ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FROM ESTUARY FOR PRODUCTION OF NEW ANTIBIOTICS

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ABSTRACT
Actinomycetes are Gram positive facultative-anaerobic fungus-like filamentous bacteria that are highly sought for by both Pharmaceutical and food processing industries due to their prolific production of antibiotics, biochemical and enzymes. Antibiotics can be sourced from different sources such as soil and medicinal herbs, but most researchers, targeted soil because most of the microorganisms live in soil. The aim of this study was to isolate and characterized, Actinomycetes from estuary for new antibiotics production. Ninety (90) soil samples were collected from different sites at depth of 5, 8 and 11 centimeters. Membrane filter technique by Ravi and Vasantha was employed and 2gm of soil samples were sprinkled on 0.45µm pore size membrane filter aseptically placed on surface of Starch Casein Agar (SCA), and incubated at 30°C for two weeks.The isolates were identified on the basis of Cultural, morphological and biochemical characterization. API Oxioid Microbact reagent 12A and 12B containing 24 different reagents and sugars were used for biochemical characterization. The isolates were further characterized by genomic DNA using 16S rDNA sequencing technique and post sequencing activities were carry out. BLAST was done and the assertion numbers were deposited in gene bank. Phylogenetic tree was also done. Thirty (30) Actinomyceycetes was isolated which belong to three families, Streptomyces 16(53.3%), Micromonospora 10(33.3%) and Nocardia 4(13.3%). And each of these species produces secondary metabolites that can be used to produce novel antibiotics.
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Introduction

The role of antimicrobial agents in the treatment of bacterial infections cannot be over emphasized (Bhatti *et al.,* 2017; Stabrouski and Chuad 2019). A lot of efforts are being made to discover novel antimicrobial agents that are produced by microorganisms (Bawazir and Shantaram 2018). As a result of multi-drug resistance (MDR) therefore, there is need for continuous discovery of new antimicrobial agents that are effective against emerging and reemerging multi-resistance pathogens that are causing infections on daily basis both in the developed and developing countries of the world (Bhatti *et al*., 2017; Ahmed Ahmed *et al.,* 2018).
Majority of the antibiotics were derived from a relatively small group of microorganisms of the genera *Penicillium, Streptomyces, Cephalosporium, Micromonospora and Bacillus* (Ayari *et al.,* 2016 ;Jose and Jha, 2016).
Antibiotics can be sourced from different sources such as soil and medicinal herbs. Most researchers have targeted the soil (Iang *et al*., 2016). The reason is being that many micro-organisms live in the soil and have the potential of producing biologically active Secondary metabolites that could be used for characterization of novel antibiotics, which could be useful for treatment of infectious diseases. These bacteria produce these secondary metabolites in their abode where they live and can be relied on to prevent other bacteria species from entering into their territory and means of communication between cells as signaling molecules (Dylag *et al*., 2022).

METHODOLOGY

This study was carried out in Oghara along the river Ethiope, in Delta State, Nigeria. Geographically, Oghara is located at Latitude $5^0$35' 11.99" N and Longtitude 6^0 06 0.60 E. the land mass is approximately 1,175 Square kilometers with a population of over 203,592 people as at 2006.. Oghara is an Island most of the land is flat and Swampy with a dense forest growth.

Sample Collection

A total of ninety (90) soil samples were collected from three regions of the estuary that include: Oghara, Ogaharefe and Oghareki using standard method (Bawizir *et al*., 2018). The samples were collected into a sterile biohazard bags and were taken to the Laboratory for analysis. The samples were labeled and dried on the bench for 7 days at room temperature in other to preserve both Gram positive and the Gram negative bacteria (Sengupta *et al.,* 2015). The soil samples were sieved with the aid of fine pore size sieve of 250µm (Rotich *et al*., 2022; Bundale *et al*., 2022 and Fisher *et al*., 2022).

Isolation of Actinomycetes

Starch casein agar(SCA) (Composition: CaCO3 0.02g, FeSO4. 7H2O 0.01g, MaSO4 7H2O 0.05g, KNO2 2g, K2HPO4 2g, NaCl 2g, Soluble Starch 10g, Casein 0.3g, Agar 18g) were prepared. Nystatin and Nalidixic Acid were added to prevent growth of fungi and bacteria contaminants. Membrane filter of 0.45µm pore size was aseptically placed on the center of the agar plate. Soil samples 2gm were sprinkled on the membrane filters (Samer, 2016; Gone *et al*., 2020 and Gupta *et al.,* 2022). The plates were incubated for the duration of 4 days the membrane filter were removed (Adama *et al.,* 2017). The plates were further incubated for 2-3 weeks until the *Actinomycetes* colonies were visibly seen grown on the plates. This membrane filter technique is preferred due to its selectivity of *Actinomycetes* (Nandhini, 2015). This is because other microorganisms have no ability to produce mycelia that can penetrate the membrane filter (Jeyaraman, 2015; Ge *et al*., 2019 and Ganesan *et al*., 2022).

Macroscopic Characterization

Actinomycetes that were isolated were observed for the production of the following features aerial mycelium, submerged mycelium, colour and diffusible pigment (Hernandez *et al*., 2020).

Microscopic Characterization

Microscopic characterization was done with the aid of cover slip using Gram staining technique. The cover slip was inserted into the surface of the starch casein agar medium at an angle 45^{o}C with the agar surface. Isolate of Actinomycetes was inoculated along the surface of the medium that meets the surface of the buried cover slip. The cover slip was incubated at 30^{o}C for four days. Then the cover slip was removed with the aid of forceps and placed on a clean free grease slide, and were examined under the light microscope using the oil immersion objective (x100) lens (Liu *et al*., 2019; Mesrian *et al*., 2022 and Muhammmad *et al*., 2022.

Biochemical Characterization using Oxoid API Microbact 12A and 12B Identification Kits

A loopful of colony of the pure isolates that have been incubated for 7 days in Starch Casein Agar (SCA), was inoculated into Starch Casein Broth (SCB) and incubated at 30^{o}C for 4 days. 100µl of the broth suspension was added to each of the well of the tests strips. One drop of Microbact mineral oil reagent was added to the first and second well of 12A strip while the 12B only the broth suspensions was added. Both strips were incubated at 30^{o}C for 5 to 7 days. Kovac's reagent was added to indole well and TDA reagent was added to TDA well. The two strips were both incubated for 48hrs even up to 72hrs for full development of colour change which were read and recorded as positive (+) or negative (-) by comparing with the colour chart of the manual (Pooja *et al.,* 2017).

The Oxoid API Microbact strip 12A contain: lysine, ornithine, hydrogen sulphide (H2S), glucose, ortho-nitrohpenyl-b-D-galatopyranoside (ONPG), indole, urease, voges-proskauer (V-P), Citrate and tryptophan deaminase reagent (TDA)

Microbact reagent strips 12B include: gelatin, malonate inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose, salicin and arginine (Pooja *et al.,* 2017).

DNA Extraction

Genomic DNA was extracted from the samples using the Quick-DNA Fungal/Bacterial Miniprep Kit. **PCR Amplification** The target region was amplified using OneTaq® Quick-Load (Hu et al., 2018).

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RESULTS

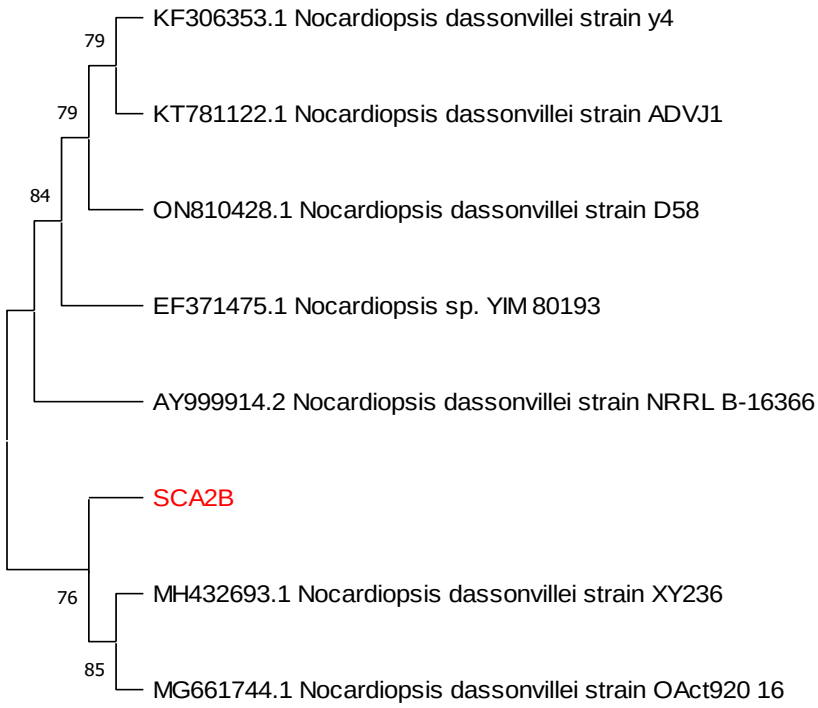
A total number of thirty (30) *Actinomyces* that comprises of three genera that include *Streptomyces species*, *Micromonospora species* and Nocardia species were isolated. The study showed that among the three families of *Actinomyces* isolated, *Streptomyces species* were most commonly isolated they accounted for 16 (53.3%), Micromonospora 10 (33.3%) and the least was *Nocardia species* which was 4 (13.3%).

THE PERCENTAGE OF ACTINOMYCETES ISOLATED

Isolates	No. of Isolates	Percentages (%)
Streptomycetes spp	16	53.33%
Micromonospora spp	10	33.33%
Norcadia spp	4	13.33%
TOTAL	**30**	**100%**

SUMMARY OF THE SEQUENCE

S/NO	SPECIES CHARACTERIZED
1	*Streptomyces subflavus*
2	*Streptomyces rimosus*
3	*Strptomyces griseus*
4	*Micromonospora terminaliae*
5	*Streptomyces antibioticus*
6	*Streptomyces microflavus*
7	*Micromonospora mangrove*
8	*Streptomyces rimosus*
9	*Micromonospora purpurea*
10	*Streptomyces rimosus*
11	*Streptomyces fradiae*
12	*Nocardiopsis dossonvillei*
13	*Micromonospora fluostatini*
14	*Streptomyces microflavus*
15	*Mcromonospora terminaliae*
16	*Streptomyces lincolnensis*
17	*Micromonospora mangrove*
18	*Micromonospora purpurea*
19	*Micromonospora mangrove*
20	*Nocardiopsis dasomillie*
21	*Micromonospora tarminaliae*
22	*Streptomyces griseus*
23	*Streptomyceces microflavus*
24	*Streptomyces subflavus*
25	*Nocardiopsis flavenscens*
26	*Sreptomyces microflavus*
27	*Streptomyces lincolnesis*
28	*Micromonospora fluostatini*
29	*Nocardiopsis dassonvillei*
30	*Streptomyces rimosus*



Phalogenetic tree of *Nocardiopsis dassonvillei*

DISCUSSION

This study was carried out to isolate and characterized Organisms from estuary for production of new antibiotics. It has become worrisome that multidrug resistant organisms are on the increase therefore, rendering treatment of infectious diseases a difficult task. Due to the continuous emerging and reemerging of multidrug resistant pathogens, this has call for urgent need for the development of new antibiotics that can be effective against these pathogens. One of the major known remedies to this ugly trend of multidrug resistant is *Actinomycetes* which have been proven beyond reasonable doubt repeatedly as potential sources of bioactive compounds and the richest known sources of secondary metabolites this was also reported by another researcher (Budhathoki, and Shrestha 2020). In this study a total number of thirty (30) *Actinomycetes* were isolated, from the ninety (90) soil samples collected from the estuary. These were characterized and comprises of three families which include *Streptomyces* 16 (53.3%), *Micromonospora* 10 (33.3%) and *Nocardia* 4 (13.3%) species.

The distribution of *Actinomycetes* in estuary was not well known, but the resent information made available from investigations revealed that *Streptomyces* species were more predominant. This study was in agreement with (Tangerian, *et al,* 2020).

Starch Casein Agar (SCA) media were used for the isolation of *Actinomyceyes*, while Nystain and Nalidixic Acid was added to prevent growth of fungi and unwanted Gram negative bacteria respectively that could cause contamination. This was in agreement with other researchers such as (Gong *et al,* 2018; and De Simeis and Serra, 2021).

In this study, we characterized the isolates using different methods that include: cultural, biochemical and physiological (Backar *et al*., 2022)

The 16S rDNA genomic sequencing also used to characterize the Actinomycetes into species levels which were able to predict each organism and their Accession numbers. Each of the species had a specific Accession number while same species had same percentage Identification number and Gene Bank Accession number (Baniya *et al*., 2019; Omura *et al*., 2021 and Tamura *et al*., 2021).

Phalogenetic tree was also done based on 16S rDNA gene sequencing and it served as an important means of identification of Actinomycetes. BLAST analysis was performed and the assertion number was deposited in the Gene bank (Ahmed H.G, 2017).

From the this study we found out that out of the thirty (30) Actinomycetes isolated, sixteen (16) were Streptomyces, ten (10) were Micromonospora and four (4) were Norcadia species. And each of these species were good producers of secondary metabolites that can be used to produce new antibiotics (Rozirwan et al., 2020; Zamora et al., 2022).

Conclusion

In this study we were able to isolate screened and characterized *Actinomycetes* from estuary as good sources of secondary metabolites that can be used for the production of new antibiotics (Selim et al., 2021). It was also established that there are different strains of *Actinomycetes* present in the estuary, thirty (30) different species have been characterized in this study and among them *Streptomyces* has the highest prevalence rate. It was established that *Streptomyces, Micromonospora* and *Nocardia* were the common species present in the environment were this research was done. And each of these can be used to produce new antibiotic that can improve the quality of therapeutic treatment of infectious diseases (Zou et al., 2020).

Conflict of interest: The authors declare no conflict of interest.

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