative *P. aeruginosa*. In conclusion, *Bacillus* sp. CFS effectively inhibits *S. mutans* through robust extracellular secretion, suggesting strong potential for development into preventative oral therapeutics.
Keywords: antibacterial activity; *Bacillus* species; endophytic bacteria; *Ocimum sanctum*

INTRODUCTION

Herbal plants are valuable sources of bioactive compounds with therapeutic potential, including antimicrobial properties. Basil (*Ocimum sanctum*), widely used in traditional medicine, is particularly rich in secondary metabolites such as essential oils, flavonoids, and phenolics, which are known to exhibit antibacterial activity against pathogens like *Streptococcus mutans* and *Pseudomonas aeruginosa*.[1–3] Beyond the plant's own metabolites, the endophytic bacteria residing within its tissues have emerged as a promising resource that contributes to the host plant's biological activities.[4] Furthermore, these endophytes are capable of producing their own antimicrobial compounds, offering a novel avenue for drug discovery.[5]

In the oral ecosystem, *S. mutans* is a primary etiological agent of dental caries, while *P. aeruginosa* is an opportunistic pathogen associated with infections in compromised oral health.[6,7] The growing challenge of antibiotic resistance among such pathogens underscores the urgent need for alternative antibacterial agents. While previous studies have reported promising antibacterial activity from basil endophytes against bacteria like *E. coli* and *S. aureus*, the efficacy of their cell-free supernatants (CFS) has shown considerable variation.[8] This suggests that antibacterial effectiveness is highly influenced by the preparation method, particularly whether live cells are present.

A critical research gap exists in the comparative assessment of cell mass suspensions versus their cell-free supernatants, specifically against oral pathogens. Recent findings indicate that the activity of CFS against *S. mutans* and *P. aeruginosa* is often only weak to moderate, contrasting with the stronger activity of live cell suspensions.[6,9] This discrepancy may be attributed to several factors, including contact-dependent inhibition mechanisms where bioactive compounds remain cell-bound,[7] the instability or volatility of metabolites in suspension,[10] their production in low concentrations,[9] or the loss of crucial synergistic interactions between the cells and their extracellular products.[7] Consequently, the mechanisms underlying the reduced activity of CFS require comprehensive investigation.

Therefore, this study aims to systematically compare the antibacterial potential CM and CFS from basil endophytic bacteria against *S. mutans* and *P. aeruginosa*. Specifically, it seeks to evaluate their relative efficacy, identify the factors responsible for the low activity in supernatants, and elucidate the contributions of contact-dependent mechanisms, metabolite stability, and cell-metabolite synergy. This comparative approach is essential for a holistic understanding of endophytic antibacterial mechanisms and for developing optimized strategies for bioactive metabolite production.

METHODS

Healthy young basil leaves (*Ocimum sanctum*) were obtained from the Department of Agriculture and Plantations of Aceh, Banda Aceh City. Young leaves were selected to minimize the degradation of bioactive compounds that typically occurs in older leaves. The pathogenic bacteria used for the assay were *Streptococcus mutans* ATCC 25175 and *Pseudomonas aeruginosa* ATCC 27853, obtained from the Veterinary Medicine Laboratory of Syiah Kuala University, Aceh Province, Indonesia.

The surface of the basil leaves (10 g) was sterilized sequentially using 70 % alcohol for 1 minute, 5% sodium hypochlorite (NaOCl) for 5 minutes, and rinsed again with 70% alcohol for 30 seconds followed by sterile distilled water. The leaf samples were then aseptically crushed in 9 mL of 0.9 % NaCl solution. The suspension was serially diluted (10^{-1} to 10^{-4}) and 0.1 mL was inoculated onto Nutrient Agar (NA) media using the spread plate method, then incubated at 37 °C for 24-48 hours. Pure isolates were characterized macroscopically (colony morphology) and microscopically (Gram and Endospore staining). Comprehensive biochemical tests were conducted, including Triple Sugar Iron Agar (TSIA), indole, motility, urease, Simon citrate, catalase, oxidase, and oxygen requirement tests for genus identification.[11]

Extracellular antibacterial compounds were produced by preparing a CFS. One loop of the

endophytic bacterial isolate was inoculated into 30 mL of Nutrient Broth (NB) and incubated in a rotary shaker (120 rpm, 37 °C) for 24 hours. The suspension was standardized using 0.9 % NaCl to reach a cell density of 10^8 CFU/mL. Thirty milliliters of this culture were then fermented into 270 mL of fresh NB media and reincubated with agitation at 120 rpm at 37 °C for 48 hours. At this point, the collection of endophytic bacterial cell mass is conducted. The cell culture was centrifuged at 10,000 rpm, 4 °C for 15 minutes to separate the cell pellet and the supernatant. The supernatant was then filtered using a 0.22 µm pore membrane filter to ensure the liquid was completely free of endophytic bacterial cells.[6,9]

The antibacterial activity of the CFS was evaluated using the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA), in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines.[12] The test pathogenic bacteria (*S. mutans* and *P. aeruginosa*) were suspended to a density equivalent to the 0.5 Mc Farland standard (approximately 1.5×10^8 CFU/mL) and swabbed evenly across the surface of the MHA plates. Sterile paper discs with 6 mm in diameter were placed on the inoculated media, followed by the addition of 20 µL of the cell-free supernatant onto each disc and the cell mass in distinct test dishes. A dose of 30 micrograms of chloramphenicol disc was used as a positive control. The petri dishes were incubated at 37 °C for 24 hours. The antibacterial efficacy was determined by measuring the vertical and horizontal diameters of the clear inhibition zones using a digital vernier caliper.

The extracellular antibacterial potential of the *Bacillus* sp. isolates was evaluated by testing their cell mass and cell-free supernatants against *S. mutans* ATCC 25175 and *P. aeruginosa* ATCC 27853 as pathogenic bacteria using the disc diffusion method. The inhibition zones were classified based on standard responses: weak (≤ 5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (≥ 20 mm) (13).

Quantitative data from independent triplicates are expressed as mean ± standard deviation (SD). After verifying data normality (Shapiro-Wilk test) and variance homogeneity (Levene's test), a Two-Way ANOVA was performed to evaluate the effects of bacterial isolates and fraction treatments. Significant differences were determined using Tukey's HSD post-hoc test ($p < 0.05$). All analyses were conducted using SPSS.

RESULTS

The isolation process from healthy young basil leaves (*Ocimum sanctum*) successfully yielded six distinct endophytic bacterial isolates, designated as EO1 through EO6. Figure 1 and Table 1 showed macroscopic observation of the colonies on NA media revealing morphological variations. The colony shapes varied from filamentous to circular and root-like, with flat to convex elevations, irregular to entire margins, and colors ranging from white and cream to transparent cream.

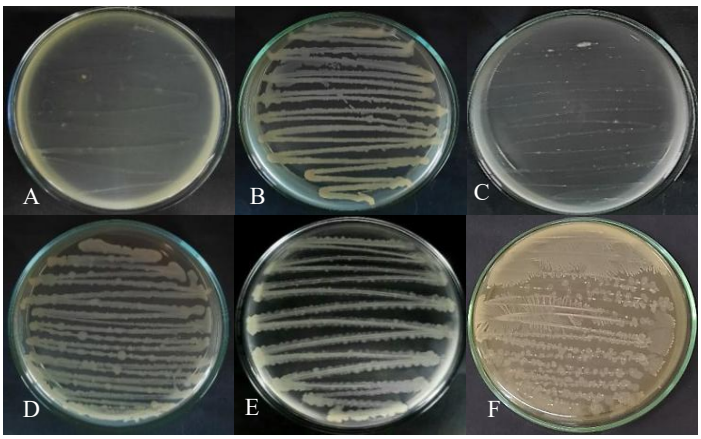

Figure 1. Macroscopic colony of endophytic bacteria from basil leaves (*Ocimum sanctum*), namely isolate: A. EO1; B. EO2; C. EO3; D. EO4; E. EO5; F. EO6

Table 1. Morphological characterization of endophytic bacteria from basil leaves (*Ocimum sanctum*)

No.	Isolate	Shape	Margin	Elevation	Color
1.	EO1	Filamentous	Filamentous	Flat	Cream
2.	EO2	Circular	Entire	Flat	Transparent cream
3.	EO3	Dotted	Irregular	Convex	White
4.	EO4	Circular	Entire	Flat	Cream
5.	EO5	Rhizoid	Filamentous	Flat	Cream
6.	EO6	Filamentous	Filamentous	Flat	Creamy white

Table 2 showed that biochemically all isolates exhibited negative results for the indole test, while demonstrating positive activities for both catalase and urease enzymes. The oxidase test was also positive across all isolates. In terms of motility, isolates EO2, EO3, EO5, and EO6 were motile, whereas EO1 and EO4 were non-motile. The Simon citrate test was positive only for isolate EO5. Furthermore, the TSIA test indicated that all isolates were capable of fermenting glucose, evidenced by a red slant and yellow butt. Oxygen requirement tests categorized all six isolates as facultative anaerobes. Based on these morphological, microscopic, and comprehensive biochemical characteristics, the six endophytic isolates were strongly identified as members of the genus *Bacillus* sp.

Table 2. Biochemical test of endophytic bacteria from basil leaves (*Ocimum sanctum*)

No.	Isolate	Gram	Indole	Motility	Catalase	Urease	Oxidase	Simmons citrate	Oxygen Req.	TSIA	Endo-spore	Genus
1.	EO1	+	-	-	+	+	+	-	FA	Ak/A	+	*Bacillus* sp. EO1
2.	EO2	+	-	+	+	+	+	-	FA	Ak/A	+	*Bacillus* sp. EO2
3.	EO3	+	-	+	+	+	+	-	FA	Ak/A	+	*Bacillus* sp. EO3
4.	EO4	+	-	-	+	+	+	-	FA	Ak/A	+	*Bacillus* sp. EO4
5.	EO5	+	-	+	+	+	+	+	FA	Ak/A	+	*Bacillus* sp. EO5
6.	EO6	+	-	+	+	+	+	-	FA	Ak/A	+	*Bacillus* sp. EO6

* +: positive test; -: negative test; FA: facultative anaerobic; Ak/A: Alkaline/Acid

Further microscopic and biochemical characterizations were systematically conducted to identify the genus of the isolates. Gram staining and microscopic observation showed that all six isolates (EO1 to EO6) were Gram-positive, rod-shaped bacteria (bacilli). The endospore staining test confirmed that all isolates were capable of forming green-stained endospores (Figure 2).

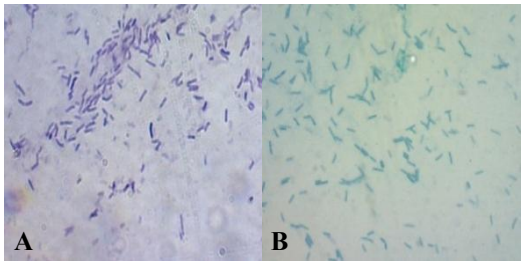

Figure 2. A. Positive Gram staining test; B. Positive endospora test

The activity assays demonstrated varying degrees of inhibition depending on the targeted pathogen. Against the Gram-positive *S. mutans*, the CFS of isolates EO5 and EO6 exhibited the highest efficacy, producing moderate inhibition zones with average diameters of 5.685 mm and 5.49 mm, respectively. The other isolates EO1, EO2, EO3, and EO4 displayed weak inhibitory activity against *S. mutans*, with zones ranging from 1.695 mm to 3.91 mm. The positive control (chloramphenicol 30 µg) showed strong to very strong inhibition zones ranging from 18.60 mm

to 21.24 mm against *S. mutans*.

Conversely, the CFS of all six endophytic isolates demonstrated weak antibacterial activity against the Gram-negative pathogen *P. aeruginosa*. The recorded inhibition zones across all treatments (EO1 to EO6) were less than 5 mm, ranging specifically from an average of 2.37 mm (EO5) to 3.41 mm (EO2). The positive control for *P. aeruginosa* produced moderate inhibition zones ranging from 5.45 mm to 8.13 mm.

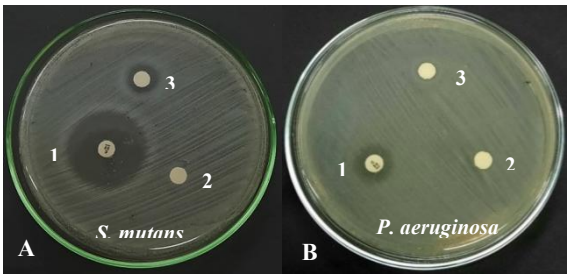

Figure 3. Best results inhibition by supernatant (3) A. *Bacillus* sp. EO5 and B. *Bacillus* sp. EO1 compared with aquadest/negative control (2) and chloramphenicol 30 µg/ positive control (1)

Table 3 showed the antibacterial efficacy of the CM and CFS fractions from six endophytic *Bacillus* sp. isolates (EO1–EO6), quantitatively assessed against two pathogenic bacteria. Data were expressed as the mean ± standard deviation (SD). Means sharing at least one common superscript letter within the same column were not significantly different ($p>0.05$), whereas different letters indicated significant differences ($p<0.05$) according to Tukey's HSD test.

Table 3. Antibacterial activity of two fractions of basil (*Ocimum sanctum*) leaf endophytic bacteria against *S. mutans* ATCC 25175 and *P. aeruginosa* ATCC 27853

Endophytic bacteria	Type of fractions	Inhibition zone diameter (mm) ± SD*	
		S. mutans ATCC 25175	***P. aeruginosa*** ATCC 27853
Bacillus sp. EO1	Cell mass	3.51 ± 1.06 ^{bcd}	3.16 ± 1.66 ^{b}
	Cell free supernatant	4.10 ± 0.99 ^{bcd}	3.34 ± 0.61 ^{b}
Bacillus sp. EO2	Cell mass	3.06 ± 0.81 ^{de}	2.99 ± 0.67 ^{b}
	Cell free supernatant	1.69 ± 0.63 ^{def}	3.42 ± 1.91 ^{b}
Bacillus sp. EO3	Cell mass	3.24 ± 1.34 ^{cd}	2.52 ± 0.34 ^{bc}
	Cell free supernatant	3.92 ± 1.65 ^{bcd}	2.71 ± 1.04 ^{b}
Bacillus sp. EO4	Cell mass	2.65 ± 1.29 ^{de}	1.68 ± 0.77 ^{bc}
	Cell free supernatant	3.05 ± 0.77 ^{de}	3.04 ± 0.40 ^{b}
Bacillus sp. EO5	Cell mass	1.67 ± 1.16 ^{def}	2.08 ± 0.81 ^{bc}
	Cell free supernatant	5.69 ± 0.83 ^{b}	2.37 ± 0.56 ^{bc}
Bacillus sp. EO6	Cell mass	0.66 ± 0.85 ^{ef}	2.21 ± 1.73 ^{bc}
	Cell free supernatant	5.54 ± 0.55 ^{bc}	2.83 ± 0.48 ^{b}
Positive control	Chloramphenicol 30 µg	20.84 ± 0.43 ^{a}	7.50 ± 0.97 ^{a}
Negative control	Aquadest	0.00 ± 0.00 ^{f}	0.00 ± 0.00 ^{c}

The data reveals a highly pathogen-specific and fraction-dependent trend. Against *S. mutans*, the CFS fractions of *Bacillus* sp. EO5 and EO6 exhibited the most prominent antibacterial activity, which was significantly superior ($p<0.05$) to their respective cell mass fractions and other isolates, as indicated by the distinct superscript letters. Conversely, a uniformly weak inhibitory trend was observed against *P. aeruginosa*, where neither the CM nor the CFS fractions of any isolate displayed statistically significant differences in their antibacterial efficacy, sharing common superscript notations across all treatments.

DISCUSSION

Bacillus species frequently interact synergistically with *Ocimum sanctum* leaf tissues, exhibiting robust tolerance to host-produced antimicrobial secondary metabolites, such as essential oils and eugenol. By forming environmentally resilient endospores and biofilms, these bacteria establish a stable symbiosis and produce diverse bioactive extracellular metabolites.[14] This highly adaptive microbial consortium not only demonstrates effective biocontrol capabilities against destructive pathogens[15] but also actively enhances the antioxidant capacity and essential oil yields of *Ocimum* species.[3]

This study systematically compared the antibacterial potential of CM and CFS from basil endophytic bacteria to address a critical gap in understanding their localized efficacy. As highlighted previously, several studies have reported that the activity of endophytic CFS against oral pathogens is often weak to moderate compared to whole-cell suspensions.[9] This discrepancy is frequently attributed to contact-dependent inhibition mechanisms,[16,17] low extracellular metabolite concentrations, or the instability of secreted compounds. However, our findings present a compelling paradigm shift.

Specifically, against the Gram-positive oral pathogen *S. mutans*, the CFS of *Bacillus* sp. isolates EO5 and EO6 exhibited significantly superior efficacy ($p<0.05$) compared to their CM fractions. This robust extracellular activity indicates that these specific strains do not rely on cell-metabolite synergy or contact-dependent mechanisms. Members of the genus Bacillus are a group of microbes with a large genomic capacity for secondary metabolite biosynthesis, enabling the synthesis of various bioactive secondary metabolites, including ribosomally synthesized bacteriocins and non-ribosomal cyclic lipopeptides with strong amphiphilic properties, such as surfactin and biosurfactants.[18,19] The pronounced susceptibility of *S. mutans* to these CFS fractions is fundamentally linked to its Gram-positive cell envelope architecture. While *S. mutans* possesses a thick peptidoglycan layer, this three-dimensional polymer network is highly porous and completely lacks an outer membrane lipid bilayer.[20] Consequently, the bioactive secondary metabolites typically secreted by *Bacillus* can readily diffuse through this porous barrier to reach the cytoplasmic membrane, inducing rapid cell lysis.

Furthermore, recent findings demonstrate that *Bacillus* CFSs not only exert direct bactericidal effects but also severely disrupt the cariogenic virulence of *S. mutans*. A 2023 study confirmed that the supernatant of *Bacillus velezensis* can strongly inhibit the biofilm formation of *S. mutans* and suppress its lactic acid production (acidogenesis), which is the primary driver of tooth enamel demineralization.[6] Therefore, from a clinical perspective, formulating a highly active, sterile cell-free supernatant into mouthwashes or topical dental therapeutics circumvents the safety and stability concerns associated with administering live bacterial cell suspensions, while directly neutralizing the primary etiological agent of dental caries.

In stark contrast to the results observed with *S. mutans*, all tested endophytic fractions displayed uniformly weak and statistically non-significant differences in their antibacterial activity against the Gram-negative *P. aeruginosa*. This disparity underscores that antibacterial effectiveness is heavily dictated by the fundamental anatomical and physiological defense systems of the target pathogen. The intrinsic multidrug resistance of *P. aeruginosa* is notoriously robust and relies on the synergistic action of its restricted outer membrane permeability and highly active efflux systems.[21] Gram-negative bacteria possess an asymmetrical outer membrane composed of a phospholipid inner leaflet and a lipopolysaccharide (LPS) outer leaflet. This dense, negatively charged structure creates a formidable, selective permeability barrier that drastically reduces the influx of large, hydrophobic, and amphipathic antimicrobial compounds, which are the primary constituents of *Bacillus* exudates.[22]

Furthermore, even if a fraction of these endophytic metabolites successfully breaches the outer membrane porins or lipid bilayer, *P. aeruginosa* is equipped to rapidly expel them via constitutive and inducible multidrug efflux pumps. The most prominent of these is the MexAB-OprM system, a tripartite complex belonging to the Resistance-Nodulation-Division (RND)

family. This complex spans the inner membrane, the periplasmic space, and the outer membrane, actively capturing diverse antimicrobial molecules including naturally occurring secondary metabolites and signaling molecules and extruding them directly into the extracellular medium.[23,24] This aggressive, continuous extrusion essentially prevents the endophytic metabolites from reaching their lethal threshold concentrations inside the cell. Consequently, this synergistic barrier-efflux mechanism effectively neutralizes both the cell-bound (cell mass) and extracellular (CFS) metabolites of the endophytic isolates, explaining the uniformly low inhibition zones observed against this opportunistic pathogen.

Ultimately, these findings elucidate that the localization of bioactive compounds (intracellularly bound versus extracellularly secreted) is highly strain-dependent. The successful pharmacological application of these endophytic metabolites requires a holistic understanding of both the producer's secretion capabilities and the formidable structural defenses of specific target pathogens.

Although the *Bacillus* sp. isolates from basil leaves show potential as antibacterial agents, the inhibitory activity, mostly classified as "moderate" to "weak," is highly likely due to a lack of growth curve optimization. In this study, the supernatant was harvested at the 48th hour without determining the specific growth phase. The highest production of secondary metabolites ideally occurs when the bacteria reach the stationary phase. Therefore, future research must map the growth kinetics of isolates EO5 and EO6 to determine the most optimal harvesting time for the CFS, as well as conduct further molecular identification.

CONCLUSION

This study demonstrates that the antibacterial efficacy of *Ocimum sanctum* endophytes is highly strain-dependent and target-specific. Notably, the cell-free supernatants of *Bacillus* sp. EO5 and EO6 significantly outperformed their cell mass against the cariogenic *Streptococcus mutans*. This indicates the active secretion of potent extracellular metabolites that directly disrupt the peptidoglycan layer without requiring cell-to-cell interaction. Conversely, *Pseudomonas aeruginosa* uniformly resisted all fractions, as its outer membrane barrier and efflux pumps effectively blocked these secreted metabolites. Ultimately, these findings position specific endophytic supernatants as promising, scalable candidates for preventative oral therapeutics.

Conflict of Interest

The authors declare that they have no competing interests.

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