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Research

Antimicrobial and Enzymatic Activities of Mangrove-associated Actinomycetes

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ABSTRACT

This study delves into the enzymatic and antimicrobial capabilities of actinomycetes isolated from the Setiu Wetland mangrove in Terengganu, Malaysia. A total of eighteen actinomycete bacteria were isolated and characterized from the site. These isolates underwent antimicrobial assessments targeting a representative range of Gram-positive bacteria, Gram-negative bacteria and a fungus were employed for the testing. The results of the antimicrobial evaluations demonstrated pronounced effectiveness of the majority of isolated actinomycetes against Gram-negative bacterial strains. Intriguingly, a notable observation was the inhibition against *Streptococcus uberis* on nutrient agar by 27.7% of the isolates. In conjunction with the antimicrobial investigations, an array of enzymatic assays encompassing amylase, protease, lipase, phosphate solubilization, urease, and cellulase were executed. The outcomes revealed that a substantial portion of the examined actinomycetes exhibited positive reactions in at least half of the conducted assays, with amylase and protease production being particularly prominent, were observed from 94% of the isolates. These findings, drawn from the amassed dataset, underscore the remarkable diversity of antimicrobial and enzymatic activities within the actinomycetes thriving in the mangrove environment. This diversity exemplifies the adaptability of these mangrove-associated actinomycetes, underscoring their capacity to generate a versatile spectrum of secondary metabolites and biochemical responses as a strategy for survival within this unique ecosystem.

Key words: Actinomycetes, antimicrobial, environment, enzyme, mangrove

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INTRODUCTION

Mangrove ecosystems boast extraordinary ecological value due to their rich organic matter, nitrogen, and sulfur content (Malek *et al*., 2014). This abundance fosters competition among soil bacteria, driving the production of secondary metabolites to impede rivals (Chaudhary *et al*., 2013). While marine environments have emerged as prolific sources of bioactive compounds (Barbosa *et al*., 2020; Ibrahimi *et al*., 2020; Goel *et al*., 2021), brackish mangroves offer a unique blend of both freshwater and marine microbial potential. Actinomycetes thrive within this dynamic habitat, inhabiting diverse niches such as aqueous sediments, fluctuating salinity zones, arid soils, air, and compost (Lestari *et al*., 2019).

Prior studies in marine and coastal ecosystems in Malaysia have also documented actinomycete diversity beyond Terengganu. Notably, *Streptomyces colonosanans* MUSC 93JT was isolated from a Sarawak mangrove ecosystem through bioactive screening (Law *et al*., 2017; Law *et al*., 2019). Additionally, Pahang, Malaysia yielded a prominent group of *Streptomyces* and *Micromonospora* (Malek *et al*., 2014; Zainal Abidin *et al*., 2016).

The recent decades have witnessed an alarming rise in antibiotic resistance, posing a significant threat to global public health. This issue manifests in various bacterial diseases, and the rapid spread of multidrug-resistant pathogens raises

particular concern (Marchese *et al*., 2012; WHO, 2021). Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) exemplify the dire consequences of this phenomenon. In this context, actinomycetes have emerged as promising candidates for combating multidrug-resistant pathogens, with their potential to limit the proliferation of pathogenic microbes (Velayudham & Murugan, 2012; Ryandini *et al*., 2018). The vast array of bioactive metabolites produced by actinomycetes offers a rich source of novel antibiotics with diverse chemical structures, properties, and modes of action, alongside varying toxicity profiles (Waksman *et al*., 2010). This remarkable potential positions actinomycetes as valuable contributors to the development of innovative therapeutic solutions in the ever-evolving landscape of medicine, offering hope for tackling the challenge of drug-resistant pathogens. Notably, their potential contributes to addressing the projected ten million fatalities by 2050 due to antimicrobial resistance, as highlighted by the World Health Organization (WHO, 2022).

Beyond potential medical applications, actinomycetes play an equally critical role in environmental processes by contributing to biogeochemical cycles. Within the mangrove ecosystem, they represent a key microbial component responsible for the decomposition of complex organic matter like cellulose and lignin through their diverse enzymatic repertoire. This essential function directly supports the growth of mangrove vegetation, fostering the ecosystem's sustainability (Rashmi & Satpute, 2012).

Furthermore, their remarkable ability to produce antimicrobial compounds offers a promising avenue for addressing the global challenge of antibiotic resistance. Moreover, their prowess in enzyme production extends their potential to various industrial and biotechnological applications. This versatility is underscored by their proven capacity to synthesize a wide range of bioactive metabolites with diverse chemical structures and functionalities, including antimicrobials, catalysts, anti-tumor agents, immunomodulators, and organic degradation enzymes (Velayudham & Murugan, 2012; Gopinath *et al*., 2013; Deepa *et al*., 2014; Abedinlou *et al*., 2022).

Focusing on the ecologically valuable Setiu Wetland region of Terengganu, Malaysia, this research seeks to unravel the multifaceted contributions of mangrove-associated actinomycetes by characterizing their antimicrobial and enzymatic capabilities.

MATERIALS AND METHODS

Screening of antimicrobial activity

Sixteen samples of actinomycetes were identified using the 16s rRNA sequencing method, while two samples were identified through morphological characterization and cross-referenced with Bergey's Manual of Bacteriology and Determinative Bacteriology Biology. The actinomycetes identified were *S. cinerochromogenes* strain PT2, *Micromonospora* sp. strain M1, *S. olivaceus* strain R1M, *S. olivaceus* strain 9X, *S. althioticus* strain MP1, *S. globisporus* strain SPX, *S. olivaceus* strain R2, *S. olivaceus* strain R1, *M. chalcea* strain MP2, *Streptomyces* sp. strain MPX, *S. globisporus* strain BV, *S. olivaceus* strain M2R, *S. olivaceus* strain PT2IS, *S. globisporus* strain SP2, *S. globisporus* strain PPT, *S. globisporus* strain UNK, *S. badius* strain R1S, *S. olivaceus* strain M3R.

Antimicrobial screening of isolated actinomycetes was conducted using the cross-streak test, a well-established preliminary method for this purpose (Zainal Abidin *et al*., 2016). This assay investigated potential activity against a diverse panel of pathogens, encompassing Gram-negative bacteria (*Vibrio alginolyticus*, *Escherichia coli*, *Pseudomonas aeruginosa*), Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus uberis*, *Micrococcus luteus*), and the fungus *Candida albicans*. Mueller-Hinton agar (MHA) was chosen as the primary growth medium for this study due to its several advantages. Its defined composition facilitates the diffusion of diverse antimicrobial compounds, resulting in clearer and more readily interpretable zones of inhibition (Aryal, 2015). Additionally, MHA aligns with the recommended standard agar for antibiotic susceptibility testing outlined by the Kirby-Bauer method (CLSI, 2013). For comparative purposes and to explore a broader spectrum of bacterial responses, nutrient agar was also employed. This rich medium supports the unrestricted growth of various bacteria, enabling the observation of diverse antimicrobial reactions. The specific protocol for nutrient agar in this study was adapted from established methods, incorporating the agar plug technique by Eccleston *et al*. (2008) and the double agar overlay technique by Donkor *et al*. (2007). Following inoculation of MHA and nutrient agar plates with actinomycetes and incubation at 30°C for seven days, test microorganisms were streaked perpendicularly and incubated further at 37°C for 24 hr (bacteria) and 30°C for 48 hr (fungus). A positive antimicrobial response was identified by the presence of a clear zone of inhibition adjacent to the actinomycete streak. This zone signifies reciprocal interactions between the actinomycetes and test organisms, often indicated by visible morphological changes (Singh *et al*., 2014). Observed zones of inhibition were classified based on area: less than 50% as a low reaction, greater than 50% as a high reaction, and intermediate areas as a medium reaction.

Screening enzymatic activity

The identical set of actinomycetes isolated from the Setiu mangrove was employed for subsequent testing. Enzymatic assays were performed using specific agars matched with their respective substrates: amylase activity was assessed on starch agar (Al-Dhabi *et al*., 2020) indicated by the development of colorless zone after staining with iodine, protease activity on skim milk agar (Ozturkoglu-Budak *et al*., 2016), with the presence of transparent halo serving as an indicator of activity (Mayerhof *et al*., 1973; lipase activity on spirit blue agar (Ozturkoglu-Budak *et al*., 2016), assessed by the loss of color in the spirit blue dye due to acid produced by the consumed lipid; cellulase activity on cellulose agar (Saha & Dhanasekaran, 2006), determined by staining with 0.1% Congo red and 1M Sodium Chloride (NaCl) after incubation; urease activity on Christensen urea agar (Andrew & Hammack, 2001); and the phosphate solubilization test performed on Pikovskaya agar (Xiao *et al*., 2013), observed through the presence of a clear zone. For the gelatine liquefaction test, solidification was noted after 30 minutes of immersion in ice (Mossel & De Bruin, 1954). The enzymatic media were inoculated with the actinomycetes and subsequently incubated at 30°C for seven days for enzymatic reactions to take place.

RESULTS AND DISCUSSION

Screening of antimicrobial activity

To assess the broad-spectrum potential of the isolated actinomycetes, their antimicrobial activity was investigated against a panel of microorganisms encompassing Gram-positive and Gram-negative bacteria, as well as a yeast strain (Table 1). The presence of an outer membrane in Gram-negative bacteria can influence antibiotic efficacy, making this diverse selection crucial for evaluating potential selectivity. A representative example of a clear zone of inhibition observed following interaction with an actinomycete isolate is presented in Figure 1. From the data recorded in Table 1, analysis and comparison were conducted as charted in Figure 2.

Mueller-Hinton agar and nutrient agar exhibited subtle but interesting differences in their ability to support the antimicrobial activity of the isolated actinomycetes against both Gram-positive and Gram-negative bacteria (Figure 2). The Gram-positive *Staphylococcus aureus* displayed the highest inhibition rate (25%) on Mueller-Hinton agar, compared to 16.6% on nutrient agar. Conversely, *Streptococcus uberis* showed higher activity on nutrient agar (27.7%) compared to Mueller-Hinton agar (13.8%). While inhibition rates of the actinomycetes against *B. cereus*, *B. subtilis*, and *M. luteus* were relatively similar between the two agar types.

Alternatively, *E. coli* exhibited slightly higher inhibition rates by actinomycetes on nutrient agar (22.2%) compared to Mueller-Hinton agar (19.7%). The opposite trend was observed for *P. aeruginosa*, with higher activity on nutrient agar (16.6%) compared to Mueller-Hinton agar (13.8%). *Vibrio alginolyticus* showed higher susceptibility towards the actinomycetes on Mueller-Hinton agar (22.2%) compared to nutrient agar (13.8%).

These variations in activity suggest potential influences of the agar composition and diffusion dynamics on the efficacy of the actinomycete metabolites. Differences in nutrient availability and pore size between the two agar types could be contributing factors. The thinner peptidoglycan layer of Gram-negative bacteria might also play a role, as the outer membrane with its porin channels could be differentially affected by the agar environment (Hauser, 2015).

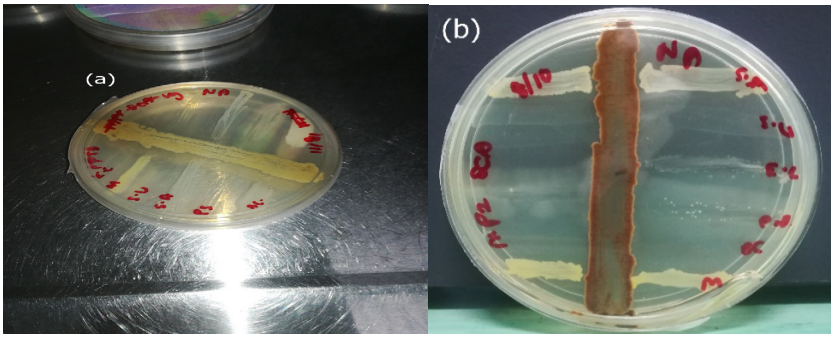

Fig. 1. Cross streak testing on nutrient agar showed a clear zone gap in (a) and an absence of bacterial growth in (b).

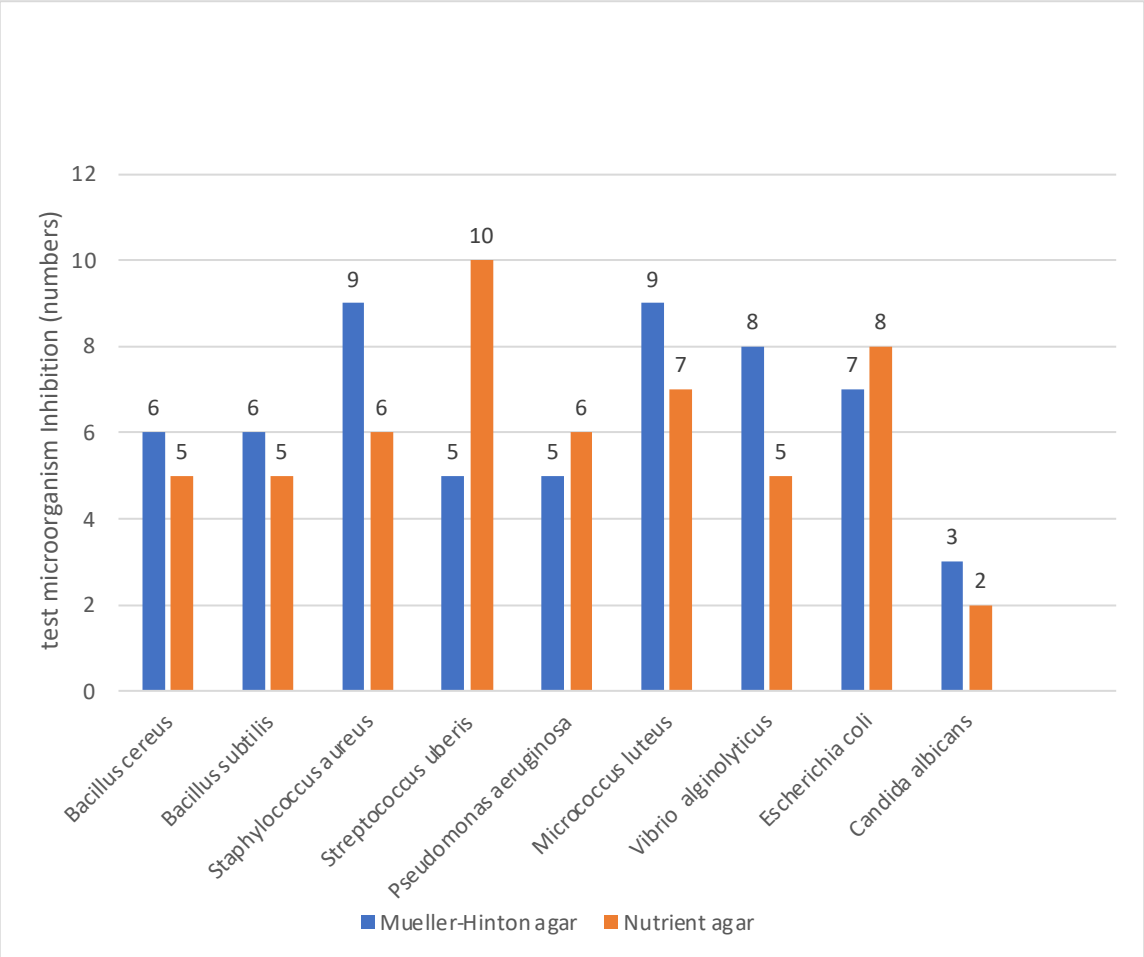

Fig. 2. Antimicrobial screening using Mueller-Hinton agar and Nutrient agar by cross streak method showing the positive result of inhibition by a clear zone of bacteria isolates against test microorganism.

The efficacy of the isolated actinomycetes against the fungal pathogen *Candida albicans* was modest, with only 8.3% and 5.5% of isolates exhibiting inhibition on Mueller-Hinton and nutrient agar, respectively. While these rates indicate limited antifungal activity, our findings align with previous reports demonstrating the potential of specific *Streptomyces* strains to inhibit *P. aeruginosa, E. coli*, and *C. albicans* (Cho *et al*., 1999). Notably, *S. olivaceus* strain PT2IS displayed inhibition against *C. albicans* on both agar types, while strains R1 and R2 exhibited activity only on Mueller-Hinton or nutrient agar, respectively. *Micromonospora chalcea* strain MP2 also demonstrated antifungal activity against *C. albicans* specifically on Mueller-Hinton agar. These observations support evidence in the literature suggesting that certain *Micromonospora* and *Nocardia* species hold promise for tackling both bacterial and fungal pathogens (Kavitha, 2010). This finding further echoes prior research documenting the inhibitory activity of *Micromonospora* isolates against *S. aureus* and *B. subtilis* (Rao *et al*., 2015), reinforcing the genus's broader antimicrobial capabilities known to target human pathogens (Lee, 2012; Talukdar, 2012).

Our data revealed several isolates exhibiting notable antibacterial activity against both Gram-positive and Gram-negative bacteria, aligning with previously reported potentials of these strains (Kaaniche *et al*., 2020). While no inhibition against Candida albicans was observed, *S. olivaceus* strain 9X demonstrated the highest level of antibacterial activity among all isolates, with 10 positive test results. This finding reinforces the known diverse antibiotic production capabilities of the *Streptomyces* genus (Kumari *et al*., 2013), highlighting the potential antimicrobial properties of this specific isolate.

The observed inhibitions likely stem from the varied spectra and activities displayed by different actinomycetes, resulting from the secretion of diverse bioactive compounds rather than single inhibitory molecules (Benhadj *et al*., 2018). Interestingly, 50% of currently known antibiotics originate from *Streptomyces* and *Micromonospora* species (Pandey *et al*., 2011), further underlining their vast potential.

Despite sharing the same genus, some isolates produced divergent antibacterial responses according to their strain and the screening agar used. This confirms that testing medium and culture

conditions can influence antimicrobial mechanisms, as reported by Garrod & Waterworth (1971) and Vijayakumar *et al*. (2012). Recent studies further emphasize the critical role of media in influencing bioactive compound secretion by affecting bacterial genetic profiles (Anteneh *et al*., 2021). This understanding allows for more reliable results and deeper comprehension of actinomycete antimicrobial responses, considering how nutrient availability impacts bacterial secondary metabolism.

Going beyond Mueller-Hinton agar, as in our methods, utilizing different agar types compared to the isolation agar can present growth challenges due to limited nutrition, potentially influencing secondary metabolism (Suryanditha, 2018). Considering the isolated habitat of decaying organic matter in Setiu

Table 1. Antimicrobial screening of isolated actinomycetes bacteria

Bacteria isolates	Test microorganisms																					
	Bacillus subtillis		*Bacillus cereus*		*Pseudomonas aeruginosa*		*Staphylococcus aureus*		*Staphylococcus uberis*		*Micrococcus luteus*		*Escherichia coli*		*Vibrio alginolyticus*		*Candida albicans*	
	MH	NA	MH	NA	MH	NA	MH	NA	MH	NA	MH	NA	MH	NA	MH	NA	MH	NA
S. cinerochromogenes strain PT2	+	+	+	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-
Micromonospora sp. strain M1	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-
S. olivaceus strain R1M	-	-	-	-	+	+	-	-	+	+	-	-	-	+	-	+	-	-
S. olivaceus strain 9X	+	+	+	-	+	+	-	-	+	+	-	-	+	+	+	-	-	-
S. althioticus strain MP1	+	-	+	+	-	-	+	+	-	-	-	+	+	-	-	-	-	-
S. globisporus strain SPX	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. olivaceus strain R2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
S. olivaceus strain R1	-	-	+	-	-	-	+	+	-	+	+	-	-	+	+	+	-	-
M. chalcea strain MP2	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
Streptomyces sp. strain MPX	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-	-
S. globisporus strain BV	-	-	-	-	-	+	+	+	+	-	+	-	-	-	+	-	-	-
S. olivaceus strain M2R	-	+	-	+	+	+	-	+	-	+	-	-	+	+	+	-	-	-
S. olivaceus strain PT2IS	+	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+
S. globisporus strain SP2	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-
S. globisporus strain PPT	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	+	-	-
S. globisporus strain UNK	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-
S. badius strain R1S	-	-	-	-	-	-	+	-	-	+	+	+	+	-	+	+	-	-
S. olivaceus strain M3R	-	+	-	+	+	-	+	+	-	-	+	+	+	+	+	-	-	-

MH: Mueller-Hinton agar; NA: nutrient agar; +: positive result; -: negative result

and the evolutionary pressure of competition, antibiotic secretion patterns can be attributed to various factors, as previously suggested by Williams *et al*. (1965).

Based on these observations, the cross-streak method effectively elucidates interactions between isolated samples and test microorganisms by assessing their antimicrobial properties. The method's effectiveness makes it a valuable tool for understanding microbial dynamics and formulating strategies to harness their antimicrobial potential, while also aligning with guidelines set by the Clinical and Laboratory Standards Institute (CLSI).

Screening of enzymatic activity

Table 2 reveals the impressive enzyme activity profiles of the isolated actinomycetes. Amylase activity, known to be present in *Streptomyces* alongside *Bacillus, Pseudomonas*, and *Clostridium* (Oyeleke *et al*., 2010; Farshid *et al*., 2012), was detected in 94% of isolates, with 5 strains exhibiting high activity. This finding aligns with the characteristic blue color observed on starch agar, indicating successful starch hydrolysis. The ability to convert carbohydrates into simple sugars holds significant potential for sustainable bioethanol and biodiesel production, offering an alternative to conventional fuels (Anteneh & Franco, 2019).

Lipase activity, another widely distributed enzyme (94% of isolates), demonstrated dominance in the middle activity range across eight isolates. Protease activity, while prevalent in 61% of isolates, was limited to low levels. Conversely, the cellulose agar assay, mimicking plant material degradation in the mangrove environment, revealed moderate activity in only 50% of isolates. Despite this, cellulases from other terrestrial actinomycetes have exhibited promising thermostability and specific activity (Xie & Pathom-Aree, 2021; Elframawy *et al*., 2022; Gong *et al*., 2020), highlighting the potential for further exploration through cellulolytic consortia (Zhang & Dong, 2022).

Gelatinase activity, linked to protease and protein digestion, showed an equal split between high and negative reactions (50% each). This enzyme aids in the breakdown of animal organic matter, crucial for decomposer organisms like actinomycetes (Leboffe & Pierce, 2010). Phosphate solubilization, a potential asset for future microbial inoculants in agriculture (Saif *et al*., 2014), was observed in 51% of isolates, albeit at low levels. Notably, urease activity, responsible for urea hydrolysis (Strope *et al*., 2011), was detected in an impressive 78% of isolates, all exhibiting high activity. Actinomycetes are recognized for their ureolytic enzyme production, alongside other diverse microbial groups (Strope *et al*., 2011; Burbank *et al*., 2012; Wen *et al*., 2015; Kang *et al*., 2015; Alizadeha *et al*., 2017). Amongst all isolates, *S. olivaceus* strain PT21S displayed remarkable versatility, exhibiting positive reactions for all enzyme activities assessed.

Table 2. Enzymatic tests

Bacteria isolates	Enzymatic tests						
	Amylase	Lipase	Protease	Cellulase	Gelatin liquefactionn	Phosphate solubilization	Urease
*S. cinerochromogene*s strain PT2	+	+++	+	-	-	-	-
Micromonospora sp. strain M1	+++	-	+	-	+++	+	+++
S. olivaceus strain R1M	-	++	-	-	+++	-	-
S. olivaceus strain 9x	+	++	-	+	+++	-	+++
S. althioticus strain MP1	+	++	-	-	-	+	+++
S. globisporus strain SPX	+	+	+	+	+++	-	+++
S. olivaceus strain R2	+	+++	+	+	-	-	+++
S. olivaceus strain R1	+	+++	-	+	+++	-	+++
M. chalcea strain MP2	++	++	+	-	+++	+	+++
Streptomyces sp. strain MPX	++	++	+	+	-	+	+++
S. globisporus strain BV	++	++	-	-	+++	+	+++
S. olivaceus strain M2R	+++	+++	-	+	+++	+	+++
S. olivaceus strain PT2IS	+	+++	+	+	+++	+	+++
S. globisporus strain SP2	+++	+++	+	-	-	+	-
S. globisporus strain PPT	+++	+++	+	+	-	-	+++
S. globisporus strain UNK	++	++	-	+	-	+	-
S. badius strain R1S	+++	+	+	-	-	-	+++
S. olivaceus strain M3R	+	+++	+	-	-	+	+++

#+++: high activity, ++ : medium activity, +: low activity, - : negative activity.

Streptomyces species are dominant actinobacteria known for their prolific enzyme secretion,

contributing to their ecological success (Dhavala & Joel, 2020). Our isolate collection from the mangrove environment showcases the remarkable diversity of enzyme bioactivities present within these fascinating bacteria. Notably, research suggests that mangrove microbial enzymes exhibit superior stability and activity compared to their animal or plant counterparts (Dhavala & Joel, 2020). This advantage, coupled with the economic scalability of bacterial enzyme production, positions them as promising alternatives to traditional enzyme sources.

In vitro enzymatic activity data is invaluable for research, allowing optimization of growth conditions for enhanced enzyme yields (Kumar & Chandra, 2020). Controlled culture environments in research and industrial settings offer precise manipulation of microbial growth media and conditions, facilitating consistent enzyme production. To bridge the gap between academic research and industry, collaborative efforts leveraging scientific expertise and industrial capabilities are crucial for maximizing knowledge transfer and new enzyme discovery (Khirennas *et al*., 2023). This synergy can not only unlock novel enzymes for commercialization but also drive the development of multienzyme complexes with broader functionalities and specificities (Balla *et al*., 2022). Such enzymes, often endowed with advantageous activities and remarkable resilience in extreme conditions, represent cutting-edge biocatalysts with significant potential.

Furthermore, isolating previously uncharacterized *Streptomyces* species from pristine ecosystems like mangroves can yield unique enzyme resources with untapped commercial possibilities (Dhavala & Joel, 2020). In support of sustainable practices, actinomycetes have even shown promise in degrading certain types of plastics, converting them into carbon sources to produce biodegradable bioplastics (Oliveira *et al*., 2022).

CONCLUSION

The antibiotic screening assays employed in this study revealed a notable ability among the isolated actinomycetes to inhibit the growth of various pathogenic bacteria. This finding demonstrates significant potential for further exploration in the realm of antibiotic research and development, offering promising avenues for novel antimicrobial discovery. Additionally, the metabolic enzyme versatility exhibited by these isolates highlights their diverse applications within the industrial sector, ranging from bioremediation to biotechnology. In conclusion, our investigation into the antimicrobial and enzymatic activities of mangrove-associated actinomycetes from the Setiu Wetland in Terengganu, Malaysia, has not only unveiled their ecological significance but also illuminated their immense potential for various medical and industrial applications. This exploration opens exciting doors for future research efforts aimed at harnessing the unique capabilities of these remarkable microorganisms.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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