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Early phase bioprospecting and phenotypic characterisation of streptomycetes in Greater Accra

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Abstract

Background: Microbial life forms, including streptomycetes, are an important source of natural products obtained from bioprospecting.

Objective: The study aimed to provide baseline data on the phenotypic diversity of streptomycetes from soils in Greater Accra and to determine the suitability of the selected areas for bioprospecting of potentially novel antimicrobial-producing strains

Methods: Twenty-one soil samples were collected from 7 areas in Greater Accra. Streptomycetes were cultured on Oatmeal Agar for sporulation. Spores were subcultured onto a variety of media to ascertain the colony morphology of the strains. Extracts of pure isolates were obtained via submerged cultures. The antimicrobial activity of the extracts was determined against clinical bacteria using the agar well diffusion method and categorised by their antimicrobial inhibition halo diameter.

Results: A total of 15 phenotypically proven diverse strains of streptomycetes were recovered from the soil samples. Two of the isolates were antimicrobial producers. Tryptone Soya Broth extract of *Streptomyces sp*. V1 showed good efficacy (++) against *Pseudomonas aeruginosa* (22 ± 2 mm) whereas *Streptomyces sp*. W2 showed good efficacy (++) against *Escherichia coli* (20 ± 8 mm) and moderate efficacy (+) against *Pseudomonas aeruginosa* (13 ± 2 mm).

Conclusion: Soils from different areas in Ghana may be potential sources of the next novel antimicrobial-producing streptomycetes.

Keywords: Bioprospecting, antimicrobial, phenotypic, streptomycetes, Ghana

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INTRODUCTION

Bioprospecting entails exploring biological material for commercially valuable genetic and biochemical properties [1]. Microbial life forms, including streptomycetes, are an important source of natural products obtained from bioprospecting activities. Natural products, for many years, have been the starting point of drug discovery. Streptomycetes are the most predominant, comprising almost 70% of the actinomyces [2,3] and account for 1 - 20% of soil microflora [4]. Streptomycetes can be found in terrestrial and marine soils, water bodies, and decaying organic matter [2,5]. Their ability to produce a plethora of secondary metabolites has been exploited in the pharmaceutical industry to produce medically important drugs used in the treatment of infections [6], cancer [7], and diseases associated with the immune system [8]. Most classes of antibiotics currently in existence were discovered in the golden era (1950 - 1970s) [9]. Unfortunately, the increasing use of antibiotics in both humans and animals has led to the development of resistant bacterial pathogens. Traditionally, bioactive compounds were discovered

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through conventional bioprospecting based on the isolation of potential producers and screening their extracts in a variety of bioassays [10]. With time, interest in the bioprospecting of streptomycetes waned because the bioactive compounds produced by most of these were not in sufficient quantities [11], coupled with consistent rediscovery of already known compounds [10].

Subsequently, multiple approaches, such as ribosome engineering, metabolic engineering, rational-based drug design, bio- and chemoinformatics, and omics, have been explored to obtain a diversity of products from novel secondary metabolites isolated from streptomycetes [10,12]. Currently, the increasing emergence of antibiotic resistance and its widespread nature are motivating a continuous search for new compounds. Considering that the "omic" approaches of creating novel compounds rely on a target base compound, it is still preferable to be able to isolate and cultivate an actual host secondary metabolite compound. Furthermore, it is also clear that several natural products cannot be produced under standard laboratory conditions [10] and hence should be isolated from the environment. Owing to the remarkable success of finding therapeutic secondary metabolites from streptomycetes [6,8], there has been a renewed interest in bioprospecting of streptomycetes [11]. In Ghana, few studies on the bioprospecting of streptomycetes have suggested that careful bioprospecting of streptomycetes from soils may lead to the discovery of novel antimicrobial-producing strains [13,14]. To achieve this, phenotyping, although preliminary, is a vital requirement to characterise the new species of streptomycetes that may be isolated [15]. This study aimed to provide baseline data on the phenotypic diversity of streptomycetes from soils in Greater Accra and to determine the suitability of the selected areas for bioprospecting of potentially novel antimicrobial-producing strains.

MATERIALS AND METHODS

Sample size and sampling technique

Soil samples were collected from seven (7) different locations, all in the Greater Accra Region of Ghana. These sites included seashores, lakesides, farmlands, and medical dumpsites. Soil samples were collected from the ground surrounding Ashaiman farmlands, Pantang dumpsite, Korle Bu dumpsite, Jamestown seashore, Dansoman seashore, Alison Green lakeside, and Ashale Botwe lakeside. For each site, the soil was collected at three separate 25 m^2 plots laying 10 m apart to obtain a representative sample of the area [16]. For each plot, the soil was sampled from 8 different points along the perimeter of each 25 m^2 area. The upper layer of the soil was removed, and the samples were collected from 5-20 cm depth. The samples from each plot were placed into three separate labelled sterile containers, giving a total of 21 composite samples. The geolocation of each plot was captured from the centre of the plot using the Ghana Post GPS software. The samples were transported to the Microbiology Laboratory of the Science Laboratory Technology Department of Accra Technical University for further analysis. Soil samples were air-dried at room temperature for 10 - 15 days [5]. Samples were processed under aseptic conditions. For each collected sample, 1 g of the soil was suspended in 9 ml of sterile distilled water and then pre-heated for 6 min at 55°C to reduce non-spore-forming bacteria. Each soil suspension was serially diluted in sterile normal saline (0.85%) up to 10 - 5 dilutions. Aliquots of 1 ml of the 10 - 2 and 10 - 3 dilutions were spread evenly over the surface of modified Starch Casein Agar (SCA) (1.2 g soluble starch,1.8 g Agar, 0.036 g Powdered milk, 0.6 mL of soil extract, 120 mL of water powdered milk) and Luria-Bertani Agar. Cultures were incubated aerobically at 28 - 30°C for 7 - 21 days [4].

Morphological characterisation of the streptomycetes colonies was done by following the methods given in the International Streptomycetes Project [17]. Colonies from the same site appearing on the same culture media that were morphologically indistinguishable were treated as being the same. Streptomycetes colonies were confirmed by microscopic examination as filamentous branching Gram-positive bacteria [4]. Isolates identified as streptomycetes were subcultured on Oatmeal Agar (0.80 g oat powder, 0.06 g agar, 20 ml water). The cultures were incubated for seven days at 28°C to 30°C. After growth, the spores were harvested and stored in cryotubes at -20°C as 25% glycerol stocks [18]. Streptomycetes spores from glycerol stocks were sub-cultured onto Mueller-Hinton Agar, Luria-Bertani Agar, Oatmeal Agar, Soil Extract Agar, Tryptone Soya Agar, Nutrient Agar, and Tryptone Glucose Yeast Agar. These were incubated at 28°C to 30°C and monitored for seven days for the occurrence of growth [18]. The colonies were described based on pigmentation, margin, and elevation. Streptomycetes spores were inoculated on Oatmeal Agar and incubated at 28°C to 30°C for the occurrence of growth. Colonies were aseptically cut from the culture and inoculated into Luria-Bertani Broth and Tryptone Soya Broth (TSB). These, alongside uninoculated Tryptone Soya Broth and Luria-Bertani Broth to be used as negative controls, were incubated at 28°C to 30°C for seven days. The broth cultures were centrifuged at 2100 rpm for 10 minutes. The supernatants were filtered and kept at -20°C until ready for testing.

Antimicrobial activity screening was determined by the agar well diffusion method [5] using previously characterised *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli* obtained from the Microbiology Laboratory of Accra Technical University, Department of Science Laboratory Technology. Gentamicin disks were included as positive controls. Negative controls comprised uninoculated Tryptone Soya Broth and Luria-Bertani Broth. Also, each extract was tested against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 control strains. After incubation, the agar plates were examined, and the zones of inhibition were measured. The test for each extract was performed in triplicates. The antimicrobial activity of the active extracts

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was categorised by the antimicrobial inhibition halo (AIH) diameter as described by Leal et al. (2020) [19]. An inhibition halo of 0 mm was categorised as non-effective (-). An inhibition halo > 0 mm but < AIH was categorised as having moderate efficacy (+). An inhibition halo > AIH but < two-fold AIH was categorised as having a good efficacy (++). An inhibition halo > two-fold AIH was categorised as having strong efficacy (+++).

Data analysis

StataCorp. 2015. Stata *Statistical Software:* Release 14 (StataMP 14). College Station, TX: StataCorp LLC was used to determine the frequency of the various phenotypic variants of streptomycetes isolates, the mean and standard deviation of the zones of inhibition produced by the extracts, and an unpaired T-test to determine whether there was any significant difference between the means of the zones of inhibition produced by the extracts and the reference antimicrobial disks. The level of statistical significance was set at $p < 0.05$.

RESULTS

Overall, 15 distinct streptomycetes were isolated from the 7 study sites (Table 1). The greatest occurrence of streptomycetes was in soil samples collected from Dansoman seashore (3) and Alison's Green lakeside (3). These were followed by Pantang dumpsite (2), Korle Bu dump site (2), Jamestown seashore (2), and Ashale-Botwe lakeside (2). The lowest occurrence was in soil samples collected from Ashaiman farmlands (1). All the isolated streptomycetes (100%) were able to utilise glucose and citrate (Figure 1). The frequency of gas producers (61.5%) and H_2S (56.3%) producers was greater than that of non-producers. The frequency of catalase-positive strains (43.8%), sucrose fermenters (30%), lactose fermenters (30%), and oxidase-positive strains (15.4%) was less than strains that did not produce these reactions. There were no urease producers (0%) among the streptomycetes isolates. A broader range of colours was presented by streptomycetes on Nutrient Agar (5/5) than Soil Extract Agar (4/5), Oatmeal Agar (3/5), Tryptone Yeast Glucose Agar (3/5), Mueller-Hinton Agar (3/5), Luria-Bertani Agar (3/5), and Tryptone Soya Agar (2/5) (Figure 2). These were white, black, brown, pink, and different shades of green and yellow. White was the most prominent colour presented by streptomycetes on Luria-Bertani Agar (85.7%), Tryptone Soya Agar (84.6%), Tryptone Glucose Yeast Agar (78.6%), Mueller-Hinton Agar (61.5%), Nutrient Agar (61.5%), and Soil Extract Agar (42.9%). Green was the most prominent colour presented on Oatmeal Agar (61.5%). Pink (7.1%) was a rare colour, and it was presented only on Luria-Bertani Agar. Colonies presented flat, raised, convex, and umbonate elevations on the culture media (Figure 3). All the elevations were presented by colonies on Tryptone Glucose Yeast Agar (4/4) and Luria-Bertani Agar (4/4) whereas some were presented by colonies on Mueller-Hinton Agar (3/4), Tryptone Soya Agar (3/4), Nutrient Agar (3/4), Oatmeal Agar (2/4), and Soil Extract Agar (2/4). Flat colonies were presented on the complete range of culture media used (7/7). This was followed by umbonate (5/7), convex (5/7), and raised (4/7) colonies. Flat colonies were prominent on Soil Extract Agar (85.7%), Nutrient Agar (69.2%), Tryptone Soya Agar (61.5%), Mueller-Hinton Agar (46.2%), and Tryptone Glucose Yeast Agar (42.9%). Raised colonies were prominent on Oatmeal Agar (77.8%).

Table 1. Streptomycetes isolated at study sites

Site	No. of Streptomycetes Isolates	Isolate Codes
Ashaiman farmlands	1	*Streptomycetes* sp. PTC2
Pantang dumpsite	2	*Streptomyces* sp. PD1 *Streptomyces* sp. PD2
Korle Bu dumpsite	2	*Streptomyces* sp. C2W *Streptomyces* sp. B2B
Dansoman seashore	3	*Streptomyces* sp. DSP01 *Streptomyces* sp. DSW01 *Streptomyces* sp. DSY01
Jamestown seashore	2	*Streptomyces* sp. V1 *Streptomyces* sp. W2
Alison's Green lakeside	3	*Streptomyces* sp. S1W3 *Streptomyces* sp. S2PT *Streptomyces* sp. S3P0
Ashale-Botwe lakeside	2	*Streptomyces* sp. PT1W *Streptomyces* sp. PT3P

Colonies presented entire, filiform, and undulate margins on all the culture media (Figure 4). The most prominent colony margin presented was undulate on Tryptone Glucose Yeast Agar (78.6%), Luria-Bertani Agar (64.3%), Mueller-Hinton Agar (53.9%), Nutrient Agar (53.9%), and Tryptone Soya Agar (46.2%). Filiform margin colonies were prominent on Oatmeal Agar (76.9%), and entire margin colonies were prominent on Soil Extract Agar (57.1%). On Luria Bertani Agar, the colony forms were irregular (92.9%) or circular (7.1%). On Oatmeal Agar, the colony forms were filamentous (61.5%), circular (30.8%), or irregular (7.7%). On soil Extract Agar, the colony forms were irregular (57.1%) or circular (42.9%). On Tryptone Soya Agar, the colony forms were irregular (53.9%) or circular (46.2%). On Mueller-Hinton Agar, the colony forms were irregular (76.9%) or circular (23.1%). On Nutrient Agar, the colony forms were irregular (61.5%) or circular (38.5%). On Tryptone Glucose Yeast Extract Agar, the colony forms were irregular (71.4%) or circular (28.6%). Overall, 5 (33.3%) of the isolates showed antimicrobial activity for metabolites extracted in Tryptone Soya Broth (TSB), and 3 (20.0%) showed antimicrobial activity for metabolites extracted in Luria-Bertani Broth (Table 2). These were Streptomyces sp.V1, *Streptomyces sp.*W2, *Streptomyces sp.*PT3, *Streptomyces sp.*PT1, and *Streptomyces sp.*S2. Against *Pseudomonas aeruginosa*, the mean zone of inhibition for *Streptomyces sp.*V1TSB extract

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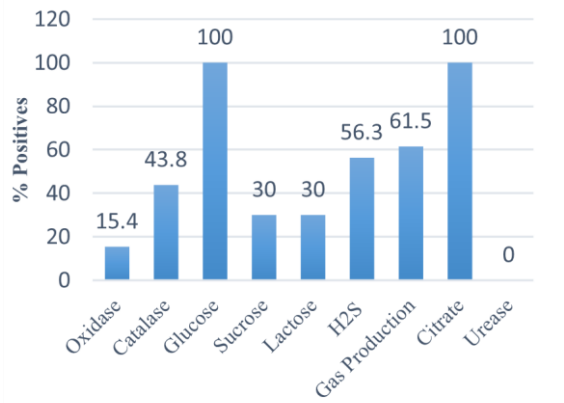

Figure 1. Biochemical identities of streptomycetes isolates

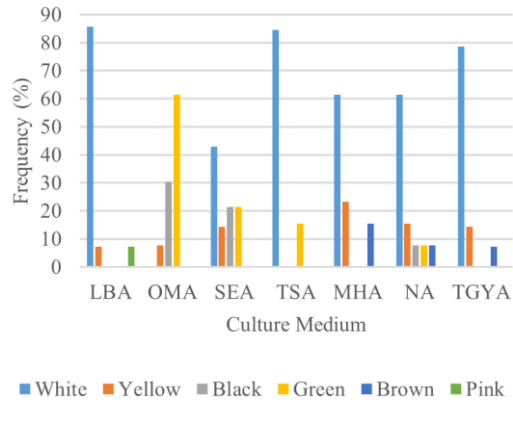

Figure 2. Colony pigmentations expressed by streptomycetes isolates on various culture media

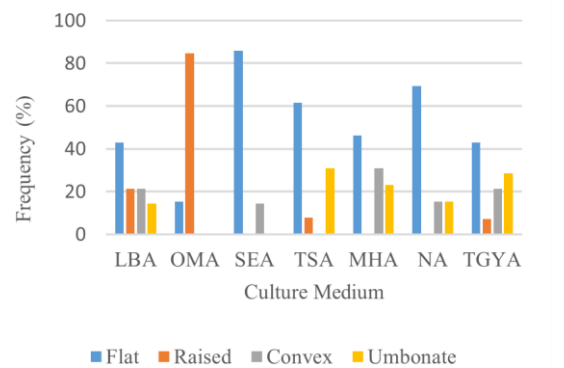

Figure 3. Colony elevations presented by streptomycetes isolates on various culture media.

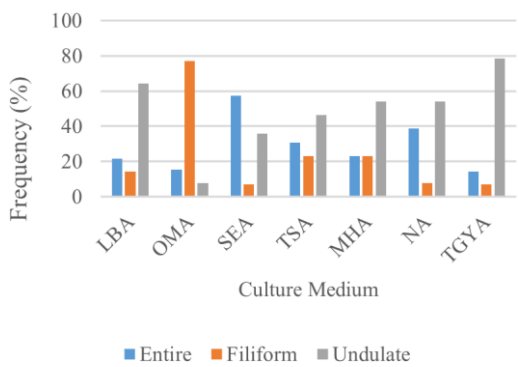

Figure 4. Colony margins presented by streptomycetes isolates on various culture media.

Luria-Bertani Agar (LBA), Oatmeal Agar (OMA), Soil Extract Agar (SEA), Tryptone Soya Agar (TSA), Mueller-Hinton Agar (MHA), Nutrient Agar (NA), and Tryptone Glucose Yeast Agar (TGYA).

Table 2. Antimicrobial activities of active extracts against isolates that showed susceptibility.

Test Isolate	Zone of Inhibition (mm)						
	V1 TSB	W2 TSB	V1 LBB	PT3 TSB	PT1 LBB	PT1 TSB	S2 LBB
P. aeruginosa	22 ± 2	13 ± 2	-	-	-	-	-
E. coli	-	20 ± 8	-	-	-	-	-
S. aureus ATCC 29213	-	-	12 ± 0	0 ± 0	11 ± 1	0 ± 0	14 ± 0
E. coli ATCC 25922	-	-	10 ± 0	10 ± 1	9 ± 0	10 ± 0	12 ± 0

*Tryptone Soya Broth (TSB), Luria-Bertani Broth (LBB

Table 3. Antimicrobial inhibition halo categorization of extracts

	TSB Extracts	
Gentamicin Reference Zone	Streptomyces sp. V1	Streptomyces sp. W2
Staphylococcus aureus (10 mm)	-	-
Escherichia coli (16 mm)	-	++
Pseudomonas aeruginosa (20 mm)	++	+
Salmonella typhi (7 mm)	-	-

*Non effective (-), moderate efficacy (+), Good efficacy (++), Strong efficacy (+++)

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(22 ± 2 mm) was not significantly greater (p = 0.2500) than the gentamicin reference zone of inhibition (20 mm). Also, the mean zone of inhibition for *Streptomyces sp.*W2 TSB extract (13 ± 2 mm) was not significantly less (p = 0.9114) than the gentamicin reference zone of inhibition (20 mm). For *Escherichia coli*, the mean zone of inhibition for *Streptomyces sp.*W2 (20 ± 2 mm) was not significantly greater (p = 0.3524) than the gentamicin reference zone of inhibition (16 mm). For the control strains, *Streptomyces sp.* S2 LBB extract had a greater inhibitory effect (14 ± 0 mm) on *S. aureus* ATCC 29213 than *Streptomyces sp.* V1 LBB extract (12 ± 0 mm) and *Streptomyces sp*. PT1 LBB extract (11 ± 1 mm). Also, *Streptomyces sp.* S2 LBB extract (12 ± 0 mm) had a greater inhibitory effect on E. coli ATCC 25922 than *Streptomyces sp.* V1 LBB extract (10 ± 0 mm), *Streptomyces sp.* PT1 (10 ± 0 mm), *Streptomyces sp.* PT3 TSB extract, and *Streptomyces sp.* PT1 LBB extract (9 ± 0 mm). The TSB extract of *Streptomyces sp.*V1 showed good efficacy (++) against *Pseudomonas aeruginosa* but was not effective (-) against the other tested clinical bacteria (Table 3). The TSB extract of *Streptomyces sp.*W2 showed good efficacy against *Escherichia coli* (++) and was moderately effective against *Pseudomonas aeruginosa* but was not effective (-) against the other tested clinical bacteria.

DISCUSSION

Owing to the remarkable success of secondary metabolites from streptomycetes as a drug source, there is a continued search for strains that may possess antimicrobial activity. This study aimed to further highlight Ghana as an area for the isolation of potent antibiotic-producing streptomycetes. Careful exploration of strains with antibiotic activity can lead to the discovery of new drug targets. Statistically, there is a 1 in 30,000 or 40,000 success rate in bioprospecting of natural products [1]. Therefore, five antimicrobial producers of 15 streptomycetes isolates are a significantly rare occurrence. This supports the bioprospecting potential of areas in Ghana, such as the coastal lines. Similarly, the antimicrobial-producing *Streptomyces sp.*1S1 [20] isolated from the Southern coast of the Red also has its origins in the seashore. The short-line biochemical test conducted in this study was able to differentiate between the morphologically distinct forms of streptomycetes strains from the different sampling sites, suggestive of the occurrence of a diversity of strains and the potential of the area for bioprospecting. The frequency of streptomycetes isolated from the different ecologies, that is, seashore, lakeside, dumpsite, and farmland, differed slightly. However, the majority were isolated from soils sampled from moist environments such as the seashore and lakeside, and this is not surprising given that streptomycetes thrive in moist environments [21,6]. Similarly, secondary metabolite-producing streptomycetes have been mostly isolated from soil and marine environments, as reported in a review by Lacey and Rutledge [22].

A limited number of media was used for the primary isolation, but these supported the growth of different strains. Using a combination and more advanced media formulations such as the International Streptomyces Project (ISP) 2 and 4 [23,4] may enhance the chances of isolating a greater diversity of streptomycetes, some of which may potentially be novel antimicrobial-producing strains. Growth capacity on the different culture media suggests that although most streptomycetes sporulate well on Oatmeal Agar [18], the growth rate is relatively slower. Therefore, other generally available media which promote quicker growth, for instance, Mueller-Hinton and Nutrient Agar, can be considered when sporulation is not the objective. Oatmeal Agar also promoted variation in pigmentation of the streptomycetes; hence, it may be good for preliminary identification. The composition of the growth medium may play a significant role in determining the culture character of streptomycetes as the colony morphology varied from one medium to the next. Colony morphology is a manifestation of the physiological processes that occur in the different strains because of the unique combination of factors in the medium that are available to the streptomycetes. Apart from pigmentation, streptomycetes isolate expressed variations in form, elevation, and margin on the different culture media used. Adeyemo et al. (2021) reported similar variations for *Streptomyces sp.* SUI and *Streptomyces sp.* SW72IV, and *Streptomyces sp.* SW72 VII is grown on various ISP media, Starch Casein Agar, and Nutrient Agar. These showed textures ranging from smooth and shiny to rough and dry, aerial colours such as whitish, cream, tan, and ivory, as well as poor or luxurious growth on different media [24].

Depending on the objective, a medium can be chosen to influence the nature of growth required when studying streptomycetes. From a bioprospecting perspective, culture media, which may appear to be appropriate for the subculturing of streptomycetes, may not necessarily be suitable for isolation. Luria-Bertani Agar was good for isolating streptomycetes from the different sampling sites but did not perform as well as others when spores were subcultured onto these other media. This supports the concept of manipulating the production of secondary metabolites by changing the composition of the media [25]. Antimicrobial metabolites were produced in either Tryptone Soya Broth, Luria-Bertani Broth, or both, depending on the source of the streptomycetes. Therefore, the choice of media for work on streptomycetes should be informed by the purpose or objective of the work. Ideally, for improving yields, be it for spore production or extraction of secondary metabolites, a range of media should be used to determine the suitability of each culture media for the strain of interest. This will reduce the time of waiting and improve yields in subsequent works once the most appropriate medium for the strain in question has been established. Working with a limited number of broth fermentation media may have contributed to the low number of antimicrobial-producing strains (33.3%) identified among the isolates. It is known that changing the media can switch on various biocryptic genes in

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streptomycetes [26] and enhance their antimicrobial production. It has been previously reported that some streptomycetes isolated from the valley of Taza in Morocco produced antimicrobial substances in Bennett medium (50%), Starch Casein medium (> 45%), and Mueller-Hinton medium (> 13%) [27]. Therefore, even the strains from the other sites that did not demonstrate any inhibitory activity against the test bacteria may possess the potential for antimicrobial production beyond the normal screening process.

Conclusion

Soils from different areas are potential sources of the next novel antimicrobial-producing streptomycetes. These streptomycetes may be morphologically diverse but may also share some colony features, which vary depending on the culture media in which their growth occurs. A range of both agar and broth culture media should be used for culture and isolation of streptomycetes during early phase bioprospecting endeavours as a means of enhancing chances of isolation and improving identification of antimicrobial-producers. Further work should be carried out on the streptomycetes isolates that were identified as antimicrobial producers by purifying the extracts for testing to ascertain the actual potencies. Also, the identified antimicrobial-producing strains should be genotyped to establish the possibility of their novelty, and the extracts of these strains should be characterised to identify the bioactive components responsible for their antimicrobial activities.

DECLARATIONS

Ethical consideration

There are no ethical issues.

Consent to publish

All authors agreed on the content of the final paper.

Funding

None

Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author contributions

REA conceived the idea. REA and DAP developed the methodology. LS, BNB, EOK, KE, AAA, PDH, NA, KNANB, VS, MA, RAY, FSA, RMA, and SO were involved in the sample collection. Laboratory analysis and drafting of the manuscript were done by all authors.

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Availability of data

Data for this work is available upon request to the corresponding author.

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