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Hyoscyamine-producing marine *Actinomycetes* from Lagos Lagoon sediment

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PEER REVIEW

Peer reviewer

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Comments

The paper provides information that Lagos Lagoon areas in Nigeria can potentially be a rich source of microorganisms which produce useful bioactive compounds. We could expect that the marine *Actinomycetes* and other organisms in the areas would provide new drugs useful in treatment of patients with infection or other illness.

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ABSTRACT

Objective: To isolate and screen *Actinomycetes* from Lagos Lagoon soil sediments for production of bioactive metabolites.

Methods: Sediment samples were collected from four different locations of Lagos Lagoon and were dried for 2 weeks after which the *Actinomycetes* were isolated by serial dilution using the spread plate method on starch casein and Kuster's agar supplemented with 80 μg/mL cycloheximide to prevent fungal growth. The plates were incubated at 28 °C for 1-2 weeks. Isolates were selected based on their colonial characteristics as well as their Gram's reaction and subcultured using the same media for isolation until pure cultures were obtained and incubated at 28 °C for 3 d. Thereafter, they were inoculated into starch casein and Kuster's broth media and incubated for 8 d. The secondary metabolites were screened for antimicrobial activity against the following microorganisms: methicillin resistant *Staphylococcus aureus*, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 29522, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* and *Enterococcus faecalis* ATCC 29212. Coagulase-negative staphylococci isolated from HIV patients were also used (*Staphylococcus warneri*, *Staphylococcus xylosus* and *Staphylococcus epidermidis*). The antimicrobial metabolites of the *Actinomycetes* isolates were identified using gas chromatography (GC).

Results: Crude extracts of isolates showed antimicrobial activity against some of the test organisms. The GC data analysis showed the antibiotic profile of these isolates.

Conclusions: Analysis of the crude extracts of the isolates using GC method, revealed the presence of antibiotics including an anticholinergic hyoscyamine among other conclusions.

KEYWORDS

Marine *Actinomycetes*, *Streptomyces*, Antimethicillin resistance, Molecular identification, Morphological characteristics, Gas chromatography

1. Introduction

There is a continuous search for safe antimicrobial agents effective against clinical infections caused by Gram-negative organisms, fungi, viruses and mycobacteria. For many years, bioprospecting for novel secondary metabolites have focused on the terrestial environment but marine environment was largely overlooked[1,2]. This is probably because scientists imagined that seawater contained very few microorganisms due to its extreme salinity which is deemed to be unfavourable habitat for many microorganisms. That impression has thus been changed as researchers have hypothesized that since conditions in both marine and terrestial ecosystem are extremely different, the microorganisms in the marine environment should possess characteristics different from those in the terrestial

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ecosystem and therefore would have the tendency to produce different bioactive compounds[3,4]. Recent discoveries from marine bacteria include Neopyrrolomycins, Salinosporamycin as well as Salinosporamide which have shown high microbial and antitumor activity.

Most known antibiotics are produced by *Actinomycetes* which are Gram-positive filamentous bacteria, which are abundant in soil, water and plants[5]. The evaluation of antibiotic-producing potential of new and less known *Actinomycetes* from new or underexplored ecosystems is therefore important in the discovery of novel therapeutic agents for use against drug-resistant microorganisms and treatment of diseases. To combat the problem of resistant microorganisms to available antibiotics, there is a need for periodic replacement of existing antibiotics with novel antibiotics through screening of unexplored habitats.

Hyoscamine is a tropane alkaloid which is a bio-active compound with therapeutic importance such as treatment of Parkinson's disease[6,7], and it has been reported to have analgesic, antispasmodic and sedative properties[8]. It is synthesized mainly by plants belonging to the family Solanaceae such as *Hyoscyamus muticus* and *Datura stramonium* L. and only few reports of its presence in crude extracts of *Actinomycetes* are available but none from African marine environment[9,10].

There is no evidence in literature that suggests that indigenous *Actinomycetes* of the marine environment in West Africa have been exploited for drug discovery. Therefore, this study was carried out to isolate and screen marine *Actinomycetes* from the unexplored Lagos Lagoon for drug discovery by possible novel strains.

2. Materials and methods

2.1. Sample collection and isolation of Actinomycetes

Sediment samples were collected from different locations of Lagos Lagoon using pre-sterilized grab. The samples were kept in sterile polythene bags and transported immediately to the laboratory. They were air-dried at ambient temperature for 2 weeks after which the *Actinomycetes* were isolated by serial dilution of 1 g of soil samples using spread plate method on starch casein and Kuster's agar supplemented with 80 μg/mL of cycloheximide to prevent fungal growth[11]. The plates were incubated at 28 ℃ for 1-2 weeks. Gram-positive bacteria were identified by Gram-staining and *Actinomycetes* were identified among Gram-positive isolates using morphological characteristics. Pure cultures were maintained on nutrient agar slants at 4 ℃[12].

2.2. Biochemical characterization of isolates

Biochemical studies were carried out on the suspected *Actinomycetes* isolates using the API 20A kit (Biomerieux, France). The tests were carried out according to the manufacturer's instructions, incubated at 28 ℃ for 24-48 h and were later read. All the positive and negative tests were recorded. Other biochemical tests such as starch hydrolysis and casein hydrolysis were carried out using standard methods[13].

2.3. DNA extraction, amplification and sequencing

DNA was extracted from the isolates using Qiagen DNA extraction kit (Qiagen, Germany) and stored at -20 ℃. A ~640 bp fragment of the 16S rRNA gene of all strains was amplified by PCR using the actinobacteria-specific primers S-C-Act-0235-a-S-20 5'CGC GGC CTA TCA GCT TGG TTG 3' and S-C-Act-0878-a-A-19 5'CCG TAC TCC CCA GGC GGG G3'[14]. PCR conditions consisted of an initial denaturation stage at 94 ℃ for 4 min, followed by 35 cycles of denaturation at 94 ℃ for 30 seconds, annealing at 60 ℃ for 45 seconds, extension at 72 ℃ for 50 seconds and a final extension at 72 ℃ for 5 min. Negative controls with no DNA template were included in all PCR experiments. Amplification was detected by agarose gel electrophoresis and visualized by ultraviolet fluorescence after ethidium bromide staining[4]. PCR products were purified using a PCR purification kit (Qiagen, U.S.A) and were sequenced by an automated sequencer using the forward primer as above. Sequences were compared with sequences from reference species of bacteria contained in genomic database banks, using the NCBI BLAST search available at http://www.ncbinlm-nih.gov/.

2.4. Screening of secondary metabolites for antimicrobial activity

A loopful of each pure actinomycete culture was inoculated into 30 mL sterile starch casein as well as Kuster's broth and incubated on a rotary shaker at 180 r/min for 8 d at 28 ℃. The culture was later centrifuged at 5000 r/min for 20 min and cell-free supernatant was collected. Using the agar well diffusion method, agar plates were seeded with test strains adjusted to 0.5 MacFarland standard and 6 mm wells were bored in the agar plates using sterile cork borer and cell-free supernatant poured into the wells. The cell-free supernatant was assayed for antimicrobial activity against the following microorganisms: methicillin resistant *Staphylococcus aureus* (*S. aureus*), *S. aureus* ATCC 29213, *Escherichia coli* ATCC 29522, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* (*C. albicans*) and *Enterococcus faecalis* ATCC 29212. Coagulase-negative staphylococci (CoNS) isolated from HIV patients were also used (*Staphylococcus warneri*, *Staphylococcus xylosus* and *Staphylococcus epidermidis*). Sterile Mueller–Hinton and Sabouraud dextrose agar plates were seeded with test bacteria and yeast respectively. The plates seeded with bacteria were incubated at 37 ℃ for 24 h while those seeded with the yeast were incubated at 37 ℃ for 48 h after which zones of inhibition indicative of antimicrobial activity were assessed.

2.5. Gas chromatography (GC) analysis of crude extract

Identification of the secondary metabolites was carried out by GC analysis using the method of Khalvati *et al.* with some modifications[15]. Twenty millilitre of cell-free crude extract was mixed with a combination of ethyl acetate/methanol (1:1) in a separating funnel and shaken vigorously for 30 min and allowed to stand without any disturbance for 15 min. The organic phase was collected into a glass beaker and concentrated to 1 mL. A standard (pure) for the antibiotic combinations was first injected into the GC to set its equivalent peak area and retention time profiles of the individual antibiotics. Afterwards, 0.1 μL was injected in to GC 6890 series (Hewlett Packard) using SE 30 column with specification (column size- 0.25 mm×30 m, carrier gas- nitrogen, flow rate- 22 mL/min, injection temperature- 220 ℃, acceleration and reflector temperature- 10 ℃/min, initial column temperature- 50 ℃, holding time- 2 min). The peak of the standard antibiotics were compared to those of the test samples.

3. Results

Three isolates (ULK2, ULK7 and ULS14) suspected to be *Actinomycetes* grew on the starch casein and Kuster's agar suplemented with cycloheximide. The mycelia of ULK2 colonies were white leathery and turned creamy with age and produced brown pigment in agar while that of ULK7 were white leathery turning

grey with age and produced dark brown pigment (Figure 1). ULS14 colonies were white leathery turning faint pink and produced brown pigment in agar.

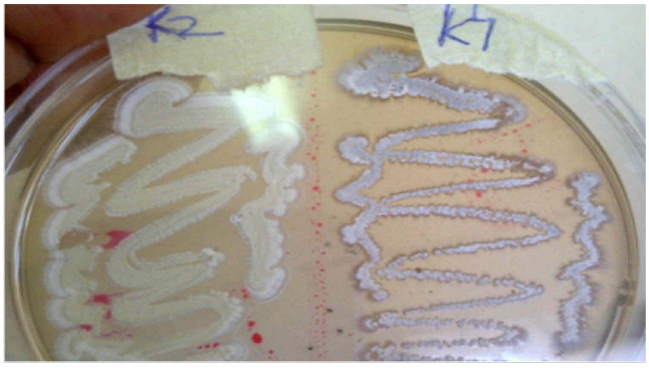

Figure 1. Isolates (ULK2 and ULK7) showing mycelia and diffusible pigment.

Figure 2. Antibacterial activity of actinomycete ULS14 and ULK2 against methicillin-resistant *S. aureus.*

The result of the physicochemical characteristics of the suspected *Actinomycetes* isolates is shown in Table 1. The organisms were non-

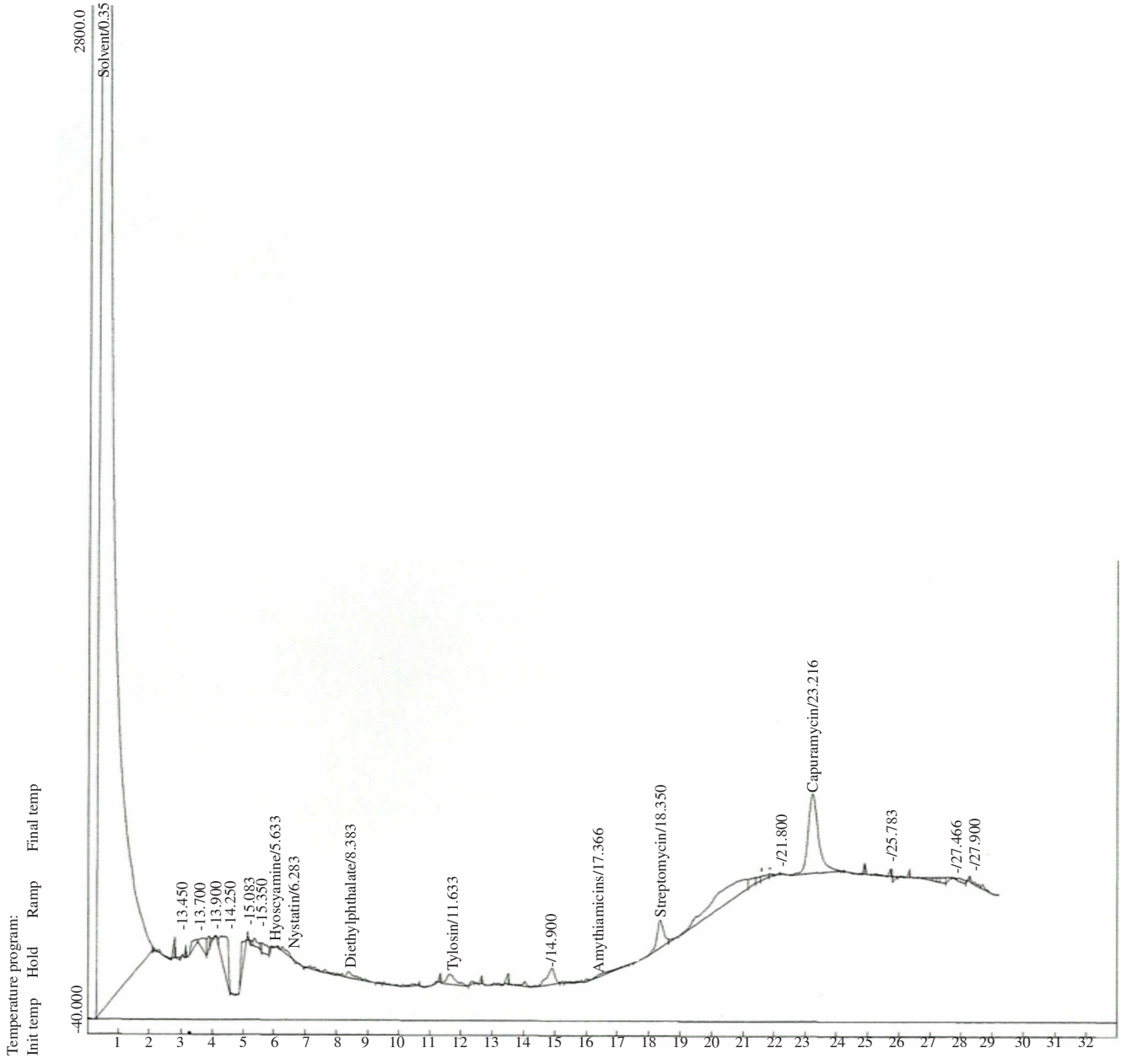

Figure 3. Detection of antibiotics present in the crude extract of ULK2.

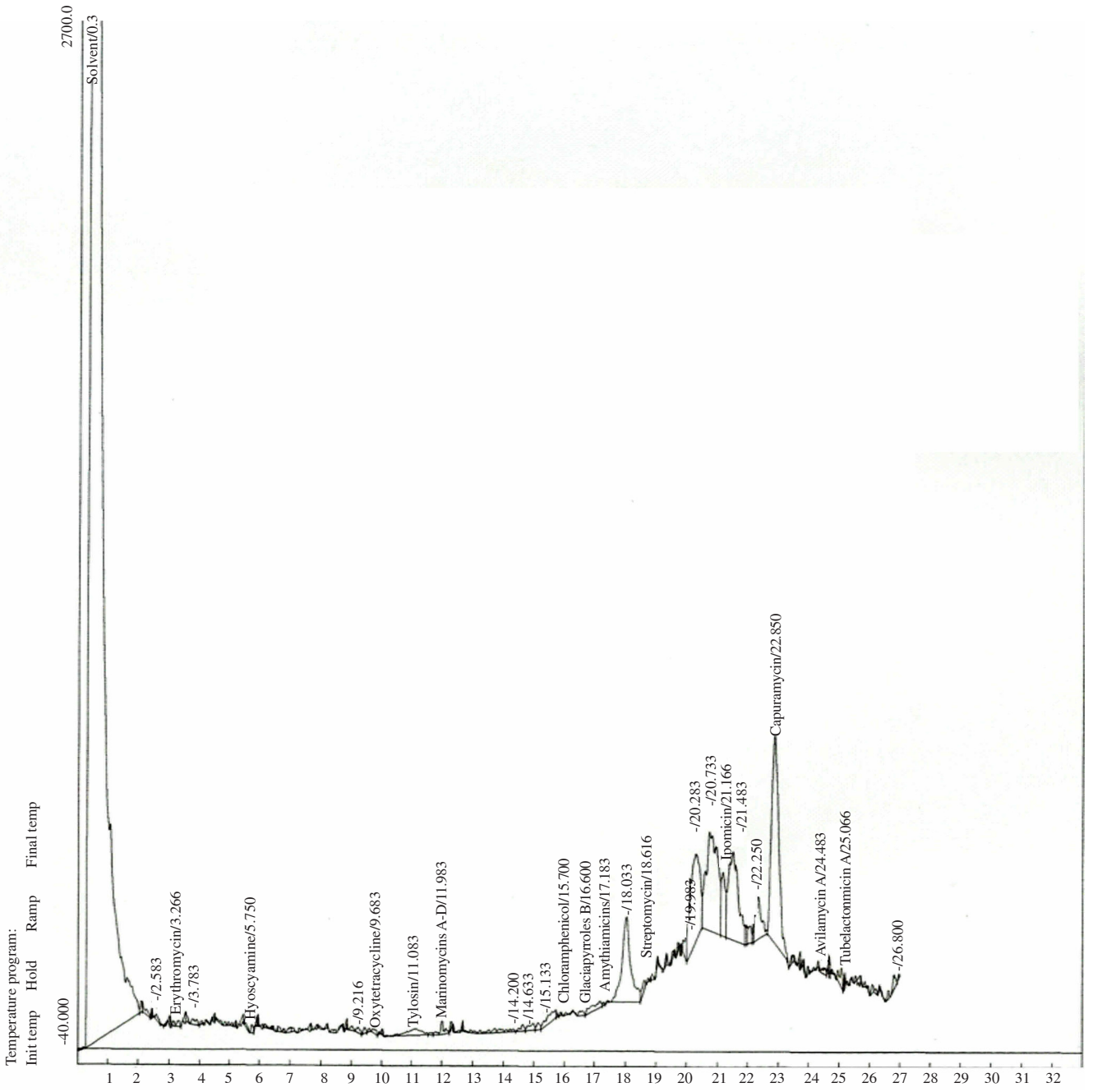

Figure 4. Detection of antibiotics present in the crude extract of ULS14.

Table 1

Physicochemical characteristics of the *Actinomycetes* isolates.

Isolates	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT	SPO	GRM	STA	CAS
ULK2	-	-	+	-	+	+	+	-	-	-	-	-	+	-	+	-	+	-	-	-	+	-	+	+	-
ULS14	+/-	-	+/-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	-	+	+	+
ULK7	-	-	+	-	+	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-

IND: Indole, URE: Urease, GLU: Glucose, MAN: Mannitol, LAC: Lactose, SAC: Saccharose, MAL: Maltose, SAL: Salicin, XYL: Xylose, ARA: Arabinose, GEL: Gelatin, ESC: Esculin, GLY: Glycerol, CEL: Cellobiose, MNE: Mannose, MLZ: Melezitose, RAF: Raffinose, SOR: Sorbitol, RHA: Rhamnose, TRE: Trehalose, CAT: Catalase, SPO-Spores, GRA: Gram reaction, STA: Starch hydrolysis, CAS: Casein hydrolysis.

sporulative and could not hydrolyse mannitol, salicin, arabinose, esculin, melezitose, sorbitol and rhamnose while all isolates showed ability to utilize glucose, glycerol and hydrolyse starch. All the isolates were found to be Gram positive and filamentous.

The 16S rRNA (640 bp) was sequenced and identities of the strains (ULK2, ULK7 and ULS14) were derived from closest matches in the BLAST search of GenBank sequences. The isolate ULK2 shared 99% similarity with *Streptomyces fulvissimus* DSM 40593, while isolates ULS14 and ULK7 were found to share 99% similarity with *Streptomyces bingchenggensis* BCW-1 and *Streptomyces albus* J1074 respectively.

Table 2 shows the result of the antimicrobial assay of cell free broth of the *Actinomycetes* on test organisms. ULK7 showed minimal activity against the CoNS. ULK2 displayed moderate

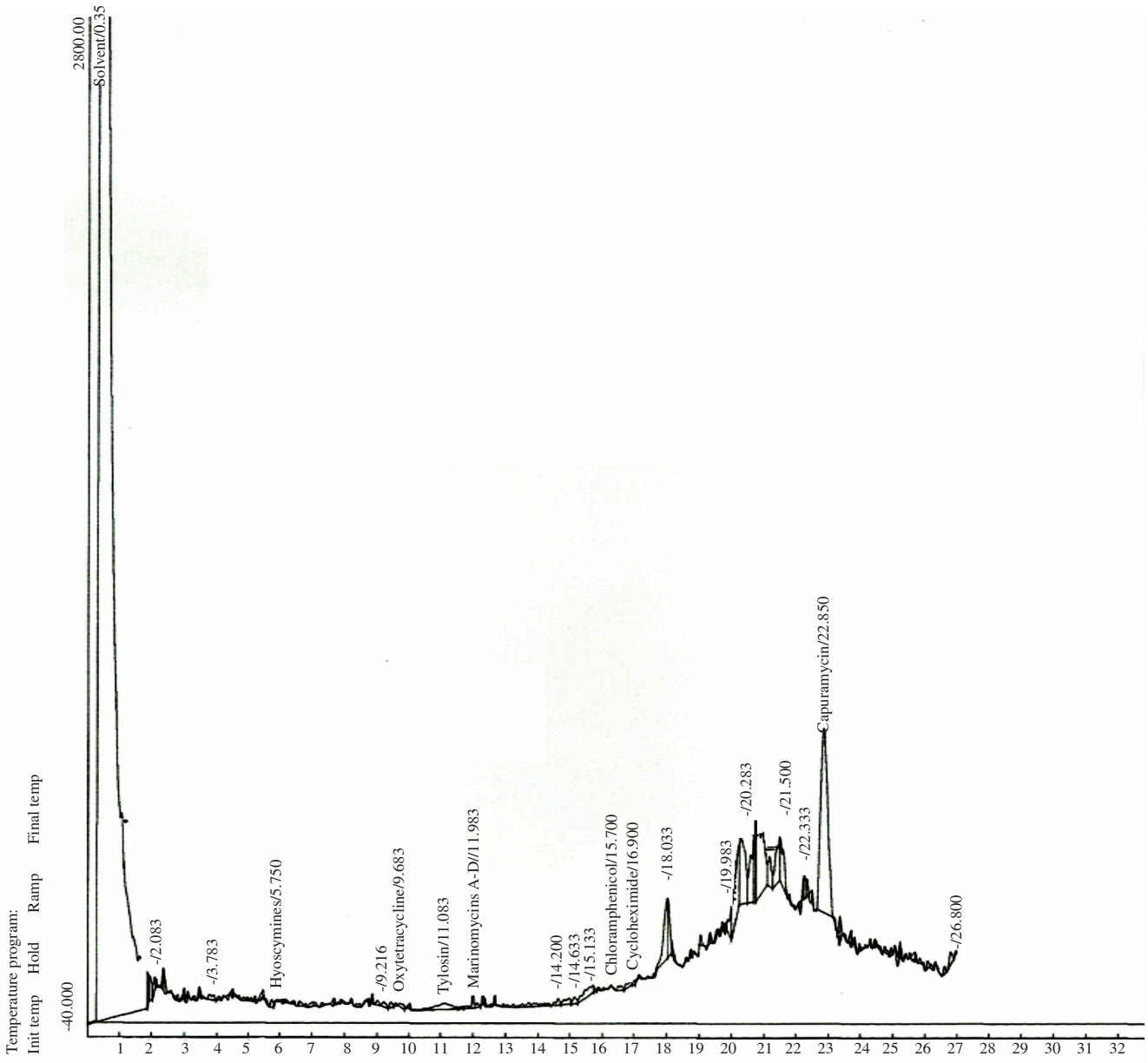

Figure 5. Detection of antibiotics present in the crude extract of ULK7.

Table 2

Antimicrobial activities of crude cell-free extract against pathogenic microorganisms.

Isolates	Zone of inhibition (mm)								
	Staphylococcus warneri	MRSA	*Staphylococcus xylosus*	*Staphylococcus epidermidis*	*Pseudomonas aeruginosa* ATCC 27853	*Escherichia coli* ATCC 29522	*Enterococcus faecalis*	*S. aureus* ATCC 29213	*C. albicans*
ULK2	4	6	-	-	-	-	-	-	7
ULS14	-	5	-	-	-	-	-	5	-
ULK7	3	-	-	-	-	-	-	-	-

MRSA: methicillin-resistant *S. aureus.*

activity against CoN *Staphylococcus warneri*, methicillin resistant *S. aureus* and *C. albicans*. ULS14 showed moderate activity against methicillin resistant *S. aureus* while ULK7 showed minimal activity against the CoNS (Figure 2). The isolate ULK2 was the only one found to display the antibacterial and antifungal activity against the test organisms.-

Figures 3, 4 and 5 show the result of the GC analysis of the crude extracts. The ethyl acetate/methanol extracts of the isolates identified 15 different types of antibiotics with varying number of peak values at different time intervals. Peaks indicating the presence of erythromycin, nystatin, oxytetracycline, tylosin, marinomycins A-D, chloramphenicol, glaciapyrroles, cycloheximide, amythiamicins, streptomycin, ipomicin, capuramycin, avilamycin, tubelactomicin and hyoscamine were detected in the crude extracts. All isolates were found to produce ipomicin, capuramycin, tylosin and hyoscamine.

4. Discussion

A large number of *Actinomycetes* have been isolated and screened largely from the underexplored marine environment for novel drug discovery. Many bioactive compounds have been discovered to be synthesized by these marine *Actinomycetes*.

The *Actinomycetes* isolates encountered in this study were all found to be Gram-positive and filamentous. This is similar to the

findings of Singh *et al.* who also identified *Actinomycetes* based on Gram reaction and morphology[13].

The PCR amplification of 16S rRNA gene was detected by agarose gel electrophoresis. The actinobacteria-specific primers designated S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A-1, which are used to improve the detection and identification of actinobacteria[14], amplified 640 bp stretch of the 16S rRNA gene from all strains thereby confirming them as *Actinomycetes*.

Only extracts of ULK2 and ULS14 were found to have moderate inhibitory activity against methicillin-resistant *S. aureus* and this could be attributed to the presence of amythiamicins which are quite effective against drug-resistant strains. Isolate ULK2 was also observed to have antifungal properties which is similar to a previous report of Ceylan *et al*[16].

Hyoscyamine which is usually found in plants was identified in the crude extracts of all the isolates through GC. Its presence in the ethyl acetate/methanolic extract of all the isolates is suggestive of these *Actinomycetes* isolates as new sources of this tropane alkaloid. Christhudas *et al.* also reported similar findings in a study whereby hyoscyamine was isolated from methanolic extract of *Streptomyces* spp, an endophytic actinomycete isolated from *Datura stramonium* L[10].

A significant number of unidentified peaks were present in the extracts with the highest (18 peaks) being found in ethyl acetate/methanolic extract of ULS14. This development could be indicative of the presence of some novel antimicrobials in the extract and further study could help in the isolation, purification and structure elucidation of the unidentified bioactive compounds.

This study has helped to highlight the significance of the marine *Actinomycetes* of the Lagos marine environment as a potential source of novel bioactive compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Emergence and increase of drug-resistant pathogenic bacteria, fungi and parasites provides strong incentive to find and develop new anti microbial agents. Although much efforts have been put into finding new bioactive drug from *Actinomycetes*, the authors think that not enough works have been performed for marine *Actinomycetes* of Lagos Lagoon areas.

Research frontiers

The authors found at least three *Streptomyces* strains isolated from Logos Lagoon area produce bioactive substances including erythromycin, nystatin, oxytetracycline, tylosin, marinomycins, chloramphenicol, glaciapyrroles, cycloheximide, amythiamicins, streptomycin, hyoscyamine and so on. New bioactive compounds are expected to be found in the crude extracts.

Innovations and breakthroughs

The authors showed a potential of Lagos Lagoon areas as a collecting (hunting) area for microorganisms which produce useful bioactive compounds.

Applications

It is helpful in finding novel and useful bioactive compounds from microorganisms in the lagoon areas.

Peer review

The paper provides information that Lagos Lagoon areas in Nigeria can potentially be a rich source of microorganisms which produce useful bioactive compounds. We could expect that the marine *Actinomycetes* and other organisms in the areas would provide new drugs useful in treatment of patients with infection or other illness.

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