Antibacterial and antifungal activities of *Bacillus* RM-10 isolated from the Ngurah Rai Mangrove Forest

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Abstract. The escalating global health crisis due to antibiotic and antifungal resistance necessitates discovering novel antimicrobial agents. Extreme environment microorganisms, like those in mangrove soil, offer promising bioactive secondary metabolites. This study investigated the antibacterial and antifungal potential of *Bacillus* sp. RM10, an isolate from Ngurah Rai Mangrove Forest soil, via ethyl acetate extraction. *Bacillus* sp. RM10 was fermented in 100 mL ISP-2 liquid medium for 7 days. The supernatant was extracted twice with 100 mL ethyl acetate (1:1 v/v), and the extract was evaporated at 40°C. The viscous extract was tested against four bacteria (*Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 70060, *Escherichia coli* ATCC 25922, and *Streptococcus mutans* FNCC 0405) and two fungi (*Candida albicans* and *Aspergillus flavus*) using the Kirby-Bauer method. Results showed the ethyl acetate extract inhibited all tested microorganisms. Strong antibacterial activity was observed against *S. aureus* (15.00±1.30 mm), *K. pneumoniae* (14.50±0.90 mm), and *E. coli* (14.10±1.95 mm). Moderate inhibition was noted against *S. mutans* (9.00±1.43 mm). Strong antifungal activity was evident against *C. albicans* (10.50±1.40 mm) and *A. flavus* (10.80±0.60 mm). These findings confirm *Bacillus* sp. RM10 as a potential source of novel antibacterial and antifungal compounds, highlighting the therapeutic potential of mangrove ecosystems.

1 Introduction

Infectious diseases are caused by microorganisms that infect the human body. Bacteria and fungi are microorganisms responsible for the majority of infectious diseases. The annual increase in infectious diseases is attributed to several factors such as low public sanitation awareness, a shortage of trained healthcare personnel, and a general lack of public understanding regarding the basics of infectious diseases [1]. These issues are exacerbated

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by the inappropriate use of antibiotics, which leads to bacterial resistance. Inappropriate antibiotic use is categorized by incorrect dosage, erroneous diagnosis, and targeting the wrong pathogen [2]. Bacterial resistance to antibiotics, resulting from genetic mutations, enables bacteria to reduce or even eliminate antibiotic effectiveness. Some bacterial species proven to develop antibiotic resistance include vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and methicillin-resistant coagulase-negative *Staphylococcus* (MRCNS) [3].

Beyond bacterial resistance, antifungal drugs are also considered to have several limitations in treating fungal infectious diseases, such as a limited antifungal spectrum, the development of fungal resistance, poor tissue penetration, and serious side effects [4]. Similar to the search for new antibiotic compounds, efforts to explore antifungal compounds sourced from microorganisms also need to be intensified. This aims to obtain a wider selection of new antifungal compound candidates to be developed into therapies for diseases caused by fungal infections.

Mangrove forests are a unique type of forest found in coastal ecosystems, acting as the interface between terrestrial estuaries and the ocean. Within this ecosystem, elements of the mangrove forest are significantly influenced by tidal fluctuations. Given these environmental conditions, the distinctive mangrove ecosystem possesses highly potential natural resources due to the integration of physical and biological elements from both land and sea within the mangrove forest area [5]. Mangrove forests are one of the natural ecosystems rich in microorganisms. The ability of mangroves to produce secondary metabolites is associated with the microorganisms living within them, one of which is endophytes. Endophytic bacteria are bacterial colonies that utilize nutrients produced by the host plant's metabolism to sustain their colony without damaging the plant's tissue structure [6].

Co-evolution and genetic transmission from host plants are hypothesized to enable endophytic bacteria to produce secondary metabolites that structurally resemble those of their host plants [7]. Endophytic bacteria demonstrate significant potential as a substitute for plants as a source of bioactive compounds. This is primarily due to the substantial biomass quantities required for direct secondary metabolite extraction from plants. The diverse array of bacteria within this group holds promise for the development of antibiotics and antifungals, as numerous bacterial species actively synthesize secondary metabolites capable of mitigating bacterial and fungal infectious agents [8].

An imperative to search for novel antibacterial producers from marine bacteria, rather than focusing solely on terrestrial samples, is driven by several compelling factors. Firstly, a vastly underexplored realm compared to terrestrial ecosystems, the marine environment represents an immense trove of microbial diversity. This diversity is crucial, as many marine organisms have adapted to extreme conditions, leading to the production of unique metabolites with potent antibacterial properties. Marine bacteria, evolving in high-stress environments, often synthesize secondary metabolites that differ significantly from those produced by land-based counterparts due to their unique evolutionary adaptations and ecological niches [7].

The Ngurah Rai Forest Park in Bali is a significant mangrove ecosystem, encompassing 1373.5 hectares, thus representing the province's largest mangrove expanse [9]. Previous investigations reported the isolation of 22 out of 68 bacterial isolates from the Ngurah Rai mangrove soil, demonstrating antibacterial potential [10]. Seminal works utilizing chemical extraction have indeed confirmed the bioactivity capabilities of individual Bacillus isolates from the Ngurah Rai mangrove forest, demonstrating antibacterial, antifungal, and cytotoxic properties [11, 12]. Among these active isolates, a Gram-positive, rod-shaped bacterium, subsequently identified as *Bacillus* sp. RM-10, was purified from the soil habitat of the mangrove species *Rhizophora mucronata*. This particular isolate has not been thoroughly investigated regarding its full range of bioactivities.

Preliminary screening indicated that *Bacillus* sp. RM-10 inhibited the growth of *Streptococcus mutans* and *Staphylococcus aureus*, exhibiting an 8 mm inhibition zone, and *Escherichia coli*, with a 6 mm inhibition zone [10]. However, this prior research employed the perpendicular streak method, which is recognized for its limitation in providing accurate quantitative data due to the diffuse and indistinct nature of its inhibition zone edges. Consequently, further verification of the antibacterial and antifungal potential of the *Bacillus* sp. RM-10 isolate is warranted, utilizing chemical solvent extraction methodologies to enhance precision.

2 Material and methods

2.1. Cell culture refreshment

A fresh culture of *B. cereus* RM10 was prepared from a slant culture that had been stored at 4°C on ISP-2 agar. One loopful of these *Bacillus* RM-10 cells was then added to 100 mL of sterile ISP-2 broth. This mixture was agitated at 150 rpm and left to incubate at room temperature for 7 days.

2.2. Bacterial fermentation and extraction

The supernatant from the pure culture was first separated from the cell mass by filtering it through Whatman paper no. 1. The collected supernatant was then extracted twice with pro-analysis ethyl acetate solvent, maintaining a 1:1 volume ratio. The combined extracts were subsequently separated using a separating funnel.

2.3. Antibacterial screening

The obtained extract was tested by employing Kirby-Bauer disk diffusion method, performed under aseptic conditions within a biosafety cabinet. Briefly, 200 µL each of *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* FNCC 0405, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 were suspended in LB agar media and then evenly spread using a sterile cotton swab. Each agar plate was divided into three sections, with each section accommodating three discs. Subsequently, sterile 6 mm diameter paper discs, were individually impregnated with 20 µL of the *Bacillus* sp. RM-10 extract. These discs were then positioned on the LB media containing the bacterial suspension and subsequently incubated at 37°C for 24 hours. Paper discs containing the test substance were placed on agar media already inoculated with the target bacteria. This arrangement allowed for triplicate testing of the extract, a positive control, and a negative control for each bacterial strain. Ethyl acetate served as the negative control, while the broad-spectrum antibiotic levofloxacin was used as the positive control. These discs were then positioned on the LB media containing the bacterial suspension and subsequently incubated at 37°C for 24 hours. After 24 hours, the diameter of the inhibition zone (the clear halo around the paper disc) was measured using digital calliper. To ensure accuracy, four measurements were taken for each inhibition zone (vertical, horizontal, and both diagonal positions) and then averaged.

2.4. Antifungal screening

Antifungal testing was performed by evenly spreading 200 µL suspensions of C. albicans and *Aspergillus flavus* onto Petri dishes containing Saboraud Dextrose Agar (SDA) using a sterile cotton swab. The antifungal activity of the *Bacillus* sp. RM-10 ethyl acetate extract

was tested using the Kirby-Bauer method, following the same procedure as the bacterial tests. This involved using three paper discs for each antifungal test. The positive control for antifungal testing was nystatin.

2.5. Data analysis

The inhibition zone diameter data obtained in this study were analysed descriptively. The interpretation of antibacterial test results will be categorized into four groups based on their inhibition zone diameter: weak (0-5 mm), medium (5-10 mm), strong (10-20 mm), and very strong (>20 mm). The inhibition zone diameter was analysed using an independent t-test.

3 Results

Antibacterial screening of the viscous ethyl acetate extract of *Bacillus* sp. RM-10 demonstrated that the extract could inhibit the tested bacteria. The complete screening results for both Gram-positive and Gram-negative extracts are summarized in Table 1.

Table 1. Antibacterial activity test of Bacillus sp. RM10 extract

Tested Bacteria	Sample	Diameter zone of inhibition (mm) ± SD	Interpretation	P-value
Staphylococcus aureus ATCC 25923	*Bacillus* sp. RM10 Extract	15.00±1.30	Strong	p<0.05
	Levofloxacin	23.28±0.09	Strong	
	Ethyl acetate	0±0	-	
Streptococcus mutans FNCC 0405	*Bacillus* sp. RM10 Extract	9.00±1.43	Moderate	p<0.05
	Levofloxacin	27.38±0.98	Strong	
	Ethyl acetate	0±0	-	
Klebsiella pneumoniae ATCC 700603	*Bacillus* sp. RM10 Extract	14.50±0.90	Strong	p<0.05
	Levofloxacin	27.14±0.81	Strong	
	Ethyl acetate	0±0	-	
Escherichia coli ATCC 25922	*Bacillus* sp. RM10 Extract	14.10±1.95	Strong	p<0.05
	Levofloxacin	26.31±1.07	Strong	
	Ethyl acetate	0±0	-	

The antibacterial activity test results showed that the Bacillus sp. RM10 extract had average inhibition zone diameters of 15.00±1.30 mm and 9.00±1.43 mm against *S. aureus* ATCC 25923 and *S. mutans* FNCC 0405, respectively. The inhibition zone diameters formed were 14.50±0.90 mm and 14.10±1.95 mm against *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922. The Bacillus sp. RM10 extract falls into the strong antibacterial activity

category (>10 mm) for three of the tested bacteria: *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 700603, and *E. coli* ATCC 25922. It showed moderate antibacterial activity against *S. mutans* FNCC 0405 (Figure 1). However, the antibacterial activity of the *Bacillus* sp. RM10 extract was still smaller than that of levofloxacin (>25 mm), which served as the positive control in this study. Ethyl acetate, used as the negative control in this antibacterial test, did not produce any inhibition zones on the paper discs inoculated with the tested bacteria. The independent t-test results indicated that the ethyl acetate extract of Bacillus sp. RM10 had a significance value similar to levofloxacin ($p<0.05$). This suggests that the extract treatment possesses significant antibacterial activity, comparable to levofloxacin.

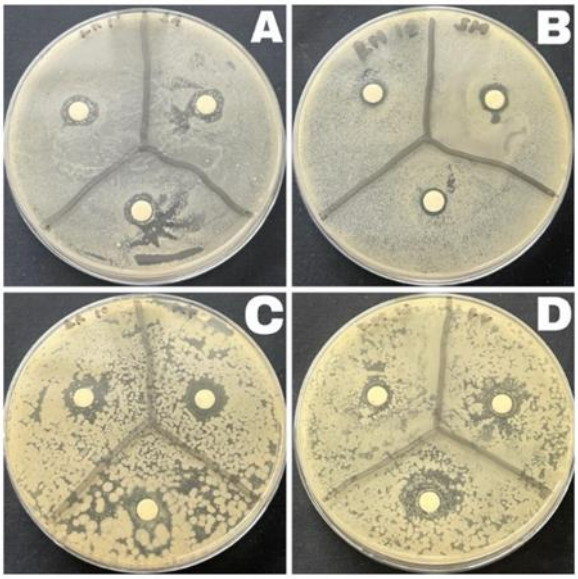

Fig. 1. Antibacterial test results of *Bacillus* sp. RM10 extract against four tested bacteria. A: *S. aureus* ATCC 25923, B: *S. mutans* FNCC 0405, C: *K. pneumoniae* ATCC 70060, D: *E. coli* ATCC 25922

The results of testing the ethyl acetate extract of *Bacillus* sp. RM10 against the tested fungi, *C. albicans* and *A. flavus*, are presented in Table 2 below. Based on general observation, the *Bacillus* sp. RM10 ethyl acetate extract was able to inhibit the tested fungi.

Table 2. Antifungal activity test of *Bacillus* sp. RM-10 Extract

Tested Microorganism	Sample	Diameter zone of inhibition (mm) ± SD	Interpretation	P-value
Candida albicans	*Bacillus* sp. RM10 Extract	10.50±1.40	Strong	p<0.05
	Nystatin	15.12±1.24	Strong	
	Ethyl acetate	0±0	-	
Aspergillus flavus	*Bacillus* sp. RM10 Extract	10.80±0.60	Strong	p<0.05
	Nystatin	19.33±1.75	Strong	
	Ethyl acetate	0±0	-	

The antifungal activity test results show that the Bacillus sp. RM10 extract had average inhibition zone diameters of 10.50±1.40 mm and 10.80±0.60 mm against *C. albicans* and *A. flavus,* respectively (Figure 2). The *Bacillus* sp. RM-10 extract falls into the strong antifungal activity category (>10 mm) for both tested fungi: *C. albicans* and *A. flavus*. However, the inhibition zones produced by the *Bacillus* sp. RM-10 extract were smaller than those of nystatin (>15 mm), which was used as the positive control in this study. On the other hand, ethyl acetate, serving as the negative control, did not form any inhibition zones against the tested fungi. Independent t-test analysis revealed that the ethyl acetate extract of *Bacillus* sp. RM-10 yielded a significance value of p>0.05. This indicates that the extract treatment possesses significant antifungal activity, similar to nystatin.

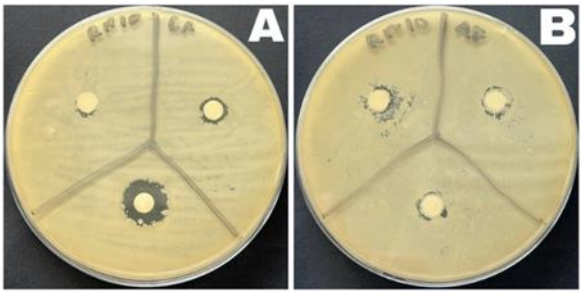

Fig. 2. Antifungal test results of *Bacillus* sp. RM10 extract against two fungal test bacteria. A: *C. albicans* B: *A. flavus*

4 Discussion

The *Bacillus* sp. RM10 extract is categorized as having strong inhibitory activity against three tested bacteria: *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *K. pneumoniae* ATCC 70060. For *S. mutans*, the *Bacillus* sp. RM-10 extract demonstrated moderate inhibitory activity. Based on its inhibitory capacity, the *Bacillus* sp. RM10 extract exhibits broad-spectrum inhibition activity. Broad-spectrum inhibition refers to a compound's ability to broadly inhibit and kill bacteria from both Gram-positive and Gram-negative groups. Some antibiotics classified as broad-spectrum antibiotics include tetracycline, chloramphenicol, and ampicillin.

Bacillus species are known to synthesize a variety of antibiotic compounds, including bacteriocins and other secondary metabolites like alkaloids, terpenoids, lactones, phenols, quinones, and more [13]. Specifically, *Bacillus* is recognized for producing the antibacterial bacteriocin Zwittermicin A, a peptide with a molecular formula similar to tetracycline-group antibiotics, which acts as a bacteriostatic agent. However, at this stage of the research, the specific type of antibacterial compound produced by *Bacillus* sp. RM-10 remains unidentified. This is because the extraction process focused on obtaining a crude extract rather than isolating specific molecules. The antibacterial mechanisms of *Bacillus* spp. can be broadly categorized into direct and indirect actions. Direct antibacterial mechanisms involve negative ecological interactions, where microbes are harmed or destroyed through antibiosis, often due to competition for nutrients. Indirect bacterial inhibition, on the other hand, occurs when antimicrobial agents produce bioactive compounds that hinder bacterial growth.

When comparing the inhibition zone of *Bacillus* sp. RM-10 to that of the positive control, the average diameter of the inhibition zone produced by the extract was smaller. Levofloxacin, as a pure compound, provides significantly more optimal bacterial inhibition compared to *Bacillus* sp. RM-10, which is a mixture of various compounds. Factors such as

the quantity, quality of secondary metabolites, and antibacterial substances produced by the *Bacillus* sp. RM-10 bacterial isolate may not be sufficient to achieve an inhibition zone as large as that of the positive control. Additionally, levofloxacin exhibits a high inhibition zone value due to its ability to inhibit DNA gyrase. DNA gyrase is an enzyme essential for DNA replication, transcription, and DNA repair. This inhibition prevents the relaxation of supercoiled DNA and damages the bacterial DNA strands. Conversely, the negative control, ethyl acetate, did not produce any inhibition zones because this compound does not yield secondary metabolites, thus it cannot inhibit bacterial growth on the disc. This confirms that the negative control is crucial for ensuring that the observed inhibition diameter genuinely originates from the secondary metabolites produced by the bacterial isolate, and not from the solvent used (ethyl acetate).

The *Bacillus* sp. RM-10 extract falls into the strong antifungal category against both tested fungi. The formation of clear zones around the discs containing the tested fungi indicates that the *Bacillus* sp. RM-10 isolate synthesizes secondary metabolites capable of inhibiting fungal growth. This finding aligns with a previous study that reported a formation of an inhibition zone around discs with tested fungi signifies the bacterial isolate's ability to produce antibiotic secondary metabolites or extracellular compounds that can inhibit or kill other fungi such as bacilysin, bacillomycin, iturin, and surfactin [14]. Additionally, tannin compounds present in *Bacillus* spp. contribute to their antifungal activity, leading to large inhibition zones against tested fungi. The mechanism of action for tannin compounds involves inhibiting the biosynthesis of ergosterol, which is the primary sterol composing fungal cell membranes.

When compared to the positive control antifungal, nystatin, the inhibition zone diameter formed was higher than that of the *Bacillus* sp. RM-10 extract. Nystatin, a pure compound, exhibits a larger inhibition zone because its active compounds can inhibit the synthesis of fungal cell wall polymers by blocking the action of (1,3)-ß-glucan synthase [15]. This enzyme is crucial for the normal growth and development of *Candida albicans*. In contrast, the *Bacillus* sp. RM-10 ethyl acetate extract is a mixture of various compounds, not a single pure substance. The negative control, ethyl acetate, showed no antifungal activity against either of the tested fungi. This is because it lacks secondary metabolites that could inhibit fungal growth. Therefore, this finding confirms that the inhibition zones produced by the *Bacillu*s sp. RM-10 ethyl acetate extract are indeed due to the secondary metabolites generated by the bacterial isolate itself, and not from the solvent used during the extraction process. These reported results also provide an initial indication that the *Bacillus* sp. RM-10 ethyl acetate extract does contain antifungal compounds.

Conclusion

In conclusion, the *Bacillus* sp. RM10 ethyl acetate extract, isolated from the unique environment of Ngurah Rai mangrove forest soils, demonstrated broad-spectrum inhibitory activity against both bacterial and fungal pathogens. It exhibited strong antibacterial effects against *S. aureus*, *E. coli*, and *K. pneumoniae*, while showing moderate activity against *S. mutans*. Furthermore, the extract displayed strong antifungal activity against *C. albicans* and *A. flavus*. While its inhibitory zones were generally smaller compared to pure positive controls like levofloxacin and nystatin, this is expected given that the extract is a crude mixture of various secondary metabolites. The lack of inhibition from the ethyl acetate negative control confirms that the observed antimicrobial activity is genuinely derived from the bioactive compounds produced by *Bacillus* sp. RM10, highlighting the unravelled potential of endophytic bacteria from this specific ecological niche as a source of novel antimicrobial agents.

Future research should focus on isolating and characterizing the specific bioactive compounds responsible for the observed broad-spectrum antimicrobial activity of *Bacillus* sp. RM10. Further purification and chemical identification using techniques such as chromatography and mass spectrometry would elucidate the exact nature of these secondary metabolites. Additionally, in-depth studies on the mechanisms of action of these isolated compounds, beyond general antibiosis or ergosterol synthesis inhibition, would be beneficial. Investigating the extract's cytotoxicity and efficacy in *in vivo* models would also be crucial to determine its potential for therapeutic applications.

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