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Phylogenetic relationship and biotechnological use potential of epiphytic Actinomycetota species isolated from seagrasses from the coast of Tanzania

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Abstract. *Mbusi LD, Lyantagaye SL, Lyimo TJ. 2024. Phylogenetic relationship and biotechnological use potential of epiphytic Actinomycetota species isolated from seagrasses from the coast of Tanzania. Asian J Trop Biotechnol 21: 41-51.* Actinomycetota, previously recognized as actinobacteria, has demonstrated considerable promise as a valuable reservoir of secondary metabolites with potential pharmaceutical applications. This study examined the diversity, antimicrobial properties, and cytotoxic effects of epiphytic Actinomycetota species isolated from the seagrasses *Thalassia hemprichii* and *Syringodium isoetifolium*. Therefore, 12 strains of Actinomycetota were isolated through a process involving assessment of their morphological and biochemical characteristics, along with phylogenetic analysis using partial sequences of the 16S rRNA gene. The identified strains were associated with eight phylogenetic genera: *Cellulosimicrobium*, *Corynebacterium*, *Microbacterium*, *Rhodococcus*, *Arthrobacter*, *Leucobacter*, *Dietzia,* and *Micrococcus*. The findings unveiled five potential new species within Actinomycetota. Seven of the 12 strains displayed antimicrobial effects against at least one human pathogen tested. Notably, the *Microbacterium* strain SIP6 exhibited extensive antimicrobial efficacy against all the pathogens under examination. Toxicity tests revealed that only two strains (*Microbacterium* (THP6) and *Micrococcus* (SIP14)) were nontoxic, with the lowest LC_{50} values of 3836.7 and 1243.4 µg/mL, respectively, while the remaining extracts were toxic. This study marks the initial discovery of epiphytic Actinomycetota strains from seagrasses in the western Indian Ocean. The isolated epiphytic Actinomycetota strains, some of which are novel, showed potential for bioactive metabolites that hold promise for biotechnological use.

Keywords: Bioactivity, epiphytic bacteria, marine Actinomycetota, phylogenetic analysis, seagrass species

INTRODUCTION

Actinomycetota is a phylum that primarily consists of Gram-positive bacteria. This phylum was reclassified from Actinobacteria to Actinomycetota due to amendments in the International Code of Nomenclature of Prokaryotes concerning phylum-level classifications (Whitman et al. 2018). The Actinomycetota phylum is categorized into six classes, specifically, Actinomycetia, Nitriliruptoria, Acidimicrobiia, Thermoleophilia, Rubrobacteria, and Coriobacteria according to Bergey's Manual of Systematic Bacteriology. Actinomycetota genomes stand out due to their elevated Guanine-Cytosine (GC) content and include genes linked to the production of antibiotics (Mast and Stegmann 2019). Their reputation stems from producing more than 20,000 natural compounds widely used in pharmaceuticals and agrochemicals (Charousová et al. 2017). With the rise of bacterial resistance to antibiotics and the emergence of various diseases like cancer, AIDS, and other severe ailments, there is a worldwide urgency to uncover new drugs (Aslam et al. 2018). Infections caused by resistant microbes lead to high illness and death rates, incur greater treatment costs, and extend hospital stays, imposing significant strains on healthcare networks (Frost et al. 2019). Furthermore, 50% of the drugs currently accessible on sale are derived from natural compounds (Cita et al. 2017), including those sourced from marine organisms (Boontanom and Chantarasiri 2020). Although most Actinomycetota secondary metabolites are powerful antibiotics, this phylum also has clinical significance in synthesizing antitumor drugs. These comprise drugs like aclarubicin, actinomycin D, neocarzinostatin, doxorubicin, bleomycin, and numerous others (Ngamcharungchit et al. 2023). Nonetheless, the quest for new medications remains a crucial objective in cancer treatment, given the swift emergence of resistance to numerous chemotherapy drugs.

Actinomycetota is mainly distributed in soil, freshwater, and marine environments (Passari et al. 2017). Nevertheless, in contrast to terrestrial settings, the marine environment remains underexplored in terms of its potential as a reservoir for antimicrobial substances (Boontanom and Chantarasiri 2020). Marine Actinomycetota had to adapt from the deepsea floor's extremely high pressure, anaerobic conditions, and temperatures just below 0-8°C to the highly acidic environments with temperatures ranging from over 8°C to 100°C near hydrothermal vents at mid-ocean ridges. Consequently, very few new metabolites synthesized by marine Actinomycetota have been harnessed for pharmaceutical purposes. Marine environments include seagrass meadows distributed in coastal areas worldwide, excluding Antarctica (Piñeiro-Juncal et al. 2022). There are 13 genera, and 72 species of these seagrasses found globally. Seven genera (*Halodule*, *Cymodocea*, *Halophila*, *Enhalus*, *Syringodium*, *Thalassia*, and *Thalassodendron*)

are distributed in tropical seas (Ravikumar et al. 2010). In Tanzania, a total of 12 seagrass species, namely, *Thalassodendron ciliatum* (Forssk.) Hartog, *Thalassia hemprichii* (Ehrenb. ex Solms) Asch., *Zostera capensis* Setch., *Halophila stipulacea* (Forssk.) Asch., *Syringodium isoetifolium* (Asch.) Dandy, *Cymodocea serrulata* (R.Br.) Asch. & Magnus, *Halophila ovalis* (R.Br.) Hook.f., *Cymodocea rotundata* Asch. & Schweinf., *Halophila minor* (Zoll.) Hartog, *Halodule uninervis* (Forssk.) Boiss., *Enhalus acoroides* (L.f.) Royle and *Halodule pinifolia* (Miki) Hartog, have been recognized (Lugendo et al. 2024). Most large seagrass meadows are between shores or cliffs and nearby fringing reefs. These seagrasses host diverse bacterial communities, including epiphytic Actinomycetota bacteria that form symbiotic relationships within their leaves and roots. Mishra and Mohanraju (2018) reported *Micrococcus* as epiphytic in the seagrasses *C. rotundata* and *Thalassia testudinum* Banks & Sol. ex K.D.Koenig. These bacterial strains differ from those found in the surrounding environment, particularly those dominant in the water column (Roth-Schulze et al. 2016). Epiphytic bacteria, as described by Tarquinio et al. (2019), are benign bacteria that reside on the surface of different plant organs. The symbiotic relationship between bacteria and plants has also been associated with the ability of bacteria to produce metabolites that protect seagrasses from pathogens, nitrogen fixation, and nutrient cycling (Mishra and Mohanraju 2018; Su et al. 2023).

Among bacteria, Actinomycetota species have been described as important colonizers of seagrasses (Siro and Pipite 2024). Currently, there is no research on the population of epiphytic Actinomycetota in seagrasses in the Western Indian Ocean region. In this research, we isolated and characterized culturable epiphytic Actinomycetota strains from the seagrasses *T. hemprichii* and *S. isoetifolium* in seagrass meadows of Mjimwema, Dar es Salaam, Tanzania. Additionally, we assessed the antimicrobial effects and cytotoxicity of raw extracts derived from isolated epiphytic Actinomycetota strains, which may contribute to antibacterial or antifungal activities.

MATERIALS AND METHODS

Study area

Seagrass samples were collected at Mjimwema Beach, located 4 km south of the Dar es Salaam harbor, Tanzania, at approximately 06°50'S and 39°21'E (Figure 1). Leaf samples of the seagrass species *T. hemprichii* (THP) and *S. isoetifolium* (SIP), which are among the dominant seagrass species in this area, were collected during low tides at approximately 10 m intervals within the seagrass-dominated by each species, resulting in 10 sampling points per species. Seagrass species were identified based on the field guidebook by Oliveira et al. (2015). The samples of each species were collected in a sterile plastic bag; they were kept in a cool box with ice cubes and transported to the Molecular Biology and Biotechnology Department Laboratory, University of Dar es Salaam, Tanzania, for further analysis. The Actinomycetota isolation procedures were conducted on the same day as the sample collection.

Isolation and identification of epiphytic Actinomycetota

The procedure for isolating marine epiphytic Actinomycetota from seagrass samples was adhered to the methodology outlined by Boontanom and Chantarasiri (2020) with minor modifications. The surfaces of the seagrass leaf samples were softly rubbed using a sterile cotton swab and then placed into 2 mL of sterilized seawater. Next, 1 mL of suspension was applied to cover the surface of a Starch Nitrate Agar (SNA), while an additional 1 mL was spread onto actinomycete isolation agar. Both media were enriched with nystatin (50 µg/mL) and nalidixic acid (20 µg/mL) to prevent non actinomycetota bacterial and fungal, respectively. The plates were incubated at 28°C for 7-14 days until colonies appeared. The cultures were monitored daily to observe the growth of Actinomycetota. The epiphytic Actinomycetota were identified through morphological assessment (macroscopic and microscopic examination) and biochemical analysis using the techniques outlined by Lema et al. (2022). Biochemical characterization included methyl red tests, catalase tests, gelatin hydrolysis tests, Voges-Proskauer tests, oxidase tests, urea hydrolysis tests, indole tests, citrate utilization tests, hydrogen sulfide production tests, and starch hydrolysis tests.

Molecular characterization and phylogenetic analysis

The genomic DNA of the isolated epiphytic Actinomycetota was extracted according to the manufacturer's instructions using the ZymoBIOMICS DNA Fungal/Bacterial MinPrep kit. The extracted DNA's quality and quantity were evaluated using a Nanodrop One device from Thermo Fisher Scientific in the USA. The primers 235F-CGCGGCCTATCAGCTTGTTG and 878R-CCGTACTCCCCAGGCGGGG were used to determine whether the isolates belonged to the Actinomycetota phylum following the procedure outlined by Kaale et al. (2022). PCR was performed in PCR tubes of 200 µL, starting with an initial denaturation at 94°C for 30 minutes. This was followed by 35 cycles at the same temperature regimen, each lasting 30 seconds, primer annealing at 60°C for 50 seconds, and primer extension at 68°C for 1 minute. The reaction was then held at 68°C for 5 minutes before cooling to 4°C. The PCR products were analyzed on a 1.5% agarose gel and visualized using a UV transilluminator (Electron, VWR International Ltd.). Gel imaging was carried out using a gel documentation system (Zenith).

The PCR products underwent sequencing with the standard pair of primers (235F' and 878R') using an Applied Biosystems instrument from the USA. This sequencing was performed at the commercial facility Macrogen Europe (www.macrogen-europe.com). The partial sequences of these 16S rDNA sequences were subjected to BLAST analysis against the GenBank sequence database on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov). Phylogenetic analyses were performed using MEGA version 11 (Tamura et al. 2021). The MUSCLE program in MEGA version 11 was employed to align the sequences, and the evolutionary history was deduced using the maximum likelihood method (Tamura et al. 2021). A consensus tree based on 1000

replicates using bootstrap was created to depict the evolutionary lineage of the studied taxa.

Determination of the bioactivity potential

Extraction of bioactive compounds

The culture broth (1500 mL) of Actinomycetota isolates underwent solvent extraction to collect the crude metabolites. Initially, the culture broth was filtered through cotton wool and subsequently centrifuged at 15,000 rpm for 10 minutes. Ethyl acetate was introduced into the filtrate at a 1:1 (v/v) ratio, and the blend was vigorously shaken for 24 hours to achieve thorough extraction. The extracts obtained were dried to completion using a rotary evaporator (BUCHI Labortechnik AG, Switzerland) at 45°C, following the procedure described by Lema et al. (2022).

Antimicrobial activity

The extracts were evaluated for their antimicrobial activity against four specific reference microorganisms: the Gram-positive bacterium *Staphylococcus aureus* (ATCC-25923) Rosenbach, 1884, the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC-27853) A, and *Escherichia coli* (ATCC-35218) E, and the fungus *Candida albicans* (ATCC-14053) (C.P.Robin) Berkhout. The evaluation was conducted using the agar well diffusion technique, following the protocol described by Hajizadeh et al. (2023) with slight adjustments. The pathogenic bacteria were cultured on Muller Hinton agar, whereas the fungal pathogen was cultivated on Sabouraud's dextrose agar. Once the media had solidified, wells were formed, and the bacterial and fungal cultures were introduced using a sterile micro tip (1 mL), after which 100 µL of the extracts dissolved in dimethyl sulfoxide (DMSO) was added. The bacteria were incubated at 37°C, while the fungi were kept at 28°C. After 24 hours of incubation, the diameter of the inhibition zones was measured to assess the antimicrobial effectiveness of the Actinomycetota isolates.

Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined using the micro broth dilution method, following the procedure described by Hamisi et al. (2023). The extracts were serially diluted in sterile Mueller Hinton broth (Liofilchem, Italy) and subsequently added to 96-well microtiter plates containing bacterial cultures. This resulted in final concentrations ranging from 25 mg/mL to 0.195 mg/mL. The plates were then incubated at 37°C for 24 hours, with each test performed in duplicate. After incubation, an indicator dye of p-iodonitrotetrazolium (0.02%/40 µL) was added, and the mixture was incubated for an additional hour at 37°C. A pink color change indicated microbial growth, while the indicator remained colorless without growth. The MIC was identified as the lowest concentration, causing a color change.

Cytotoxicity test

The Brine Shrimp Lethality Test (BSLT) was used to evaluate the cytotoxicity of the extracts following the method outlined by Hamisi et al. (2023). The crude extracts were dissolved in DMSO to prepare a stock solution with a 40 mg/mL concentration for each sample. Brine shrimp eggs were hatched by placing one teaspoonful of 300 mL of filtered seawater in a container, which was then illuminated using an electric bulb for 48 hours. Next, 10 shrimp larvae were carefully chosen and transferred into individual sample vials using 100 µL pipettes. The volume in each vial was then adjusted to 5 mL using artificial seawater, which was made by dissolving 3.8 g of sea salt in 1 liter of distilled water. Different amounts of the extract from the stock solution were introduced into the wells, achieving concentrations of 240, 120, 80, 40, 24, and 10 µg/mL. Each concentration of the extract was evaluated in duplicate. The negative control sample was DMSO (0.6%) without extract treatment. Following 24 hours, the surviving larvae were tallied, and the percentage mortality was computed for each dosage. The concentration causing 50% mortality of the brine shrimp (LC_{50} in µg/mL with 95% confidence intervals) was calculated using the Microsoft Excel computer program. The brine-shrimp percentage mortality rates were graphed against the logarithm of the concentration using the Microsoft Excel program; furthermore, the regression equation was determined. The LC_{50} (µg/mL) was determined from the logarithmic plot. The following formula calculated the percentage mortality:

$$\% \text{ Mortality} = \frac{\text{Number of dead brine shrimp larvae}}{\text{Total number of brine shrimp larvae}} \times 100$$

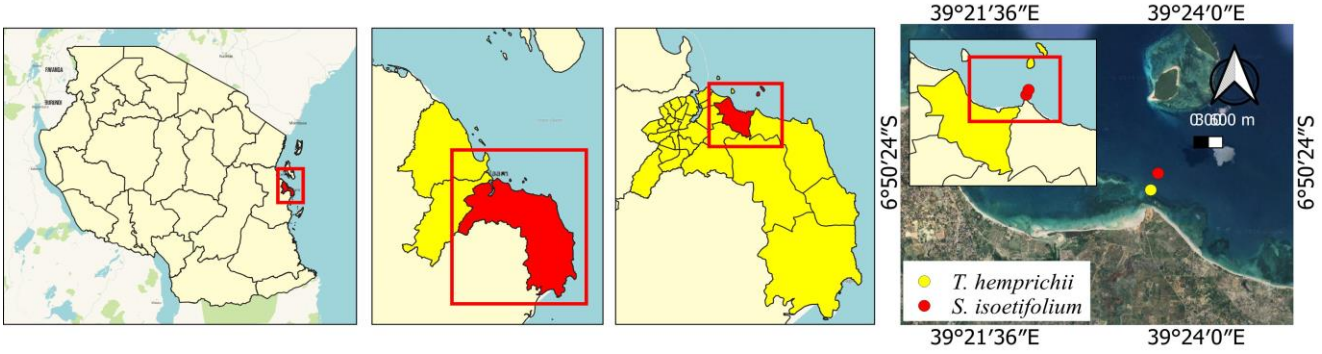


Figure 1. Map of Mjimwema, Dar es Salaam, Tanzania, illustrating sampling points for each seagrass species

The toxicity criteria for the fractions were established based on the guidelines by Hamidi et al. (2014): LC_{50} values greater than 1000 µg/mL were deemed nontoxic, LC_{50} values between 500 and 1000 µg/mL were considered weakly toxic, and LC_{50} values below 500 µg/mL were classified as toxic.

RESULTS AND DISCUSSION

Morphological and biochemical identification of isolated Actinomycetota strains

Moreover, 12 isolates (8 from *T. hemprichii* and 4 from *S. isoetifolium* seagrasses) were identified as Actinomycetota based on their phenotypic characteristics. All the isolates were Gram-positive and rod, cocci, or rod-filamentous in shape with different colony colors, including yellow, cream, and orange (Figure 2, Table 1). Morphological traits such as the color and texture of colonies have traditionally served as key criteria for categorizing Actinomycetota (Hasani et al. 2014; Lema et al. 2022). According to these observations, we inferred that our isolates could be assigned to eight distinct Actinomycetota genera, as detailed in Table 1. *Microbacterium* was the most dominant among the genera identified, followed by *Micrococcus*. Biochemical analysis revealed that all 12 isolates were Voges-Proskauer negative and could produce catalase, oligo-1,6-glucosidase (starch hydrolysis), gelatinase, and a-amylase enzymes. Most isolates were Indole- and citrate-negative, and other biochemical test results are explained in Table 2.

Phylogenetic analysis of the isolated epiphytic Actinomycetota strains

Genotyping revealed that the marine epiphytic strains isolated from the seagrass *T. hemprichii* belonged to six genera of the phylum Actinomycetota, namely, *Cellulosimicrobium*, *Corynebacterium*, *Microbacterium*, *Rhodococcus*, *Arthrobacter* and *Micrococcus*, while the strains from the seagrass *S. isoetifolium* belonged to four genera, namely, *Leucobacter*, *Microbacterium*, *Dietzia* and *Micrococcus*. Seven Actinomycetota isolates in this study exhibited sequence similarities ranging from 98.96% to 100% with type strains documented in the NCBI database. According to Kim et al. (2014), differentiating species with higher GC content is possible when their 16S rRNA gene sequence similarity decreases to less than 98.65%. Therefore, five of the acquired Actinomycetota strains in this study were classified as potentially new species (Table 3).

Table 1. Morphological characteristics of epiphytic Actinomycetota strains obtained from Mjimwema seagrasses, Tanzania

Isolates code	Aerial mass color	Reserve color	Texture	Elevation	Cell shapes (microscopic)	Probable genera	Reference
THP1	Cream yellow	Cream yellow	Smooth	Flat	Rod	*Cellulosimicrobium*	Zhang et al. (2020)
THP5	Whitish grey	Whitish grey	Rough	Raised	Rod	*Corynebacterium*	Sunbul (2000)
THP6	Cream	Cream	Smooth	Raised	Rod	*Microbacterium*	Meddeb-Mouelhi et al. (2016)
THP7	Pale yellow	Pale yellow	Rough	Flat	Rod	*Rhodococcus*	Ward et al. (2018)
THP9	Cream	Cream	Smooth	Flat	Rod, filamentous	*Arthrobacter*	Siddiqi et al. (2023)
THP10	Pale yellow	Pale yellow	Smooth	Raised	Cocci	*Microbacterium*	Cheng et al. (2019)
THP12	Orange	Orange	Rough	Flat	Rod	*Microbacterium*	Suzuki and Moriyuki (2015)
THP13	Cream	Cream	Rough	Flat	Rod, filamentous	*Micrococcus*	David et al. (2017)
SIP3	Yellow	Yellow	Smooth	Raised	Cocci	*Leucobacter*	Clark and Hodgkin (2015)
SIP6	Yellow	Yellow	Smooth	Flat	Cocci	*Microbacterium*	Cheng et al. (2019)
SIP12	Pale yellow	Pale yellow	Smooth	Flat	Cocci	*Dietzia*	Hirvonen et al. (2012)
SIP14	Creamy yellow	Creamy yellow	Rough	Raised	Cocci	*Micrococcus*	David et al. (2017)

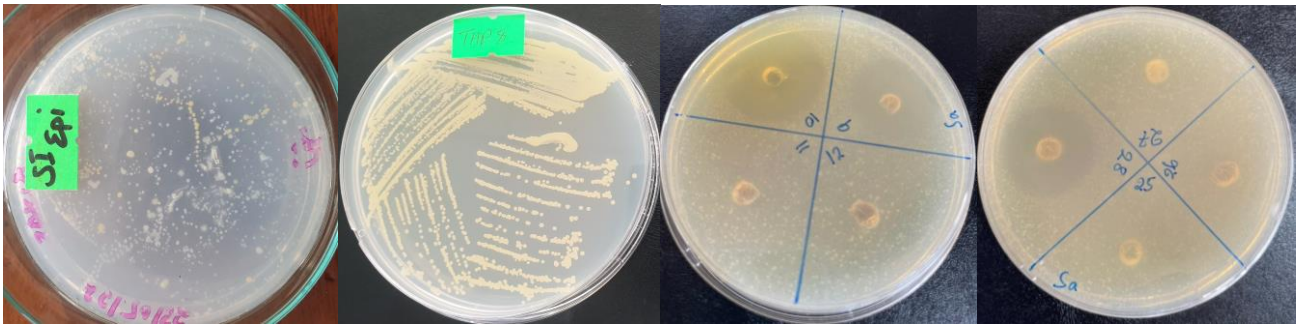
Table 2. Biochemical characterization of epiphytic Actinomycetota from Mjimwema seagrasses, Tanzania

Isolates code	Oxidase	MR test	Urea hydrolysis	Indole test	Citrate	Casein
THP1	-	+	+	-	-	+
THP5	-	+	-	-	-	-
THP6	-	+	+	-	-	-
THP7	-	+	+	-	-	-
THP9	+	-	+	-	-	+
THP10	+	-	-	-	-	+
THP12	+	-	+	-	-	-
THP13	+	+	-	-	+	-
SIP3	+	+	+	+	+	+
SIP6	-	+	+	+	+	-
SIP12	+	+	+	-	+	+
SIP14	+	-	+	-	+	+

Table 3. BLAST results for epiphytic Actinomycetota Isolated from seagrasses with their closest match from the GENBANK database

Cluster	Isolate (Accession number)	Size (bp)	Closest match (accession number)	%	Source	Reference microorganism
1	THP1 (OR936744)	590	*Cellulosimicrobium funkei* (ON678198)	100	Lake	Glekas et al. (2022)
2	THP5 (OR936745)	591	*Corynebacterium casei* (DQ361013)	100	Cheese	Monnet et al. (2006)
3	THP6 (OR936746)*	585	*Microbacterium paraoxydans* (MK403853)	97.09	Desert Soil	Belov et al. (2019)
4	THP7 (OR936747)*	585	*Rhodococcus rhodnii* (X80622)	96.36	Culture	Rainey et al. (1995)
5	THP9 (OR936748)*	580	*Arthrobacter* sp. M44 (AF235113)	81.36	North sea	Eilers et al. (2000)
6	THP10 (OR936749)*	597	*Microbacterium indicum* (NR_042459)	84.35	Sea sediment	Shivaji et al. (2007)
7	THP12 (OR939738)*	601	*Microbacterium indicum* (NR_042459)	85.64	Sea sediment	Shivaji et al. (2007)
8	THP13 (OR936750)	605	*Micrococcus luteus* (OP986538)	100	Soybean plant	Twizeyimana et al. (2023)
9	SIP3(OR936740)	600	*Leucobacter chromiireducens* (NR_042287)	100	sludge of a treatment plant	Morais et al. (2004)
10	SIP6 (OR936741)	599	*Microbacterium* sp. U50 (KU598706)	99.15	Juncus acutus	Syranidou et al. (2017)
11	SIP12 (OR936742)	604	*Dietzia timorensis* (NR_112775)	99.65	Soil	Yamamura et al. (2010)
12	SIP14 (OR936743)	597	*Micrococcus luteus* (MG603678)	98.96	Soft coral	Saranya et al. (2018)

Note: *: Putative novel isolate, OR: Accession number, % sequence similarity: percentage sequence



A **B** **C** **D**

Figure 2. Representative plates showing A. Mixed of Actinomycetota colonies from *Syringodium isoetifolium* epiphytes (SI epi) and B. Pure culture colonies of Actinomycetota from *Thalassia hemprichii* (THP8) as well as C and D showing antimicrobial activities of different strains. Numbers in the plates represent different Actinomycetota crude extracts and controls, i.e., in Figure 2.C, 9: SIP6, 10: Positive Control, 11: Negative control, 12: THP6, in Figure 2.D, 25: THP9, 26: THP13, 27: THP12, 28: THP3

These potential new strains were *Microbacterium* THP6 (OR936746), *Microbacterium* THP7 (OR936747), *Microbacterium* THP9 (OR936748), *Arthrobacter* THP10 (OR936749), and *Rhodococcus* THP12 (OR939738). Most isolated strains showed similarities to type strains isolated from various sources, such as soils and lakes, with few from marine environments (i.e., from soft coral and sea sediment), and none of them have been reported before in seagrasses (Table 3). Figure 2 displays the phylogenetic rebuilding of partial 16S rRNA gene sequences of the isolated epiphytic Actinomycetota strains alongside their closely related matches from the database. The phylogenetic tree illustrates the relationships and placement of 7 known Actinomycetota species, and the 5 newly discovered strains were shown to form unique clusters within their respective taxa, as depicted in Figure 3.

Antimicrobial activity of the isolated epiphytic Actinomycetota strains

Twelve strains of epiphytic Actinomycetota were examined to determine their potential to produce antimicrobial substances against four test microorganisms. The findings revealed that a significant proportion of isolates (7 out of 12, accounting for 58%) exhibited antibacterial activity against at least one of the test organisms, with inhibition zones varying from 8 to 30 mm (Table 4). Among these strains, only one (*Microbacterium* SIP6) displayed antifungal activity against *C. albicans*, with an inhibition zone measuring 15 mm. Seven strains showed antagonistic effects against the Gram-positive bacterium *S. aureus*. However, fewer isolates demonstrated antibacterial activity against the Gram-negative bacteria *P. aeruginosa* (3) and *E. coli* (2) (as delineated in Table 4). Remarkably, the antimicrobial agent *Microbacterium* SIP6 exhibited the most substantial inhibitory impact on *E. coli* (26 mm) and *S. aureus* (30 mm). In comparison, the inhibition zones for the positive controls (ciprofloxacin at 4 mg/mL) were 25 mm, 26 mm, and 22 mm for *S. aureus*, *P. aeruginosa*, and *E. coli*, respectively, while the antifungal agent fluconazole at 64 µg/mL resulted in a 15 mm inhibition zone.

Minimum Inhibitory Concentration (MIC)

Of the 12 extracts from the isolated epiphytic Actinomycetota strains, only four demonstrated antimicrobial activities characterized by an estimated

minimum inhibitory concentration (MICs) at or below the defined cut-off value of 3.13 mg/mL. The strain *Dietzia* (SIP12) displayed inhibition to all three tested organisms, followed by *Microbacterium* (THP10) and *Microbacterium* (SIP6) (Figure 4). Only two strains, *Micrococcus* (THP13) and *Dietzia* (SIP12), exhibited MICs of 1.56 mg/mL and 3.13 mg/mL against *C. albicans*, respectively. The MICs of the positive control (ciprofloxacin) were 0.015, 0.5, and 0.25 µg/mL for *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively, while those of the negative control were ≥25 mg/mL.

The Brine Shrimp Lethality Assay

The Brine Shrimp Lethality Test (BSLT) findings revealed that most of the extracts from the isolates were toxic, with LC_{50} values ranging from 134.40 to 399.24 µg/mL (Table 5). Extracts from the two strains THP6 (*Microbacterium* sp.) and SIP14 (*Micrococcus* sp.) exhibited low cytotoxicity and were classified as nontoxic, with LC_{50} values of ≥ 3836.7 µg/mL and 1243.4 µg/mL, respectively. Cyclophosphamide was used as a toxic standard drug and exhibited an LC_{50} value of 16.367 µg/mL, showing that all our strains had lower toxicity.

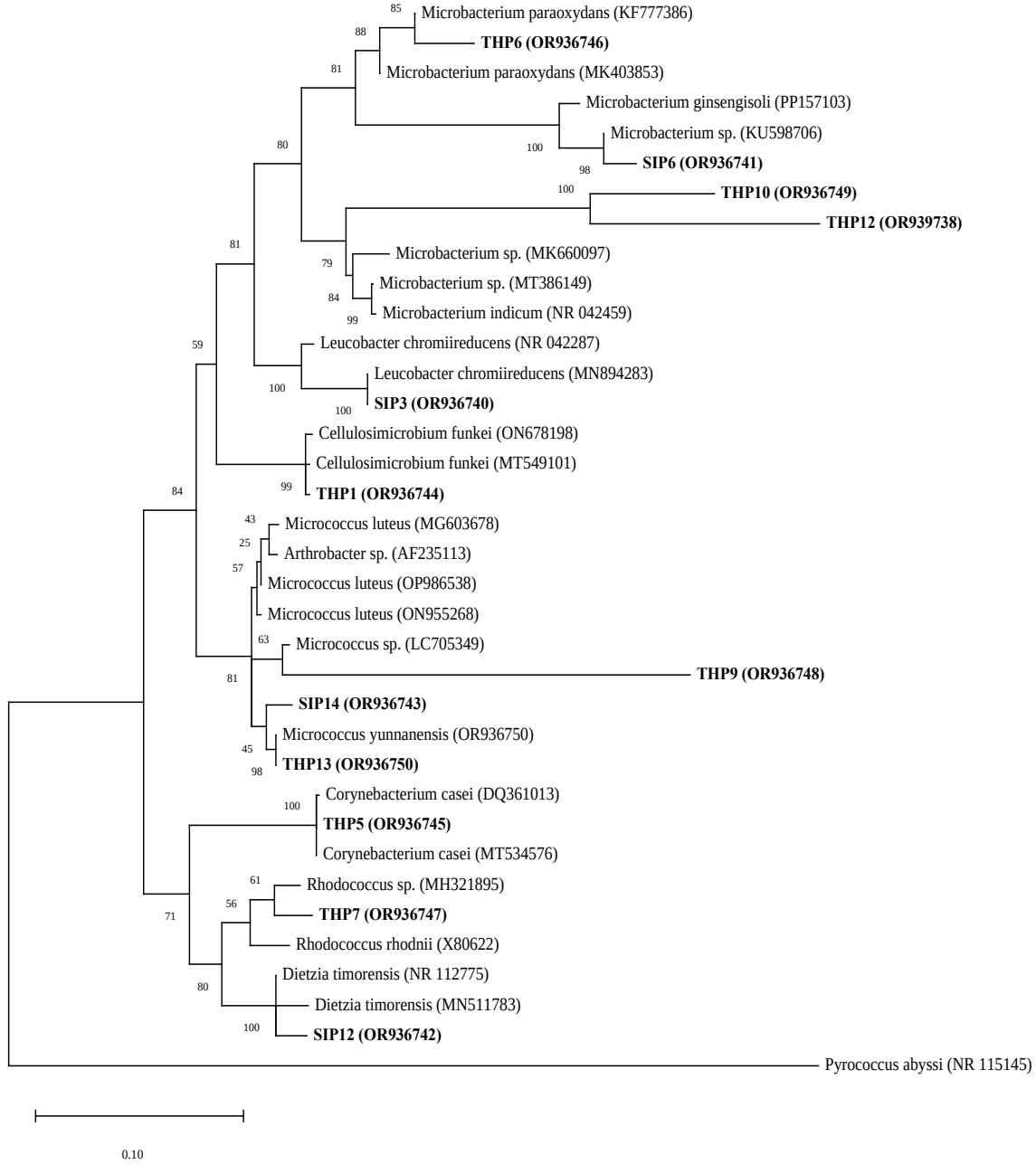


Figure 3. The construction of the phylogenetic tree involved using partial 16S rRNA gene sequences derived from epiphytic Actinomycetota found on the seagrasses *T. hemprichii* (THP) and *S. isoetifolium* (SIP) from Mjimwema, Dar es Salaam, Tanzania. This analysis also included type strains of closely related genera. The maximum likelihood heuristic method was applied for tree generation using nearest neighbor interchange. Bootstrap values were calculated through 1,000 data resamplings, with branch points showing bootstrap values exceeding 22%. The scale indicated on the tree represents a rate of 0.10 substitutions per nucleotide position

Table 4. Diameters of the zone of inhibition of the crude extracts of epiphytic Actinomycetota

Isolates	Zone of Inhibition (mm) at 100 mg/mL			
	E.coli	***S. aureus***	***P. aeruginosa***	***C. albicans***
Cellulosimicrobium (THP1)	NA	10	NA	NA
Corynebacterium (THP5)	17	12	NA	NA
Microbacterium (THP6)	NA	NA	NA	NA
Rhodococcus (THP7)	NA	10	NA	NA
Arthrobacter (THP9)	NA	NA	NA	NA
Microbacterium (THP10)	20	8	NA	NA
Microbacterium (THP12)	NA	NA	NA	NA
Micrococcus (THP13)	NA	25	16	NA
Leucobacter (SIP3)	NA	NA	NA	NA
Microbacterium (SIP6)	26	30	21	15
Dietzia (SIP12)	NA	NA	NA	NA
Micrococcus (SIP14)	NA	20	NA	NA
Ciprofloxacin	22	25	26	NA
Fluconazole	NA	NA	NA	15

Note: NA: Not active

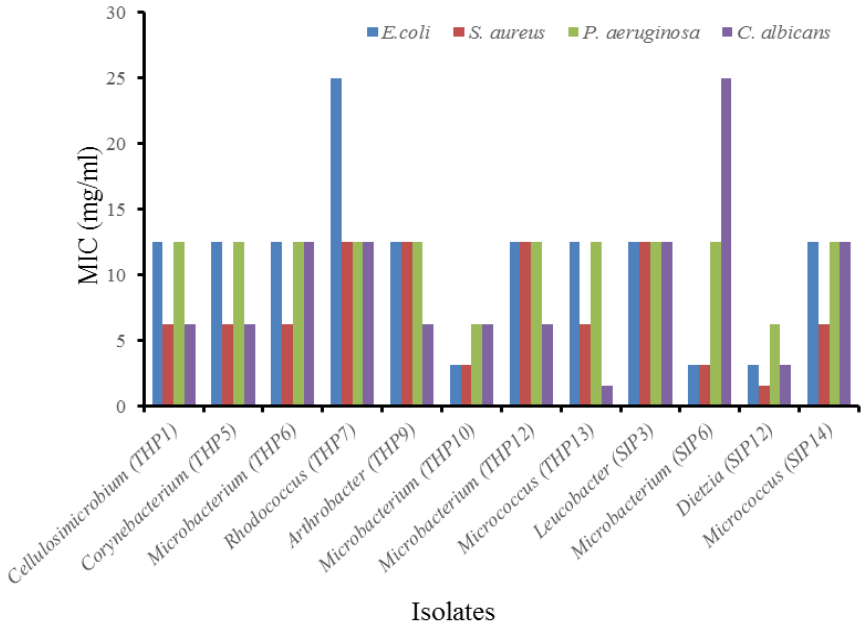


Figure 4. Minimum inhibitory concentration for marine epiphytic Actinomycetota

Table 5. Brine shrimp activity of epiphytic Actinomycetota extracts from *T. hemprichii* and *S. isoetifolium* seagrasses

Isolate extracts	LC_{50} (µg/mL)	95% Confidence interval (CI)	Regression equation	Regression coefficient (r)
Cellulosimicrobium (THP1)	165.50	150.89 -181.53	y=65.715logx-95.808	0.9248
Corynebacterium (THP5)	235.44	175.87 -315.19	y=49.693logx-67.866	0.9519
Microbacterium (THP6)	3836.7	830.11 -17733.6	y=33.223logx-69.07	1
Rhodococcus (THP7)	369.56	342.25 -399.06	y=70.838logx-131.89	0.9469
Arthrobacter (THP9)	168.64	134.23 -211.89	y=74.168logx-115.17	0.9589
Microbacterium (THP10)	399.24	62.789 -703.17	y=42.323logx-60.092	0.9864
Microbacterium (THP12)	134.40	91.048 -194.40	y=100.01logx-162.86	0.9734
Micrococcus (THP13)	323.11	294.05 -355.08	y=72.628logx-132.35	0.9954
Leucobacter (SIP3)	139.32	100.65 -192.85	y=81.665logx-125.09	0.9326
Microbacterium (SIP6)	201.88	134.03 -304.08	y=126.55logx-241.71	0.9984
Dietzia (SIP12)	164.55	89.329 -303.15	y=139.48logx-259.13	0.9773
Micrococcus (SIP14)	1243.4	557.76 -2771.7	y=42.781logx-82.39	0.983
Cyclophosphamide	16.367	12.006 - 22.305	y=69.968logx-34.93	0.995

Discussion

Epiphytic microbial communities in seagrasses have previously been reported in *C. rotundata*, *T. testudinum*, and *Zostera marina* L. (Mishra and Mohanraju 2018; Tasdemir et al. 2024). This study reports for the first time epiphytic Actinomycetota species diversity within seagrass *T. hemprichii* and *S. isoetifolium* meadows of the coast of Tanzania. All the isolated strains in this study were found to belong to rare Actinomycetota genera. The term "rare Actinomycetota" generally denotes less frequently isolated strains than *Streptomyces* strains (Martin-Pozas et al. 2023). Research conducted by Parra et al. (2023) has demonstrated that most marine ecosystems are abundant in rare Actinomycetota species. These species have been studied relatively less than the genus *Streptomyces*, often due to their slower growth rates under laboratory conditions (Parra et al. 2023).

Most Actinomycetota isolates in this study exhibited sequence similarities ranging from 98.96% to 100% with type strains documented in the NCBI database, showing that they were already known from other ecosystems. According to Kim et al. (2014), species with higher GC content can be differentiated when their 16S rRNA gene sequence similarity drops below 98.65%. The predominant genus encountered was *Microbacterium* spp., with two strains (THP6 (OR936746) and THP10 (OR936749)) out of four that were notably distinct from type strains, with percentage similarities ranging from 84.36-97.06%. *Microbacterium* species have been found in the seagrass species *T. hemprichii* (Siro and Pipite 2024) and *Z. marina* (Tasdemir et al. 2024) and various other marine environments (Xie et al. 2021; Zhu et al. 2021a). However, our findings mark the first report showing the occurrence of *Microbacterium* species as epiphytes of the seagrass *S. isoetifolium*. *Microbacterium* spp. are recognized for their enzymatic production (Li et al. 2019), capacity for bioremediation, and biomolecule transport (e.g., glucose, fructose, and galactose) (Avramov et al. 2016). Other researchers documented the antimicrobial properties of *Microbacterium* species, e.g., Graça et al. (2013). For the first time, our findings demonstrated the strong antimicrobial activity of *Microbacterium* (SIP6) and the antibacterial effects of a novel strain, *Microbacterium* (THP10). This new strain exhibits potential for use in pharmaceutical applications. Three of the four *Microbacterium* strains demonstrated toxicity in the brine shrimp test, indicating their potential as anticancer agents (Yusriadi et al. 2019).

The genus *Micrococcus* emerged in both seagrass species, with our isolated strains showing greater than 98.96% similarity to known type strains in GenBank, meaning that they were all already isolated in other studies (Saranya et al. 2018; Twizeyimana et al. 2023). They have been reported in various seagrass species, such as *H. uninervis* (Wahbeh et al. 1984), *H. stipulacea* (Pereg et al. 1994), *C. rotundata* and *T. testudinum* (Mishra and Mohanraju 2018). Nevertheless, their occurrence in seagrass meadows of *T. hemprichii* and *S. isoetifolium* has never been reported. We have demonstrated that these strains exhibit antibacterial properties, consistent with the findings of Tizabi and Hill (2023), who documented the antibacterial effects of *Micrococcus luteus* (Schroeter, 1872) Cohn, 1872 against various other bacteria. Furthermore, in our study, one strain (*Micrococcus* THP13) was found to be nontoxic, whereas the other exhibited toxicity in brine shrimp, making it a potential candidate for further investigation regarding its anticancer or cytotoxic properties (Omeke et al. 2018; Yusriadi et al. 2019). In another study, a strain of *M. luteus* was reported to be capable of causing infections such as bacteremia, hepatic and brain abscesses, and septic arthritis in immunocompromised patients (Zhu et al. 2021b). This also calls for additional investigations to evaluate the clinical potential of our isolate as an opportunistic pathogen in clinical settings.

The other two isolated strains (*Rhodococcus* THP7 and *Arthrobacter* THP9) appeared distantly related to *Rhodococcus rhodnii* Goodfellow & Alderson, 1979 (X80622) and *Arthrobacter* sp. M44 (AF235113) species might be novel species, showing similarity identities of 96.36% and 81.36%, respectively (Kim et al. 2014). *Rhodococcus* species have been successfully isolated from seagrasses such as *Z. marina* (Ettinger and Eisen 2021), *Halodule wrightii* Asch., *T. testudinum*, and *H. stipulacea* (Aires et al. 2021). However, these species have not yet been isolated from the seagrasses *T. hemprichii* and *S. isoetifolium*. We demonstrated the antibacterial effects of the isolated novel strain of *Rhodococcus* (THP7) against *S. aureus*, similar to the findings of other studies (e.g., Naragani et al. 2014) documenting the antimicrobial properties of *Rhodococcus* species. In addition, some species of *Rhodococcus* sp. can break down various compounds, produce bioactive steroids, and play a role in the biodesulfurization of fossil fuels (Zappaterra et al. 2021). However, a few species within this genus are pathogenic (Savory et al. 2020). Species of the genus *Arthrobacter* have been reported from different seagrass species, such as *H. uninervis*, *H. ovalis*, *H. stipulacea*, *Z. marina* and *Zostera japonica* Asch. & Graebn. (Bibi et al. 2018: Siro and Pipite 2024). Nonetheless, we are documenting their presence in the seagrasses *T. hemprichii* and *S. isoetifolium* for the first time. Our new strain, *Arthrobacter* (THP9), did not exhibit any antimicrobial activity against the tested human pathogens, unlike some other reports, such as that of Ramlawi et al. (2021), who documented the antimicrobial effects of *Arthrobacter* species isolated from disease-suppressive compost. Additional research has indicated that Arthrobacter species can break down polymeric compounds, contributing significantly to the biodegradation of agrochemicals and pollutants (Gobbetti and Rizzello 2014). Our strains were also identified as toxic, but further investigations of their antitumor, cytotoxic, and pesticide activities are warranted (Omeke et al. 2018).

Other species identified in this study, including *Cellulosimicrobium*, *Corynebacterium*, *Leucobacter*, and *Dietzia*, showed similarity scores ranging from 99.65% to 100% with type strains from GenBank. These species have previously been isolated from various seagrasses, for example, *Cellulosimicrobium* in *T. testudinum* (Couto-

Rodríguez 2009) and *Corynebacterium* in *H. uninervis* (Bibi et al. 2018). However, our study marks the first report of their presence in the seagrasses *T. hemprichii* and *S. isoetifolium*. Notably, this research marks the initial documentation of the *Leucobacter* and *Dietzia* genera within seagrass species. Tanveer et al. (2021) discovered the antioxidant properties of marine *Cellulosimicrobium funkei* Brown et al. 2006. In contrast, our study marks the first report of its antimicrobial activity. Researchers have predominantly focused on reporting the antimicrobial activities of *Corynebacterium* species apart from *Corynebacterium casei* Brennan et al. 2001 (Gowramma et al. 2015; Menberu et al. 2021). Our study, however, presents the antimicrobial activity of Actinomycetota *C. casei* for the first time. Our study is the first to report on the antimicrobial activities of strains *Leucobacter chromiireducens* A and *Dietzia timorensis* Yamamura et al., 2010. None of the strains exhibited any activity against the tested human pathogens. The isolated strains of the genera *Cellulosimicrobium*, *Corynebacterium*, *Leucobacter*, and *Dietzia* exhibited toxicity, indicating their potential use in anticancer applications.

In conclusion, this investigation aimed to assess the diversity and biotechnological applications of epiphytic Actinomycetota species obtained from seagrasses along the coastline of Dar es Salaam. The research identifies and describes 12 rare Actinomycetota species, 5 of which show promise as potentially new species. The isolated Actinomycetota can produce extracellular enzymes that may be used in drug development to target diseases involving enzyme dysregulation. Most isolated Actinomycetota species showed antibacterial activities against at least one tested human pathogen. Moreover, the *Microbacterium* (SIP6) strain displayed potent antimicrobial effects against all examined human pathogens, suggesting its possible pharmaceutical value. *Micrococcus* (THP13) and *Dietzia* (SIP12) exhibited activity against *C. albicans*, indicating their potential utility in the production of antifungal agents. Furthermore, most isolated strains in this study are toxic, making them potential candidates for further exploration of their anticancer and cytotoxic activities. These findings highlight the diverse biotechnological potential of marine epiphytic Actinomycetota strains and warrant further investigation into their medicinal and therapeutic applications. It is necessary to conduct additional studies, possibly involving in vitro and in vivo experiments, to evaluate the isolated strains' potential clinical applications, safety, and effectiveness in a clinical context. Additionally, comprehensive toxicity studies and risk assessments should be carried out to assess the safety profile of the extracts for potential biotechnological and pharmaceutical applications. Furthermore, future research should investigate how these findings can be extended to other marine environments, enhancing the overall generalizability of the results.

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