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Himalomycin A and cycloheximide-producing marine actinomycete from Lagos Lagoon soil sediment

Davies Olabisi Flora^{1,2*}, Adeleye Isaac Adeyemi¹, Wang Peng George²

¹Department of Microbiology, University of Lagos, Akoka, Lagos State, Nigeria

²Department of Chemistry, Georgia State University, Atlanta, Georgia, U.S.A

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ABSTRACT

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

Methods: Soil samples were collected from four different locations of Lagos Lagoon and were dried for 2 weeks. *Actinomycetes* were isolated by serial dilution using 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 cultural characteristics as well as their Gram's reaction and subcultured on same media for isolation and incubated at 28 °C for 3 days. Pure cultures were maintained on nutrient agar slants at 4 °C. Thereafter, they were inoculated into starch casein and Kuster's broth media and incubated at 28 °C for 8 days. The resulting crude extracts 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*). Extraction of secondary metabolites was carried out and analysed using gas chromatography-mass spectrometer.

Results: All the isolates displayed varying antimicrobial activity against at least one of the test organisms. Himalomycin A was identified in the extract from isolate ULS7. The gas chromatography-mass spectrometer data analysis showed the antibiotic profile of these isolates.

Conclusions: The isolate ULS7 was found to display the highest antimicrobial activity against the test organisms.

1. Introduction

Actinomycetes are a group of Gram positive, branching unicellular microorganisms with an unrivalled capacity to synthesize novel bioactive metabolites[1,2]. This group accounts

for the highest synthesis of bioactive metabolites (over 10000) which makes them the highest source in comparison to bacteria and fungi[1]. However, it is estimated that approximately 90% of all bioactive secondary metabolites which can be derived from *Actinomycetes* are yet to be discovered[3].

The surface of the earth is covered by 70% oceans and life on earth originates from the sea, it has been therefore estimated that microbial diversity in marine environment is higher than in terrestrial ecosystem[4]. Most currently used antibiotics are isolated from terrestrial sources but with the recent upsurge of diseases caused by antibiotics-resistant pathogens. There is

*Corresponding author: Davies Olabisi Flora, Department of Microbiology, University of Lagos, Akoka, Lagos State, Nigeria; Department of Chemistry, Georgia State University, Atlanta, Georgia, U.S.A.

Tel: +1-(678)467-6311

E-mail: bizdave1@gmail.com

renewed interest in marine bioprospecting which is generally an underexplored environment for novel drug discovery and this interest has resulted in the discovery of some novel antibiotics such as himalomycin A which was first isolated from marine *Streptomyces* sp. This antibiotic was found to possess very strong antibacterial properties[5].

Until recently, the marine ecosystem of the African continent have been neglected in the quest for novel drug discovery. A study carried out by Ogunmwoyi *et al.* showed high prospects for antibiotic production from marine *Streptomyces* isolated from Nahoon beach, South Africa[6]. However, there are no reports available for marine bioprospecting from West African marine environment. This study, therefore aims to discover the antimicrobial potentials of marine *Actinomycetes* from Lagos Lagoon, as part of current worldwide research on novel drug discovery.

2. Materials and methods

2.1. Sample collection and isolation of *Actinomycetes*

Soil 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 for 2 weeks after which the *Actinomycetes* were isolated by serial dilution using spread plate method on starch casein and Kuster's agar supplemented with 80 µg/mL of cycloheximide to prevent fungal growth[7]. The plates were incubated at 28 °C for 1-2 weeks. Isolates were selected based on their cultural characteristics as well as their Gram's reaction and subcultured. Pure cultures were stored on nutrient agar slants at 4 °C[8].

2.2. Biochemical characterization of isolates

Biochemical studies were carried out on the putative *Actinomycetes* isolates using API 20A kit (Biomerieux). The tests were carried out according to the manufacturer's instructions, incubated at 28 °C for 24-48 h and later read. Other biochemical tests such as starch hydrolysis and casein hydrolysis were carried out using standard methods[9].

2.3. DNA extraction and amplification

The isolates identified phenotypically as *Actinomycetes* were subjected to genotypic characterization using PCR techniques. DNA was extracted from the isolates and

stored at -20 °C. The primers S-C-Act-0235-a-S-20 5'CGCGGCTATCAGCTTGGTTG3' and S-C-Act-0878-a-A-19 5'CCGTACTCCCCAGGCGGG3', specific for actinobacteria were used to amplify an ~640 bp stretch of the 16S rRNA gene of all the strains using PCR method[10]. The PCR conditions were an initial denaturation stage at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55.5 °C for 45 seconds, extension at 72 °C for 50 seconds and a final extension at 72 °C 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]. Sequencing analysis (ABI 3730 DNA Analyzer) was carried out after purification of the PCR products (Qiagen PCR purification kit) and the results run on BLAST program (NCBI).

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 for 8 days at 28 °C. The culture was later centrifuged at 5000 r/min for 20 min and cell-free supernatant was collected. Using agar well diffusion method[11], 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 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 °C for 24 h while those seeded with the yeast were incubated at 28 °C for 48 h.

2.5. Gas chromatography-mass spectrometer (GC-MS) analysis of crude extract

Extraction of secondary metabolites was carried out using the method of Rajesh *et al.* with modifications[12]. 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 afterwards, was allowed to stand without any disturbance for 15 min. The lower aqueous phase was discarded and 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 uL was injected in to GC 6890 series (Hewlett Packard) with specification (column size: 0.25 mm × 30 m, carrier gas: nitrogen, flow rate: 22 mL/min, injection temperature: 220 °C, acceleration and reflector temperature: 10 °C/min, initial column temperature: 50 °C and holding time: 2 min). The peak areas of the standard antibiotics were compared to those of the test samples.

3. Results

Using colonial morphology, ULS1, ULS7 and ULK11 were suspected to be *Actinomycetes*. The colonies of ULS1 and ULS7 were red waxy with white powdery aerial mycelium and produced no pigment in agar while that of ULK11 colonies were light green, powdery surface and produced light green non-diffusible pigment in agar (Figure 1). They also grew on starch casein agar.



Figure 1. Isolate ULK11 showing mycelia and insoluble metabolite.

The result of the physicochemical characteristics of the

suspected *Actinomycetes* isolates is shown in Table 1. The organisms were non-sporulative, catalase negative and could neither hydrolyse gelatin nor produce indole and urease. All isolates showed ability to utilize glucose, lactose, saccharose, maltose, salicin and hydrolyse starch.

All the strains were identified as *Actinomycetes* based on their phenotypic characteristics and amplification of the 640 bp stretch of the 16S rRNA gene also confirmed their identities as belonging to the *Streptomyces* sp. The results of the sequence analysis using BLAST identified ULK11 and ULS7 as *Streptomyces* sp. The isolate ULS1 was however not available for sequencing analysis.

Actinomycetes were isolated from marine sediments by serial dilution agar plate method and screened for their antimicrobial activity by agar well diffusion method as reported by Khan and Patel[7,11]. Table 2 shows the result of the antimicrobial assay of crude extracts of broth cultures of the *Actinomycetes* on test organisms. ULS7 displayed the highest activity against *S. aureus* ATCC 29213 (Figure 2) while ULS1 showed antibacterial and antifungal properties against *S. aureus* ATCC 29213 and *C. albicans*. The zones of inhibition ranged from 3 mm to 20 mm.



Figure 2. Antibacterial activity of actinomycete ULS7 against *S. aureus* ATCC 29213.

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	
ULS1	-	-	+	-	+	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	-	-	+	+	-	
ULS7	-	-	+	+/-	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
ULK11	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+/-	+/-	+	-	-	-	+	+	+

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.

Table 2

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

Isolates	Zone of inhibition (mm)								
	<i>Staphylococcus warneri</i>	MRSA	<i>Staphylococcus xylosum</i>	<i>Staphylococcus epidermidis</i>	<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Escherichia coli</i> ATCC 29522	<i>Enterococcus faecalis</i>	<i>S. aureus</i> ATCC 29213	<i>C. albicans</i>
ULS1	-	-	-	-	-	-	-	3	5
ULS7	-	-	-	-	-	-	-	20	-
ULK11	-	-	-	-	-	-	-	3	-

MRSA: methicillin-resistant *S. aureus*.

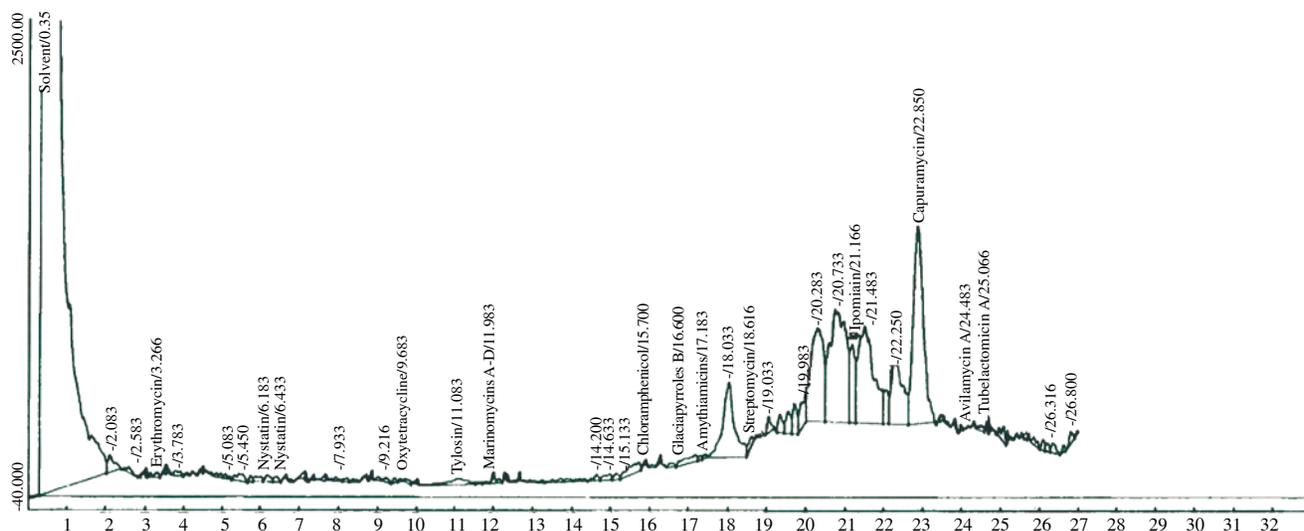


Figure 3. Detection of antibiotics present in the crude extract of ULS7.

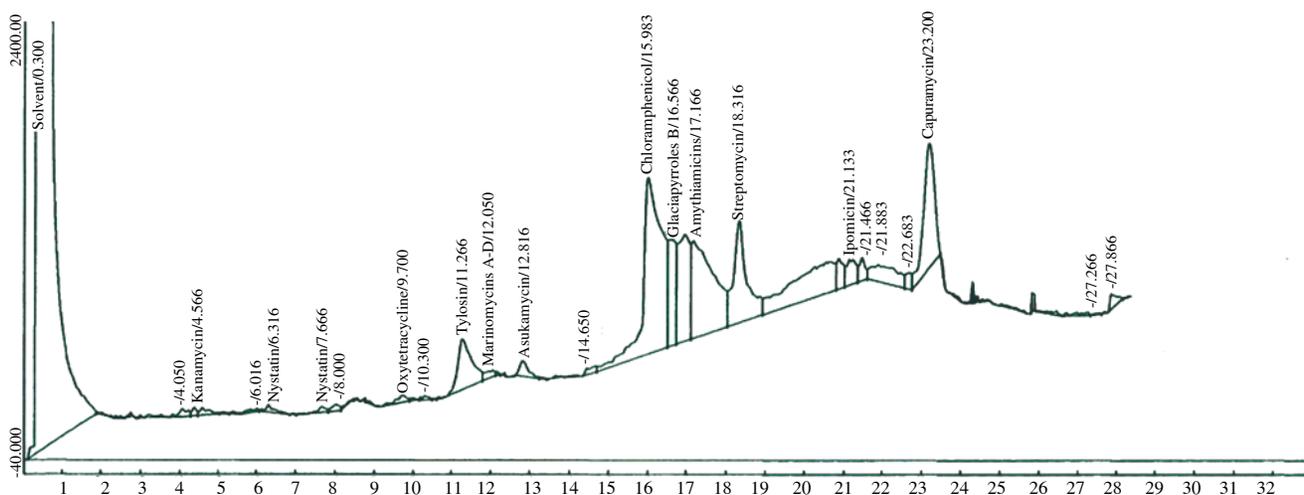


Figure 4. Detection of antibiotics present in the crude extract of ULS1.

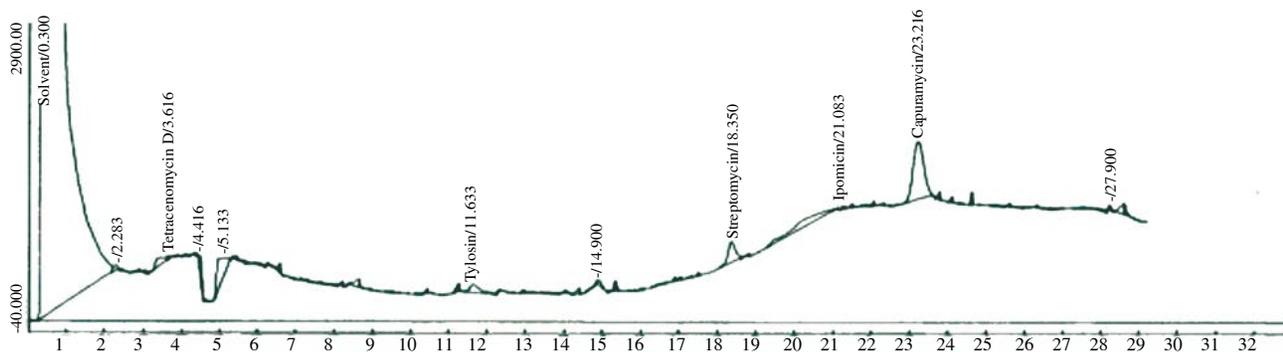


Figure 5. Detection of antibiotics present in the crude extract of ULK11.

Figures 3, 4 and 5 show the result of the GC-MS analysis of the crude extracts. The ethyl acetate/methanol extracts of the isolates identified 19 different types of antibiotics with varying number of peak values at different time intervals. Peaks indicating the presence of himalomyacin A, asukamycin, erythromycin, nystatin,

oxytetracycline, kanamycin, tylosin, marinomycins A-D, chloramphenicol, glaciapyrroles, cycloheximide, amphotericin, streptomycin, ipomicin, capuramycin, avilamycin, tubelactomicin, resistoflavin and glaciapyrroles B were detected in the crude extracts.

4. Discussion

Many bioactive compounds are synthesized by marine *Actinomycetes* that is why a large number of *Actinomycetes* have been isolated and screened largely from the underexplored marine environment for novel drug discovery. Therefore, this study was carried out to isolate and screen marine *Actinomycetes* from the unexplored Lagos Lagoon for antibiotic production.

The PCR 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 for the amplification of V3-V5 regions of 16S rRNA gene and used to improve the detection and identification of actinobacteria[10], amplified an approximately 640 bp stretch of the 16S rRNA gene from all strains thereby confirming them as *Actinomycetes*.

The isolate ULS1 was the only one that showed both antibacterial as well as antifungal properties. This could be attributed to the presence of cycloheximide, which is a potent antifungal agent synthesized by this isolate.

The highest number of peaks was observed in the chromatograph of ULS7 (41 peaks) and also shown to have the highest number of unidentified peaks which could be an indication of the presence of some novel antibiotics judging from the very high inhibitory activity the isolate displayed against *S. aureus* ATCC 29213.

The extract from ULS7 was observed to contain himalomycin A, a fridamycin-type antibiotic which has antibacterial activities. This is similar to the findings of Maskey *et al.* in a study where they isolated what they called “new quinone antibiotics” of the group, himalomycin A and B from marine *Streptomyces* isolate B6921[5]. Their study was the first report of this antibiotic and there seems not to be any other report of the isolation of this antibiotic. Some of the biochemical characteristics exhibited by the *Streptomyces* isolate B6921 and isolate ULS7 in this study show some differences such as catalase production as well as degradation of casein and gelatin. This suggests that the isolate ULS7 might be yet another source of this antibiotic.

This study shows the antimicrobial activity of crude extracts of *Actinomycetes* isolated from soil sediment from the Lagos Lagoon, which is largely an underexplored habitat for antibiotic-producing marine *Actinomycetes*.

There is the possibility of these isolates and others yet to be discovered being able to synthesize novel antibiotics which can be exploited for medical use.

Conflict of interest statement

We declare that we have no conflict of interest.

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