

In vitro antimicrobial activity of crude extracts from marine *streptomyces* isolated from mangrove sediments of Tanzania

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Received: 15 January 2012 / Received in revised form: 27 January 2012, Accepted: 5 June 2012, Published online: 30 October 2012
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Abstract

This study was undertaken to isolate actinomycetes from mangrove sediments of coastal Tanzania and evaluate their potential for production of bioactive metabolites. Starch casein agar (SCA) medium was used to isolate the actinomycetes. Extraction of actinomycetes using ethyl acetate (1:1), afforded dry extracts. The extracts were tested for antimicrobial activity and for brine shrimp toxicity test. A total of three isolates (ACTN 1, ACTN 2 and ACTN 3) were obtained by using culture medium selective for actinomycetes. Actinomycetes specific primers; S-C-Act-235-S-20 and S-C-Act-878-A-19 were used to identify two isolates as *Streptomyces* sp and one as actinomycetes sp. The strongest activity against bacterium (*Bacillus subtilis*) and fungus (*Candida albicans*) was exhibited by crude extracts of *Streptomyces* sp (ACTN 2 and ACTN 3). Crude extracts of all three isolates exhibited non-cytotoxic activity against brine shrimp larvae with LC₅₀ values ranging from 250 - 446 µg/ml respectively. The results from this study provide evidence that the streptomycetes in mangrove sediments could be promising sources for antimicrobial bioactive agents.

Key words: actinomycetes, mangrove, sediments, antimicrobial activity.

Introduction

Infectious diseases are leading health problems with high morbidity and mortality in the developing countries (Hong et al. 2009). On the other hand, the development of resistance to multiple drugs is a major problem in the treatment of infectious diseases caused by pathogenic microorganisms. This multidrug resistance is presently an urgent focus of research and therefore calls for development of new bioactive compounds in order to handle the challenge.

Actinomycetes and certain bacterial species are well known as good source of microbial secondary metabolites producers in drug discovery programs (Berdy 2005). They play a major role in recycling of organic matter, production of novel pharmaceuticals, nutritional materials, cosmetics, enzymes, antitumour agents, enzyme inhibitors, immune-modifiers and vitamins (Remya and Vijayakumar 2008). Marine ecosystems are a rich source of novel actinomycetes which have the capacity to produce interesting new bioactive compounds, including antibiotics (Hong et al. 2009). Actinomycetes of the genus *Streptomyces* are especially prolific and can produce many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites (Devi et al. 2006).

Mangroves are highly productive ecosystems (Kathiresan and Bingham 2001) though surprisingly, information on Tanzanian mangrove microbial communities is lacking. However, few studies have reported on Tanzanian mangrove microorganisms (Abbu and Lyimo 2007; Lyimo et al. 2009; Lyimo et al. 2009). There is evidence that mangrove sediments contain high populations of actinomycetes (Kathiresan and Bingham 2001). The few actinomycetes which have been isolated from mangrove habitats are a potentially rich source for discovery of anti-infection and anti-tumor compounds (Remya and Vijayakumar 2008; Hong et al. 2009). Reports from other parts of the world describe the potential role of marine actinomycetes in production of bioactive compounds (Goodfellow et al. 2007; Remya and Vijayakumar 2008; Gurung et al. 2009). In Tanzania so far, one study has reported on soil actinomycetes (Ndonde and semu 2000). In this regard then, little is known about the actinomycetes of Tanzanian marine sediments, which is a resource that has not been properly exploited. In the present study, an attempt was made to isolate the actinomycetes from the mangrove sediments of Dar es Salaam, Tanzania, and to screen for their bioactive properties.

Materials and Methods

Collection of sediment samples

Mangrove sediment samples were collected from Mbweni, a mangrove ecosystem which lies on 39° 25' E and 6° 34' S of Dar es Salaam. Sediment samples from at least ten locations were collected at a depth of 6 - 10 cm within a 100 m² area. The sediments from each location were bulked and homogenized to prepare composite

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samples. All samples were transported to the laboratory in sterile polythene bags and stored at 4°C for further study.

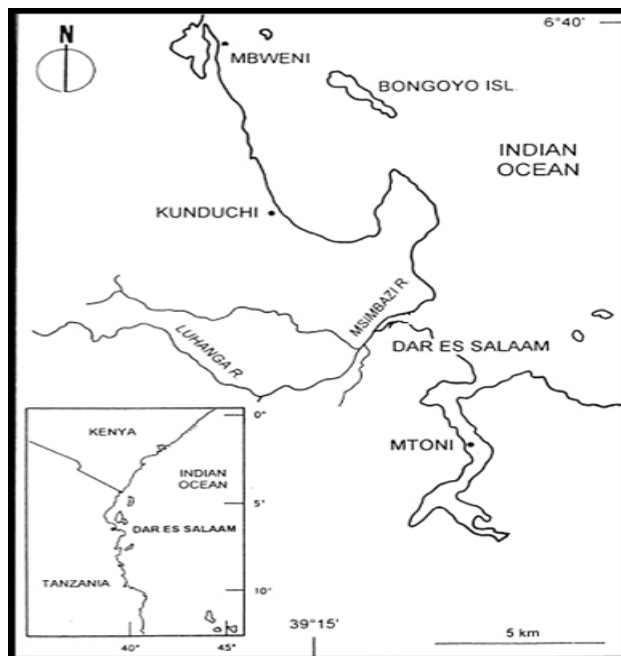


Fig. 1. Location of sampling site.

Isolation of actinomycetes

Starch casein agar (starch, 10 g; casein, 3 g; agar, 18 g; pH 7.0-7.4) medium was used for isolation and enumeration of actinomycetes (Hong et al. 2009). The medium was supplemented with 50 µg/ml cyclohexamine to inhibit the development of fungi and 50 mg/L nystatin to inhibit the bacteria capable of swarming, without affecting the growth of actinomycetes. In conventional dilution plate technique, 1g of marine sediment sample was suspended in 9 ml of sterile sea water, serially diluted to 10^{-4} and then pretreated by incubating the tubes in water bath at 56°C for 6 minutes. A volume of 0.2 ml of suspension was spread over 50% sea water starch casein agar medium (Hong et al. 2009) and incubated for 7-21 days at 28°C. After incubation, the actinomycetes colonies were purified by sub-culturing individual colonies on SC agar plates and strains were preliminarily isolated according to traditional morphological criteria. This criteria included observing characteristics of colonies on the plates, gram stain, morphology of substrate and aerial hyphae, morphology of spores and pigment production (Goodfellow et al. 2007).

Identification of isolates

(i) Extraction of DNA from pure cultures and PCR amplification.

Total genomic DNA samples from the actinomycetes isolates were isolated using a modified protocol of Goodfellow et al. 2007. Prior to DNA isolation, the pure actinomycetes isolates were cultivated on a SCA plate and incubated at room temperature for 5 - 7 days. 0.8 g of pure colonies was scooped from a plate into a sterile 2 ml eppendorf tube. 1 g of sterile glass beads and 0.8 ml of extraction buffer (2% Triton X100, 1% SDS, 100 mM NaCl, and 10 mM Tris-pH 8, 1 mM EDTA-pH 8) were added and the mixture was dismembranated (Sartorius Mikro dimembrator U) for 1.5 min. 60 µl of 20% sodium dodecyl sulfate (SDS) was added into a mixture then vortexed for 30 seconds. The mixture was then incubated into

a waterbath at 65°C for 1 h. From the water bath, the mixture was centrifuged (15000 g) for 5 minutes. The supernatant was collected into a new 2 ml eppendorf tube. This was followed by addition of equal volume of phenol/chloroform/isoamylalcohol (25: 24: 1) and incubation at 65°C (in a water bath) for 20 min. After vortexing for 10 seconds, the mixture was centrifuged (15000 g) for 10 min. The upper aqueous layer was transferred into a new sterile tube and extracted with equal volume of chloroform: isoamyl alcohol (24: 1) then centrifuged (15000 g) for 5 minutes. The supernatant was collected into a new 2 ml eppendorf tube and the DNA was precipitated overnight at -20°C by addition of two volumes of cold iso-propanol and collected by centrifugation (10 min at 15000 g). The DNA pellet was washed with 250 µl ethanol (70%). The pellet was then air dried for about 20 min, dissolved in 50 µl TE buffer and stored at -20°C.

(ii) Polymerase Chain reaction

The PCR was performed in a final volume of 25 µl which was composed of about 50ng template DNA, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 200 pM of Actinomycetes specific primers S-C-Act-235-S-20 (5'-CGCGGCCTATCAGCTTGTG-3') and S-C-Act-878-A-19 (5'-CCGTACTCCCCAGGCGGGG-3') and 1U of Taq polymerase with the appropriate reaction buffer under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 50 s, annealing at 52 °C for 50s, and 72 °C for 90s. The amplification products were separated by gel electrophoresis in 1.2% agarose gels which were stained with ethidium bromide (Stach et al. 2003a).

(iii) Nucleotide Sequencing

The PCR products were sequenced at a commercial facility in South Africa (INQABA Biotechnology Ltd) whereby, actinomycetes PCR products of sizes around 640 bp were first cleaned using standard kit and thereafter sequenced twice (forward and reverse) using an ABI 3730 automated sequencer (Applied Biosystems). The nucleotide sequences obtained were aligned using a standard Basic Alignment Search Tool (BLAST) available at the National Centre for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov>). The gene sequences were further aligned using CLUSTAL-X software.

Bioactivity assay of crude extracts

(i) Preparation of crude extract

The culture broth (50 × 200 ml) of actinomycetes isolates was subjected for solvent extraction method to recover antibacterial metabolites in pure form. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 h for complete extraction. The extracts were concentrated to dryness by using a rotary evaporator under vacuum at 40°C (Gurung et al. 2009).

(ii) Brine shrimp testing

The brine shrimp lethality test (BST) was used to predict the presence, in the extracts, of cytotoxic activity (Moshi et al. 2009). The crude extracts were dissolved in dimethylsulfoxide (DMSO) to a concentration of 4 mg/ml (stock solution) for each sample. To begin the bioassay about one teaspoonful of brine shrimp eggs (Great Salt Lake, USA) were hatched in 300ml of filtered sea water in a conical flask and allowed to shake for 48 hours with illumination using an electric bulb. Ten shrimp larvae were selected and transferred into each sample well by means of 100µl pipette and

the final volume of each vial was adjusted to 200µl using natural sea water. The extract (stock solution) was introduced into the wells by pipette in volumes of 15µl, 6µl, 5µl, 4µl, 2µl and 1.5µl to make concentrations of 240µg/ml, 120µg/ml, 100µg/ml, 80µg/ml, 40µg/ml and 24µg/ml respectively. The control sample was DMSO which was not treated with extract. The microtiter plate was maintained under illumination. Survivors were counted with the aid of a stereo microscope after 24 h and the mortality of larvae at each dose was determined. The concentration that killed 50% of the nauplii (LC₅₀ in µg/mL and Confidence Intervals 95%) was determined using probit analysis. An LC₅₀ concentration of greater than 100 is nontoxic, less than 100 is toxic and less than 20 has cancer fighting potential (Bastos et al. 2009).

(iii) Determination of Antimicrobial activity

(a) Test microorganisms

Three test microorganisms were used in the determination of antimicrobial activity. They were *Bacillus subtilis* (DSM 347), a gram positive bacteria, *Pseudomonas aeruginosa* (DSM 1117), gram negative bacteria and *Candida albicans* (ATCC 90028) a yeast. All these were obtained from the Department of Molecular Biology and Biotechnology (MBB), University of Dar es Salaam.

(b) Preparation of test microorganisms

All test bacteria were grown in Nutrient broth and incubated at 37°C for 24 h. A total of 10 ml of the pure culture was centrifuged to pellet out the cells, washed twice with sterile physiological saline and the suspension adjusted to optical density 0.1 at 600 nm which is equivalent to a cell population of about 10⁶ cells/ml on the McFarland standard. Microbial suspension was stored in test tubes and refrigerated at 4°C. These test organisms were frequently checked for viability and re-prepared when appropriate.

(c) Antimicrobial assay

Antimicrobial activities were carried out using micro dilution method to determine minimum inhibitory concentrations (MICs) (Moshi et al. 2009). The 96 well microtitre plates were used and each Actinomycetes extract was tested in duplicate at serial dilutions of 0.08, 0.16, 0.31, 0.625, 1.25, 2.5, 5 and 10 mg/ml. Columns 1 and 2 were used for solvent controls and Columns 11 and 12 for positive controls. An amount of 100 µl of clear broth was added in each well, and then, 100 µl of extract were added in the first and second rows of the microtiter plate. From the second row, a series of two fold dilutions was performed in subsequent rows (except the last row). Then, 100 µl of the broth culture of a test organism was added in the wells (except the first row). The plates were then incubated at 37°C for 24 hours. Two hours before reading the results, 40µl of the indicator (0.2% nitroterazolium chloride) were added to the plates and incubation continued for a maximum of two hours. At this point the plates were inspected for colour change; blue coloration indicating presence of microbial growth. MICs were recorded in the last well in a row where there was no colour change. All experiments were done in duplicates.

Results

Isolation of actinomycetes

A total of 3 strains were isolated from the mangrove sediment samples. Strains were grouped based on their colony morphology observed on the SC agar plates. The isolates were small to medium

sized, grayish white to pure white in colour, round, powdery, with regular margin and some were pigmented in golden yellow colour.

Identification of Actinomycetes Isolates Using DNA techniques

A BLAST search of the 640 bp 16S-rRNA gene sequence of isolates showed two of them to have 98% - 99% homology to known streptomycetes sp. A phylogenetic analysis of these isolates that shared morphological similarities with the genus *Streptomyces* revealed that, they all belonged to this genus. However, one isolate which had 84% homology with known species of the *Streptomyces* genus, clustered singly on the phylogenetic tree. This implies a distinct phylogenetic position within the radiation including representatives of the known genus (Fig. 2).

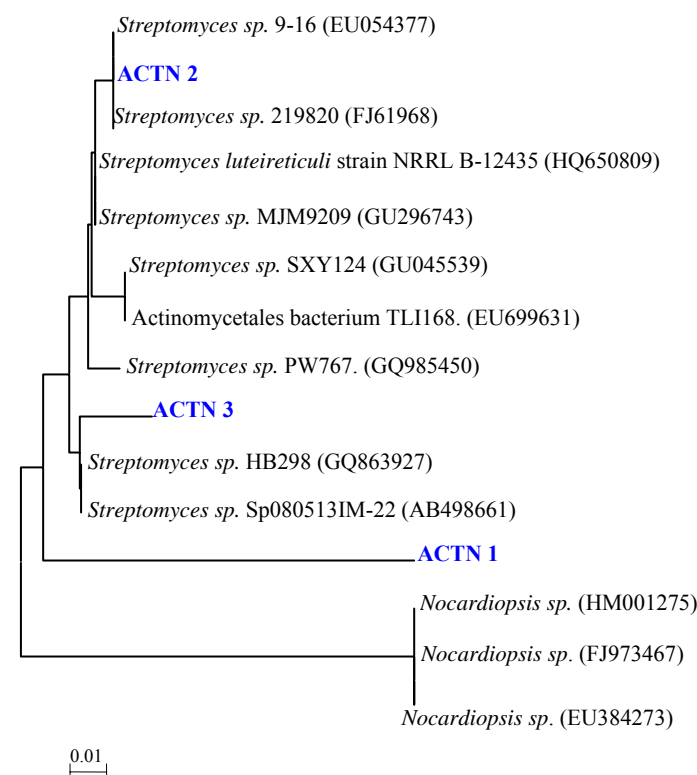


Fig. 2. Evolutionary relationships of taxa: Relationships between isolated strains and members of the genus *Streptomyces*, *Nocardioopsis* and *Actinomycetales* sp on a neighbour-joining tree based on 16S rDNA sequences. The analysis involved 15 nucleotide sequences. The scale bar indicates 0.01 substitutions per nucleotide position. Evolutionary analyses were conducted in MEGA5.

Brine shrimp toxicity

All three isolated strains were found to be non-toxic. The LC₅₀ results ranged from 250.780 – 446.954 µg/ml for all extracts of the representative actinomycetes strains. The LC₅₀ for the control was 349.546 µg/ml (Table 1)

Table 1. Brine shrimp toxicity expressed as LC₅₀ µg/ml (95% Confidence Intervals)

	Actn 1 µg/ml	Actn 2 µg/ml	Actn 3 µg/ml	Control µg/ml
LC ₅₀	446.954	255.418	250.780	349.546
95%	198.914	166.900	154.756	187.231
Confidence				

Antimicrobial activity

(a) Minimum inhibitory Concentrations (MICs)

All the three strains showed antimicrobial activity against the two test bacteria and the fungus. The MIC values of isolate ACTN 1, ACTN 2 and ACTN 3 crude extracts against *Pseudomonas aureginosa*, *Bacillus subtilis* and *Candida albicans* are shown in Table 2. The most active extract was that of isolate ACTN 2 and ACTN 3 against *Bacillus subtilis* and *Candida albicans* with MIC value of less than 0.5 mg/ml. The least active extract was that of isolate ACTN 1 against *Pseudomonas aureginosa* with MIC value of 5 mg/ml.

Table 2. Minimum inhibitory concentrations (MICs) of test organisms

Isolates	MIC (mg/ml)		
	<i>Pseudomonas aureginosa</i>	<i>Bacillus subtilis</i>	<i>Candida albicans</i>
Actn 1	5	5	1.25
Actn 2	5	0.1563	0.3125
Actn 3	5	0.1563	0.625

Discussion

Based on macro morphological characteristics that included colony characteristics and pigmentation properties of the obtained pure isolates, two isolates formed substrate mycelium and abundant aerial mycelium with powdery spore mass. One isolate produced aerial mycelium which was white in colour but lacked powdery spore mass. The isolated Actinomycetes were identified based on the colony morphology and Gram staining (Holt et al. 1994). In the present work, we have identified the actinomycetes by the presence of powdered colonies on the surface of agar plate and the gram reaction. The same patterns were observed in a previous study by Goodfellow et al. 2007. In this study, the morphological approach is however complemented with the DNA methodology taxonomy. This study observed the growth of *Streptomyces* spp. on SC agar as similarly observed during the isolation of actinomycetes from Malaysian mangrove mud and intertidal sediments by Getha et al. 2004.

The actinobacteria-specific primers (S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A- 19) used in this study have high specificity (Stach et al. 2003a). The 16S rDNA sequencing analysis of the isolate in this study; using the actinobacteria-specific primers yielded 640 base pairs. These results are in line with those from previous studies which showed that, the 16S rDNA gene in actinomycetes is specific to each species and whose ends 5' and 3' are conserved in all species (Stach et al. 2003b, Sun et al. 2010). The NCBI BLAST search analysis showed that the sequences of ACTN 2 and ACTN 3 isolates were 98% - 99% similar to the sequences of known *Streptomyces* sp. ACTN 2 had 98% homology with *Streptomyces* sp.175 (2010) (HM011273) a bacterium screened to produce antimicrobial. ACTN 3 isolate had 99% homology with *Streptomyces* sp.219820 (FJ 261968) a species found to have antimicrobial activity (Hong et al. 2009). ACTN 1 isolate had 84% similarity to *Streptomyces* sp. PW767 (GQ985450) isolated from china (Hong et al. 2009). A neighbour-joining tree based on 16S rDNA sequences showed that the ACTN 1 isolate clustered singly, suggesting that it could be a new species.

The brine shrimp test (BST) represents a rapid, inexpensive and simple bioassay for testing crude extracts which in most cases correlates with cytotoxic properties (Magadula et al. 2009). All ethyl acetate extracts of this study were found to be biologically active and non toxic (Table 1). The mortality rate of the brine shrimp

nauplii in all three samples, increased with the increase in the concentration of the sample. The same behavior was reported in a previous study (Al-Bari et al. 2007).

Minimum inhibitory concentration is imperative as it gives an indication as to what level of dilution can be achieved while still retaining the level of activity (Gurung et al. 2009). The minimum inhibitory concentrations (MICs) for ACTN 2 and ACTN 3 Isolate extracts were more active than that of ACTN 1.

The present study compares well with that of Valli et al. 2011 where *Streptomyces* species showed efficient antagonistic activity as opposed to other actinomycetes. ACTN 2 and ACTN 3 isolates were identified as *Streptomyces* species and showed significant antimicrobial activity against *B. Subtilis* compared to their activity against *P. aeruginosa* similarly reported by Kokare et al. 2004. This could be ascribed to morphological differences of the bacterial cell walls. Gram negative bacteria have an outer lipopolysaccharide membrane; hence their cell wall is impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes (Gurung et al. 2009). The Gram positive bacteria have only peptidoglycan layer which is not an effective permeability barrier for the antibacterial agents.

Previous investigations indicate the tremendous potential of marine actinomycetes, particularly *Streptomyces* species as a useful and sustainable source of new bioactive natural products (Dhanasekaran et al. 2009; Hong et al. 2009; Sun et al. 2010; Eccleston et al. 2008, Huang et al. 2008). The results of the present investigation reveal that the marine actinomycetes from mangrove environment are a potential source of novel antibiotics. It is anticipated that isolation, characterization and study of actinomycetes can be useful in the discovery of novel species of actinomycetes.

It is becoming increasingly evident that the taxonomic and metabolic diversity of streptomycetes is remarkable, as putatively novel *Streptomyces* species are being isolated from under-researched habitats and shown to be valuable sources of new bioactive compounds (Hong et al. 2009). The sequencing results of this study have provided further evidence of the trend observed in previous studies (Stach et al. 2003a; Goodfellow et al. 2007; Hong et al. 2009).

Conclusion

The present study was an attempt to identify strains of streptomycetes from the mangrove sediments that display activity against microbial pathogens. Such attempts need to be sustained so as to screen more isolates for novel therapeutics. However, the study only provided a basis for antimicrobial potential of the crude extracts of the isolates. There is need to carry out bioassay guided fractionation to isolate the actual bioactive compounds from the extracts.

Acknowledgements

This research was supported by the University of Dar es Salaam: College of Natural and Applied Sciences (CoNAS) – CIBI World Bank Project. We are grateful to Prof A. Mshandete and Dr. D. Kisangau for advises and comments. Dr. Sungh'wa of UDSM - Chemistry Department for the permission to use the Rotary Evaporator. Mr Prosper Raymond for the crude extract concentration guidance. Mr S.M. Abdallah and Mr. Kamwela L. for the mangrove sediment samples. Mr. D. Temba and Mr. F. Kimati for the Standard test organisms

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