

Full Length Research Paper

Assessment of antibiotics production potentials in four actinomycetes isolated from aquatic environments of the Eastern Cape Province of South Africa

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Crude extracts of four actinomycetes species belonging to *Actinoplane* [NB 034], *Streptomyces* [TR 007] and *Actinopolyspora* [NB 069 and TR 008] genera were screened for antibacterial activities against a wide range of bacterial strains. The extracts showed antibacterial activities against both Gram-negative and Gram-positive test bacteria with zones of inhibition ranging between 2 and 27 mm (NB 034); 9 and 15 mm (TR 007); 8 and 13 (NB 069) and 2 and 12 mm (NB 069). The minimum inhibitory concentration (MIC) of the extracts against the test bacteria ranged from 0.039 - 5 mg/ml, while the minimum bactericidal concentrations (MBC) ranged from 5 to >10 mg/ml. The extracts exhibited concentration-time dependent killing of *Staphylococcus aureus* OKOH2A and *Staphylococcus scuri* OKOH2B with a $0.73\log_{10}$ and $0.88\log_{10}$ reduction in viable counts respectively, while a re-growth phenomenon was exhibited by *Klebsiella pneumonia* ATCC 4352 and *Escherichia coli* ATCC 25922 at MIC, 2 × MIC and 4 × MIC of *Streptomyces* (TR 007) *Actinopolyspora* (TR 008) extracts. All the active extracts exhibited bacteriostatic activities.

Key words: Actinoplane, streptomyces, actinopolyspora, antibiotic production.

INTRODUCTION

The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA. They were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms which are widely distributed in soil, water and found colonizing plants (Jensen and Fenical, 2000; Ogunmwonyi, 2008). The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products and other class of biologically active secondary metabolites (Okami and Hotta 1988). In the past two decades, however, there has been a decline in

the discovery of new important compounds from common soil-derived actinomycetes as culture extracts yield unacceptably high numbers of previously described metabolites (Mincer et al., 2002). Hence, the need for increased exploration of previously unexplored habitats for new actinomycete taxa has become a major focus in the search for the next generation of pharmaceutical agents (Bull et al., 2000), especially with the increasing trend in development of antibiotic resistance in microbial pathogens.

Actinomycetes from the genera *Actinoplane*, *Streptomyces*, and *Actinopolyspora* have been reported to produce over 300 broad-spectrum antibiotic substances (Kieser et al., 2000; Wynands and van Pee, 2004) and representatives of these genera are widely abundant in aquatic ecosystems.

Each year thousands of *Streptomyces* strains are screened by pharmaceutical companies as source of new antimicrobial compounds (Zhang et al., 2002). The first

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Abbreviations: MIC, represents minimum inhibitory concentration; MBC, minimum bactericidal concentration; LPS, lipopolysaccharides.

antibiotic that was discovered from this genus was Streptothricin which was discovered in 1942. However, it was streptomycin in 1944 that triggered screening of antibiotics from *Streptomyces* and since 1942 a lot of screening efforts for more antibiotics have been made in this genus (Berdy, 1974; Vandamme, 1984). However, discovery of new *Streptomyces* strains has substantially decreased over the years, and so has the probability of discovering a new compound (Watve et al., 2001). Some *Actinoplanes* species produce enzymes and metabolites of medical relevance. Typical example is *Actinoplanes missouriensis* which produces xylose isomerase (Karimaki et al., 2004).

Actinopolyspora is said to have an elongated and circular shape with 20-30 spore-chain structures (Xiao-Yang et al., 2000). Not much research has been done concerning Actinopolyspora as a source of antibiotic producing organism.

In this paper, we report on the antibacterial potentials of antibiotic compounds produced by three actinomycetes isolates belonging to the genera *Actinoplane*, *Actinopolyspora* and *Streptomyces* and isolated from the aquatic environment of the Eastern Cape Province of South Africa as part of our ongoing search of new antimicrobial compounds.

MATERIALS AND METHODS

Test actinomycetes

Four actinomycetes strains tentatively identified as belonging to the genera *Actinoplane* [NB 034], *Streptomyces* (TR 007) and *Actinopolyspora* (NB 069 and TR 008) were obtained from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa. The strains were isolated from the Nahoon beach and Tyume River (Ogunmwonyi, 2008) in the Eastern Cape Province of South Africa. The organisms were maintained on agar slants and in 20% glycerol at -80°C. The test actinomycetes suspensions were prepared by suspending a loopful of pure actinomycetes colony in 10 ml sterile physiological saline, vortexed to homogenize and stored at 4°C until ready for use. This suspension was used as actinomycetes inoculants in all cultivations.

Test bacteria

The test bacteria used in this study were obtained from AEMREG culture collection Bank University of Fort Hare, Alice, South Africa, and include the following:

Referenced strains

Escherichia coli ATCC 25922; *Pseudomonas aeruginosa* ATCC 19582; *Staphylococcus aureus* ATCC 6538; *Streptococcus faecalis* ATCC 29212; *Bacillus cereus* ATCC 10702; *Bacillus pumilus* ATCC 14884; *Pseudomonas aeruginosa* ATCC 7700; *Enterobacter cloacae* ATCC 13047; *Klebsiella pneumoniae* ATCC 10031; *Klebsiella pneumoniae* ATCC 4352; *Proteus vulgaris* ATCC 6830; *Proteus vulgaris* CSIR 0030; *Serratia marcescens* ATCC 9986; *Acinetobacter calcoaceticus* UP; and *Acinetobacter calcoaceticus anitratus* CSIR.

Environmental strains

Klebsiella pneumoniae, *Bacillus subtilis*, *Shigella dysenteriae*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Micrococcus kristinae* and *Micrococcus luteus*.

Clinical isolates

Staphylococcus aureus OKOH1, *Staphylococcus aureus* OKOH2A, *Staphylococcus aureus* OKOH3, and *Staphylococcus aureus* OKOH2B with Gene Bank accession numbers Eu244633, Eu244634, Eu244635 and Eu244636 respectively.

Preparation of test bacteria

The test bacteria were grown in nutrient broth and incubated at 37°C for 24 h. The cells were centrifuged at 7000 rpm, washed with sterile physiological saline and standardized to OD_{600nm} 0.1 and stored at 4°C until ready for use.

Preparation and inoculation of fermentation medium

The fermentation broth was prepared as follows per litre: 10 g starch, 4 g yeast extract, 2 g peptone, 5 ml potassium bromide (20 g/L) and 5 ml Iron (III) sulphate tetrahydrate (4.76 g/L). The medium was aliquoted in 500 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C and 15 mmHg for 15 min and allowed to cool after which 100 µl of the standardized actinomycetes suspension were used to inoculate the flasks and incubated at 27°C on a shaker at 300 rpm for 10 days.

Extraction of crude antibiotic metabolites from fermentation cultures

The crude extracts of the fermentation products were recovered from the culture filtrate by solvent extraction using ethyl acetate in accordance with the description of Thakur et al. (2007). Ethyl acetate was added to the filtrate in the ratio 1:1 (v/v) and shaken vigorously for complete extraction.

The ethyl acetate phase that contains the crude antibiotic metabolites was separated from the aqueous phase and concentrated *in vacuo* at 60°C using a rotary evaporator. The residue obtained was weighed and reconstituted in 50% methanol for the antibacterial assays.

Antibacterial susceptibility test

The antibacterial activities of the crude ethyl acetate extracts were determined using agar well diffusion method as described by Pandey et al. (2004). Twenty milliliters of sterilized molten Mueller Hinton agar (MHA) was seeded with 50 µl of standardized test bacteria, swirled gently and aseptically poured into Petri dishes and allowed to solidify.

Sterile cork borer (6 mm diameter) was used to bore wells in the plate, and 100 µl of the crude ethyl acetate extract at a concentration of 10 mg/ml was then carefully dispensed into the bored holes. The extract was allowed to diffuse for about 1 h before incubating aerobically at 37°C for 24 h. The presence of a zone of inhibition around each well was indicative of antibacterial activity. Control experiments were carried out by loading 100 µl of 50% methanol in place of the extracts.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MICs were determined using test organisms that showed susceptibility to the crude extracts by the broth microdilution method as outlined by the EUCAST discussion Document (2003) and as described in our previous report (Sibanda et al., 2010). Briefly, sterile plastic, disposable microtitre plates with 96 flat-bottom wells were used. The medium used in the plates was prepared at double the final strength to allow for a 50% dilution once the inoculum and solvents/ or antimicrobial were added. A 100 µl volume of double strength Muller Hinton broth was introduced into all the 96 wells and varying concentrations of the antibiotic were added in decreasing order along the wells after which wells were loaded with 50 µl of the test organism suspension. The plates were then incubated at 37°C for 18-24 h. Wells in column 12 were used as the growth controls and contained 50 µl of test organism and 50 µl of sterile distilled water.

Results were read using a microtitre plate reader (BIO-RAD model 680) at 490 nm. Visual reading of results was also done by first adding resazurin dye into all the wells. Wells with no growth turned blue in colour while those with growth turned pink, and this helped to give a clear visual demarcation of the MIC wells. The minimum inhibitory concentration (MIC) was estimated as the lowest concentration of the extract that inhibited growth of the test organisms.

The minimum bactericidal concentration (MBC) was determined from the MIC plate following a method outlined by the CLSI (2005), and is defined as the lowest concentration of an antibiotic that under defined *in vitro* conditions reduces by 99.9% the number of organisms in a medium containing a defined inoculum of bacteria, within a defined period of time (EUCAST Definitive Document, 2000). It was determined by inoculating the broths in the MIC range into drug-free nutrient agar medium. The MBC was determined as the antibiotic concentration at which no growth was observed after incubation for 48 h.

Determination of the time kill assay

The time-kill assay of the crude extracts were done in accordance with the description of Okoli and Iroegbu (2005) by determining the rate of bacterial cell-death with respect to time using highly susceptible and minimally susceptible Gram positive and Gram negative test bacteria. The inoculum was prepared following the described guidelines of EUCAST (2003). The resultant cell suspension was diluted 1:100 with fresh sterile nutrient broth and used to inoculate 50 ml volume of Mueller Hinton broth incorporated with extract at multiples of the MIC to a final cell density of 5×10^5 cfu/ml (Sibanda and Okoh, 2008).

The flasks were incubated with shaking at 37°C in an orbital shaker at 120 rpm (Sibanda and Okoh, 2008; Sibanda et al., 2010). Samples of 100 µl were withdrawn at 6 h intervals and diluted appropriately and known volumes of the diluted samples were plated out in triplicate on Mueller Hinton agar. Plates were incubated for 37°C for 24 h after which the numbers of surviving cells were enumerated (Sibanda and Okoh, 2008; Sibanda et al., 2010). Controls consisted of extract free Mueller Hinton broth inoculated with test bacteria.

Statistical analysis

Results were analyzed using a student t-test and One-way analysis of variance (ANOVA). Mean zone diameters were compared for all the four actinomycetes extracts. Analysis was carried out at $P < 0.05$.

RESULTS

Antibacterial activities of the crude extracts

The results of the antibacterial activities of the crude extracts of the fermentation products are shown in Table 1. A total of 4 actinomycetes extracts were screened against 29 test bacteria. *Actinopolyspora* [NB069] extract showed antibacterial activities against four of the test bacteria with zones of inhibition ranging between 8-13 mm, while *Actinopolyspora* (TR 008) extract was active against five test bacteria with zones of inhibition varying between 2 and 12 mm. *Actinoplane* [NB 034] and *Streptomyces* (TR 007) extracts were active against 7 and 15 test bacteria with inhibition zones ranging from 2 to 27 mm, and 9 to 15 mm respectively. None of the extracts were active against *Proteus vulgaris* CSIR 0030, *Streptococcus faecalis* ATCC 29212, *Enterobacter cloacae* ATCC 13047, and *Shigella dysenteriae* (Environmental strains). It appears that *Actinoplane* (NB 034) produced more potent antibacterial compound as it exhibited larger inhibition zone than all other extracts, although it showed no activity against all clinical isolates.

MIC and MBC assay

The minimum inhibitory concentrations (MIC) of the four extracts are presented in Table 2. The MICs of the extract *Streptomyces* (TR 007) ranged from 0.15 to 5 mg/ml, while the MBC ranged from 5 to >10 mg/ml. For extract of *Actinopolyspora* (TR 008), MIC varied between 0.039 and 5 mg/ml while MBC ranged from 2.5 to >10 mg/ml. The MICs for *Actinopolyspora* (NB 069) and *Actinoplane* (NB 034) were 5 mg/ml and 2.5 to 5 mg/ml respectively, while their MBCs ranged between 5 and >10 mg/ml and generally >10 mg/ml respectively. It appears that the extracts are more active against Gram negative bacteria than Gram positive ones.

Time kill assay

The time kill studies were conducted for antibiotic metabolites from *Streptomyces* [TR 007] and *Actinopolyspora* (TR 008). Table 3 shows the log₁₀ change in viable counts of selected test bacteria following exposure to varying concentrations of the extracts. The rate of kill of the test bacterial cells varied with concentration of extract and duration of exposure. The viable cell count of *Streptomyces* (TR 007) extract against *Staphylococcus aureus* (OKOH2A) was observed to range from 0.22 to 0.73. Also, the viable cell count of *Streptomyces* (TR 007) extract against *Staphylococcus sciuri* (OKOH2B) ranged between 0.51 and 0.88. Re-growth in *Escherichia coli* counts was observed at all concentrations of *Streptomyces* (TR 007) extract tested at all exposure times. A similar trend was observed for

Table 1. Antibacterial activities of crude extracts of fermentation products of the four test actinomycetes.

Test organisms	Antibacterial activity [inhibition zones, (mm)]			
	NB 034	NB069	TR 007	TR008
<i>Escherichia coli</i> ATCC 25922 [®]	-	-	+ (11)	-
<i>Pseudomonas aeruginosa</i> ATCC 7700 [®]	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 6535 [®]	-	-	-	+ (5)
<i>Streptococcus faecalis</i> ATCC 29212 [®]	-	-	-	-
<i>Bacillus cereus</i> ATCC 10702 [®]	-	-	-	-
<i>Bacillus pumilus</i> ATCC 10702 [®]	-	-	+ (15)	-
<i>Pseudomonas aeruginosa</i> ATCC 19582 [®]	-	-	-	+ (12)
<i>Enterobacter cloacae</i> ATCC 13047 [®]	-	-	-	-
<i>Klebsiella pneumoniae</i> ATCC 10031 [®]	-	-	+ (12)	-
<i>Klebsiella pneumoniae</i> ATCC 4352 [®]	-	-	+ (14)	+ (2)
<i>Proteus vulgaris</i> ATCC 6830 [®]	-	-	-	-
<i>Proteus vulgaris</i> CSIR 0030 [®]	+ (27)	-	-	-
<i>Serratia marcescens</i> ATCC 9986 [®]	+ (22)	+ (12)	-	-
<i>Acinetobacter calcoaceticus</i> UP [®]	+ (12)	-	-	-
<i>Acinetobacter calcoaceticus anitratus</i> CSIR [®]	-	-	+ (10)	-
<i>Klebsiella pneumoniae</i> [®]	+ (15)	-	-	-
<i>Bacillus subtilis</i> [®]	-	-	+ (13)	-
<i>Shigella dysenteriae</i> [®]	-	-	-	-
<i>Staphylococcus epidermidis</i> [®]	+ (5)	-	+ (13)	-
<i>Pseudomonas aeruginosa</i> [®]	+ (17)	+ (11)	-	-
<i>Proteus vulgaris</i> [®]	-	-	-	-
<i>Enterococcus faecalis</i> [®]	+ (2)	+ (8)	+ (15)	-
<i>Staphylococcus aureus</i> [®]	-	-	+ (9)	-
<i>Micrococcus kristinae</i> [®]	-	-	+ (12)	-
<i>Micrococcus luteus</i> [®]	-	-	+ (11)	+ (2)
<i>Staphylococcus aureus</i> OKOH 1 [™]	-	+ (13)	+ (9)	+ (5)
<i>Staphylococcus aureus</i> OKOH2A [™]	-	-	+ (12)	-
<i>Staphylococcus sciuri</i> OKOH2B [™]	-	-	+ (13)	-
<i>Staphylococcus aureus</i> OKOH3 [™]	-	-	+ (11)	-

[™]Clinical isolates, [®]Environmental strains, [®]References strains, - no activity, + activity. *Actinoplane* [NB 034], *Streptomyces* [TR 007], and *Actinopolyspora* [NB 069 and TR 008]

Klebsiella pneumoniae (ATCC 4352) but only after 12 h of exposure to the *Streptomyces* (TR 007) and at the three concentrations tested Table 3. On the other hand, with the *Actinopolyspora* (TR 008) antibiotic metabolites only *Micrococcus luteus* was observed to reduce in cell counts within 6 h of exposure, but in 12 h the test bacteria increased in growth beyond the initial cell density at all test concentrations as observed in all concentrations and exposure times for *Klebsiella pneumoniae* Table 4.

DISCUSSION

Streptomyces are Gram positive filamentous bacteria which belong to the order Actinomycetales (Madigan and Martinko, 2005). The natural habitat of most *Streptomyces* is the soil but can be also found in aquatic

environment. The most intriguing trait of *Streptomyces* is the capacity to produce secondary metabolites including antibiotics and bioactive compounds valued in human and veterinary medicine, agriculture, and unique biochemical tools (Omura, 2001), and the now uncommonly-used streptomycin takes its name directly from *Streptomyces* (Kampfer, 2006).

Actinoplane is a genus which belongs to the family Actinoplanaceae. *Actinoplanes* species are active antibiotic producers. For example, the ramoplanin complex, teicoplanin, friulimicin, purpurumycin and pentachloropseudilin (Wynands and van Pee, 2004) are produced by *Actinoplanes* species. *Actinoplanes garbardinensis nov.* spp produces a peptide antibiotic which is active *in vitro* and *in vivo* against Gram-positive bacteria, inhibiting their growth by interfering with cell-wall synthesis (Coronelli et al., 1976).

Actinopolyspora is a group of highly halophilic

Table 2. Minimum inhibitory concentration and minimum bactericidal concentration of crude extracts of fermentation products of the four test actinomycetes.

Extract code	Test organisms	Gram reaction	MIC (mg/ml)	MBC (mg/ml)
TR 007	<i>Micrococcus Kristinae</i> [®]	+	5	5
	<i>Bacillus subtilis</i> ATCC 10702 [®]	+	2.5	5
	<i>Staphylococcus aureus</i> OKOH2A [™]	+	1.25	10
	<i>Staphylococcus aureus</i> OKOH3 [™]	+	1.25	>10
	<i>Staphylococcus sciuri</i> OKOH2B [™]	+	0.625	>10
	<i>Acinetobacter calcaoceticus anitratus</i> CSIR [®]	-	0.625	>10
	<i>Micrococcus luteus</i> [®]	+	2.5	>10
	<i>Staphylococcus epidermidis</i> [®]	+	5	5
	<i>Enterococcus faecalis</i> [®]	+	2.5	>10
	<i>Staphylococcus aureus</i> ATCC 6538 [®]	+	2.5	10
	<i>Staphylococcus aureus</i> OKOH1 [™]	+	5	>10
	<i>Escherichia coli</i> ATCC 25922 [®]	-	0.315	>10
	<i>Bacillus pumilus</i> ATCC 14884 [®]	+	0.625	>10
	<i>Klebsiella pneumoniae</i> ATCC 4352 [®]	-	0.156	>10
<i>Klebsiella pneumoniae</i> [®]	-	0.315	>10	
TR 008	<i>Micrococcus luteus</i> [®]	+	0.039	>10
	<i>Klebsiella pneumoniae</i> [®]	-	0.039	>10
	<i>Staphylococcus aureus</i> ATCC 6538 [®]	+	5	5
	<i>Staphylococcus aureus</i> OKOH2A [™]	-	2.5	2.5
NB 069	<i>Serratia marcescens</i> ATCC 9986 [®]	-	5	5
	<i>Pseudomonas aeruginosa</i> ATCC 19582 [®]	-	5	10
	<i>Enterococcus faecalis</i> [®]	+	5	>10
	<i>Staphylococcus aureus</i> OKOH1 [™]	+	5	10
TR 034	<i>Proteus vulgaris</i> CSIR 0030 [®]	-	5	>10
	<i>Serratia marcescens</i> ATCC 9986 [®]	-	2.5	>10
	<i>Acinetobacter calcaoceticus</i> UP [®]	-	2.5	>10
	<i>Klebsiella pneumoniae</i> ATCC 10031 [®]	-	5	>10
	<i>Staphylococcus epidermidis</i> [®]	+	5	>10
	<i>Enterococcus faecalis</i> [®]	+	5	>10
	<i>Pseudomonas aeruginosa</i> [®]	-	5	>10

[®]Environmental strains; [™]Clinical isolates; [®]Reference strains; - represents not Susceptible activity; + represent Susceptible; MIC- Minimum inhibitory concentration; MBC- Minimum bactericidal concentration.

Actinoplanes [NB 034], *Streptomyces* [TR 007], and *Actinopolyspora* [NB 069 and TR 008]

filamentous actinomycetes. Members of this group are isolated frequently in hypersaline soil environment. Since 1975, only two species of extremely halophilic actinomycetes have been reported, *Actinopolyspora halophila* and *Actionopolyspora mortivallis*.

In this study, 12 Gram positive and 17 Gram negative bacteria were susceptible to the antibiotic metabolites produced by the test actinomycetes with those from *Actinopolyspora* (NB 069 and TR 008) being least active in comparison with those from *Streptomyces* (TR 007) and *Actinoplanes* (NB 034). The antibiotic metabolites from *Actinopolyspora* [NB 069] which was isolated from

the marine environment was active against Gram-negative bacteria while that of *Actinopolyspora* (TR 008) isolated from freshwater was active against both Gram positive and Gram negative bacteria and in contradiction with the observation of Kokare et al. (2004) who reported that *Actinopolyspora* species usually show good activity against Gram-positive bacteria, but usually lacking activity against Gram-negative bacteria, and this report was further corroborated by other workers (Pandey et al., 2004; Oskay et al., 2004). The reason for the difference in sensitivity between Gram positive and Gram negative bacteria could be attributed to the morphological

Table 3. Rate of kill for extract *Streptomyces* [TR 007] against selected susceptible test bacteria at different MICs.

Isolates	MIC (mg/ml)	Log ₁₀ kill (MIC)		Log ₁₀ kill (2×MIC)		Log ₁₀ kill (4×MIC)	
		6 h	12 h	6 h	12 h	6 h	12 h
<i>Staphylococcus aureus</i> OKOH2A TM	1.25	0.22	0.44	0.32	0.56	0.38	0.73
<i>Klebsiella pneumoniae</i> ATCC 4352 [@]	0.156	0.04	-2.11	0.04	-2.02	0.70	-1.26
<i>Staphylococcus sciuri</i> OKOH2B TM	0.156	0.81	0.51	0.82	0.65	0.88	0.85
<i>Escherichia coli</i> ATCC 25922 [@]	0.315	-1.18	ND	-0.95	-2.20	-0.93	-1.20

MIC represents minimum inhibitory concentration; ND- not determined; TMClinical isolates; [@]Reference strains

Table 4. Rate of kill for extract *Actinopolyspora* [TR 008] against selected susceptible test bacteria at different MICs.

Isolates	MIC (mg/ml)	Log ₁₀ kill (MIC)		Log ₁₀ kill (2×MIC)		Log ₁₀ kill (4×MIC)	
		6 h	12 h	6 h	12 h	6 h	12 h
<i>Micrococcus luteus</i> [@]	0.039	0.89	-1.06	1.04	-0.95	1.11	-0.64
<i>Klebsiella pneumoniae</i> [@]	0.039	-1.14	ND	-1.07	-2.18	-1.02	-2.28

MIC represents minimum inhibitory concentration; [@]Environmental strains; ND- not determined.

differences between these microorganisms, Gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components which makes the cell wall impermeable to lipophilic solutes, unlike the Gram positive bacteria that has only an outer peptidoglycan layer which is not an effective permeability barrier.

The antibacterial activities of the antimicrobial compounds from the test actinomycetes at such low concentrations as 0.039 mg/ml supports the popular notion that actinomycetes can be sources of potent antibacterial agents that can be of value in the treatment of infections especially those caused by clinically-resistant bacteria, such as *Staphylococcus aureus* and *Klebsiella pneumoniae* (Oskay et al., 2004). Also, the susceptibility of *Klebsiella pneumoniae* to extract from *Actinopolyspora* [TR 008] at such low concentration of 0.039 mg/ml Table 2 is an important finding as this pathogen is known to rank second (after *Escherichia coli*) with regards to antibiotics resistance in urinary tract infections (Gupta et al., 2001).

Bactericidal activity was defined as a reduction of 99.9% ($\geq 3 \log_{10}$) of the total count of CFU/ml to the original inoculum while bacteriostatic activity was defined as maintenance of or a reduction of less than 99.9% ($< 3 \log_{10}$) of the total count of cfu/ml in the original inoculum (CLSI, 2005). The present study demonstrated a clear concentration-dependent rate of kill for all the test bacteria exposed to the culture extracts of *Streptomyces* (TR 007) and *Actinopolyspora* (TR 008). Some of the test organisms were susceptible during screening but resistant to killing during the time kill studies for example, extract *Streptomyces* (TR 007) against *Escherichia coli* (ATCC 25922) and extracts *Actinopolyspora* (TR 008)

against *Klebsiella pneumoniae*. Similar observation has been reported for fumaradimycine an antibiotic isolated from *Streptomyces* was found to be active only in agar medium but not in broth (Hacene et al., 2000). The results suggest the dependence of the production of antimicrobial compound(s) on medium constituents as previously corroborated by the report of Holmalahti et al., (1998) who showed that the nature of medium composition strongly affected antimicrobial compounds production in different organisms. Peterson et al. (2007) reported that a strain of *Klebsiella pneumoniae* once had growth that was higher than the initial inoculum, the mechanisms behind this observation have not been determined. Nevertheless, this may not be unrelated to the characteristics of the lipopolysaccharides (LPS) found in Gram negative bacteria which contributes to their resistances to antibiotics. The LPS blocks certain antibiotics such as penicillin, dyes, and detergents, protecting the sensitive inner membrane and cell wall (Parungao et al., 2007).

The re-growth pattern observed at 12 h at all levels of the MIC concentration for *Klebsiella pneumoniae* Table 3 and *Micrococcus luteus* Table 4 suggests a temporary inhibition of growth by the crude extract which was then lost after 6 h probably due to adaptation of the test bacteria to the extract (Tam et al., 2005). Extract TR007 had a reduction in bacterial count ranging from 0.22log₁₀ - 0.88log₁₀ cfu/ml which demonstrated a pattern of bacteriostatic activity against the two out of the four test organisms *Staphylococcus aureus* OKOH2A and *Staphylococcus sciuri* OKOH2B. The reduction in viable bacterial counts was typically less than 3log₁₀ cfu/ml indicative of bacteriostatic effect. No bactericidal activity was noted with any of the extracts studied.

Conclusion

This study shows that the test actinomycetes have potentials as sources of new antibacterial compounds or lead compounds in new drug discovery. In particular, the antibacterial activities of the crude ethyl acetate extract of some of the actinomycetes products even at very low concentrations is particularly encouraging and opens up a vista for further research opportunities in our group towards elucidating and characterizing the active compound(s) in the antibiotic metabolites.

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