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RESEARCH ARTICLE

PRELIMINARY STUDY ON THE ABILITY OF A *STREPTOMYCES SPECIE* ISOLATED FROM GARDEN SOIL TO PRODUCE ANTIMICROBIAL AGENT

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ABSTRACT

Great limitations of some antibiotics nowadays range from human and animal toxicity to narrow spectrum of efficacy. Most synthetic antibiotics have failed to satisfy the needs of their production. There is need therefore to isolate new strains of microorganisms that can yield more effective drugs to be used in tackling the erupting microbial infections. A strain of *actinomycete* was isolated from a garden soil. The strain grows at optimal temperature of 30°C and maximum temperature of 42°C in a slightly alkaline medium of *pH* 6.8 and was identified as a *Streptomyces* sp. Horikoshi JAS medium supported the highest growth and highest production of the antibiotic. Antimicrobial activity of the organism as well as its extracted and purified metabolite was tested against some microorganisms namely *Bacillus* sp, *Candida* sp, *Aspergillus* sp, *Staphylococcus aureus*, *Pseudomonas* sp and *Streptococcus* sp. Both the isolate and its metabolite showed a reasonable activity against all the test organisms except *Streptococcus* sp. Antibiotic accumulation in broth started 24hrs after incubation and increased, reaching maximum at 72 hrs and stationary phase during production was observed at 96 hrs. Maximum *pH* for good yield was 3-5 with low yield at *pH* 6. Further research on the organism and its metabolite are still in progress to enable the production and further testing of the novel antibiotic.

INTRODUCTION

Microorganisms are ubiquitous in nature. They occur freely in the air, water, soil and in many other places. Due to metabolic activities of these organisms, most of them produce toxins. In nature, there is universal distribution of antibiosis among them owing to which they exhibit antagonism. Most of these toxic metabolites produced by them are known as antibiotics. Antibiotics can be classified as broad spectrum affecting a wide range of pathogens or narrow spectrum affecting few pathogenic organisms. They can further be classified as static or cidal in nature which means that they can either inhibit microbial growth and metabolism or killing totally the pathogenic organisms respectively (Bassey and Umeh, 2007). Antibiotics are known to act characteristically at specific sites in the cells of microorganisms, which may be at the cell wall, cytoplasmic membrane, the protein synthesizing machinery or they act on the enzyme involved in nucleic acid synthesis of the organisms. The emerging incidence and diversity of microbial infections have increased considerably over recent decades. These have been reflected by the increasing number of patients at the risk of these microbial infections. Although potent microbial pathogens have been found to have decreased markedly, opportunistic infections have increased gradually with *candida* sp, *Aspergillus* sp and *Pseudomonas* sp as the major microbial pathogens (Bassey and Umeh, 2007). Antibiotics produced by microorganisms have been found useful for the cure of the diseases caused by these bacteria, fungi, viruses and many other microorganisms (Dubey, 2006). They are used therapeutically and sometimes prophylactically in the control of infectious diseases (Smith, 1996).

Over 400 different antibiotics have been produced from different microorganisms and only about 50 have achieved wide range usage. Others failed due to reasons such as toxicity to humans and animals, ineffectiveness or high cost (Okafor, 2007). There is therefore great need to produce new antibiotics that can exhibit wide range of activity (destroying both bacteria and fungi or even viruses) to increasing infectious pathogenic microorganisms. This urgency stems from the limitation of some existing antibiotics most of which display toxicity or limited spectrum of efficacy. Today, screening of novel strains of organisms cover the whole globe and include screening of organisms from soil, air water and even existing

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infectious samples. Thorough screening of these organisms will enable the production of antibiotics with new actions of non toxicity to man and animals and wide range (broad spectrum) action against all microbes; virus, fungi and bacteria. The aim of this study therefore is to screen a microbial isolate that can yield a broad spectrum antibiotic, characterize the strain isolated and determine the conditions for maximum antibiotic production.

MATERIALS AND METHODS

Collection of soil samples

A soil sample was collected from a garden near Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The sample was collected from a depth of about 20 cm and taken to the laboratory immediately for screening.

Culture media and stock culture

All the culture media, chemicals and stock cultures of organisms used were obtained from the Biotechnology Research Centre, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. Culture media used include; Microbial Type Culture collection media (MTCC) made of 4g glucose, 4g yeast extract, 10g malt extract, $CaCO_3$, 15g Agar and pH 7.2. Horikoshi JAS Medium made of 3.5g glucose, 12.5g yeast extract, 3.5g Kh_2PO_4, and 1.4g $MgSO_47H_2O$. Yeast Extract Peptone Medium (YDP) made of 10g glucose, 3g yeast extract, 5g peptone and 15g Agar. CYA Medium made of 4g Zepek conc. Solution, 5g yeast extract, 1g K_2HPO_4, 30g sucrose and 15g Agar. Preparation of these media is according to the manufacturer's instructions. Microbial stock cultures used include *Bacillus sp, Candida sp, Aspergillus sp, Staphylococcus aureus, Pseudomonas sp and Streptococcus sp.*

Microbial isolation Identification

The pour plate method of Collee and Miles (1989) was used. Soil suspension was made by dissolving 1 g of the soil in 9 ml of sterile water making 10% (w/v). After vigorous shaking, the soil suspension was filtered through a fine mesh. Ten-fold dilution of the filtrate was made (10^{-1}, 10^{-2} 10^{-3}……10^{-10}). The 10^{-6} dilution was carefully spread on the surface of the media and incubated at 30^{o}C for 5 days. Series of colonies developed on the plates and the different isolates were streaked out several times until pure colonies were obtained. All the isolated organisms were identified using the method of Waksman and Lechevaier (1962) as described by Okafor (2007). The choice strain (*Streptomyces sp*) after isolation and identification was stored as slant culture and maintained at 4^{o}C.

Antimicrobial activity testing

The method of Waksman and Lechevalier (1962) used by Okafor (2007) was employed in the determination of the antimicrobial activity of the isolate. The choice isolate was grown for 5 days at 30^{o}C on an appropriate medium while the stock cultures were reconstituted and confirmed using the method of Cheesbrough (2000) for bacteria and Barnett *et al*. (1990). The choice isolate was spread on solid media and seeded with the stock cultures. The plates were incubated at 30^{o}C for 48 hours and observed for zones of inhibition.

Antibiotic producing ability

The ability of the selected *Streptomyces* sp to produce antibiotic was checked using the method of Beheeptham and Tomita (1999). A dense inoculation of the producer strain was grown in 250 ml Erlenmeyer flask containing 100 ml of YDP broth at pH 7.2. The set up was incubated on a shaker at 2000 rpm for 4 days. At the end of the period, the broth was assayed for antibiotic accumulation using a spectrophotometer set at 420 nm and confirmed using the reconstituted stock cultures.

Media selection was done using the method of Okafor (2007). The selected strain was grown in two different media (MTCC and JAS). 100 ml of the broths were inoculated with the producer strain and incubated at 30^{o}C for 4 days and centrifuged with an ultra centrifuge at 10000 rpm for 15 minutes and the supernatant assayed for antibiotic activities against the stock organisms. Seeded agar plates were prepared and incubated at room temperature for 30 minutes. Sterile paper discs made from Whatman filter paper were soaked in the supernatant and placed on the seeded agar plates. The set up was refrigerated for 2 hours for antibiotic diffusion. The plates were then incubated at 30^{o}C for 24 hours. Control plates were set up without any organism.

Time course for Antibiotic production

Using the method of Beheeptham and Tomita (1999), 100 ml of the JAS broth was added in a 500 ml flask and inoculated with 5 ml of a 5 day old culture of the *Streptomyces sp.* Using a shaker set at 200 rpm, the flasks were incubated for six days at 30^{o}C. With a sterile pipette, 5 ml of the samples was removed every 24 hours and the rate of antibiotic accumulation determined using the stock organisms. Also observed turbidity was measures with a spectrophotometer at 420 nm as a function of cell growth.

Effects of pH on the Antibiotic produced

Effects of different pH on the antibiotic production by the *Streptomyces sp* were determined at various *p*H of 3, 5, 7, 8 and 10 with Tris-buffer according to the method of Okafor (2007).

Antibiotic extraction

Extraction of the antibiotic produced was done using the method of Okafor (2007). In a 500 ml flask containing 200 ml 4 day- old broth culture of the producer strain, 100 ml of ethyl acetate was added. The set up was agitated on a shaker at 200 rpm at room temperature for 30 minutes. The medium was then centrifuged at 700 rpm for 15 minutes at 24^{o}C. The ethyl acetate layer was removed and the extraction done using an evaporator set at 50^{o}C.

Purification of the antibiotic

The method of Thin Layer Chromatography (TLC) as described by Okafor (2007) was used to purify the antibiotic produced. Mobile phase consisting of 23.75 ml chloroform, 1.0 ml absolute methanol and 2.5 ml was formed. About 0.5 g

of the antibiotic produced was dissolved in a solvent and mixed with the mobile phase. 10 ml of the sample was loaded on to a 6.5 x 6.5 cm aluminum backed silica gel TLC plate. The plate was suspended in the solvent system and allowed to run for 5 hours. The developed bands were dried for 30 minutes and visualized by UV absorption at 254 and 365 nm.

Activity of the antibiotic

The activity of the antibiotic produced was checked using the method of Okafor (2007). The active bands that developed were scraped, dissolved and seeded on the solid plates each containing one of the test organisms. The plates were made in duplicates and incubated in a sterile humid atmosphere at 30^{o}C for 48 hours.

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and invivo studies of the antibiotic

The mean inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antibiotic was determined using the method of Okafor (2007).

RESULTS

Microbial isolation, growth characteristics, physiological and biological characteristics

About five types of organisms were isolated and identified. Morphological and biochemical characteristics of the selected strain were presented in Table 1. The selected isolate was identified as *Streptomyces sp.* The ability of the selected strain to produce antibiotic were tested. The strain showed good growth and sporulation on the media used. Branched aerial mycelium developed which formed an extensively branched substrate. Single and short chains of spores were on the aerial mycelium. The strain grows at optimal temperature of 30^{o}C and maximum temperature of 42^{o}C in a slightly alkaline medium of pH 6.8.

Antimicrobial activity assay

The producer strain showed great antimicrobial activities against the test organisms as shown in Table 2. *Aspergillus sp* showed the highest zone of inhibition of 22mm, *Staphylococcus aureus* showed the least (10mm) while *Streptococcus sp* showed no zone of inhibition.

Time course for antibiotic production

The production of antibiotic started after 24 hours of incubation and increased linearly for the next 48 hours. The yield was at maximum at 72 hrs, approximately 24 hours after the beginning of lysis and constant production was observed at 96 hours.

Effect of pH and media on growth and antibiotic production

Using the Tris – buffer at different pH of 3, 5, 6, 7, 8 and 9, the optimum pH for the growth of the organism and maximum pH for antibiotic production was within the range of pH 3 – 5 with poor yield at pH 6 with long incubation. It was also observed that Horikoshi JAS medium supported the highest growth and highest production of the antibiotic. It also showed high cell density, wider zones of inhibition on solid media, followed by YPD and then MTCC. Therefore JAS medium is recommended for the production of antibiotic using this specie of *Streptomyces.* Glucose as carbon source produced high yield of antibiotic while fructose and maltose were poor carbon sources.

Extraction and purification

Ethyl acetate was the best solvent for the extraction of the antibiotic. All the two solvents used in the development of the TLC chromatography for purification, showed the development of a single band for chloroform, methanol and ammonium in the ratio of 1:1:2. The clear band that developed showed active inhibition of the test organisms.

Table 1. Morphological and biochemical characteristics of the selected isolate

Parameters	Reaction
Gram rxn	-ve short chains
Spore	-
Capsule	-
Motility	+
Fluorescence in UV	+
pH	25 – 37^{o}C
Growth on MacConky agar	+
Rxn on glucose	Gas prod +
Catalase test	+
Esculine hydrolysis	-
Cytochrome oxidase	+
Xylose	+
Manitol	+
Dextrose	+
Starch hydrolysis	+
Nitrate reduction	Weak
Dnase test	-

Key: + Positive rxn
- Negative rxn

Table 2. Antimicrobial activity of the selected isolate on the test organisms

Test organisms	Zones of inhibition (mm)
Aspergillus sp	22
Bacillus sp	16
Candida sp	16
Pseudomonas sp	19
Staphylococcus aureus	10
Streptococcus sp	-

Table 3. Zones of inhibition of the active band against the test organisms

Test organisms	Zones of inhibition (mm)
Aspergillus sp	10
Bacillus sp	8
Candida sp	7
Pseudomonas sp	9
Staphylococcus aureus	6
Streptococcus sp	-

DISCUSSION

According to Dubey (2006), antibiotics are those complex chemical substances, the secondary metabolites, which are produced by microorganisms and act against other microorganisms. They can be used for the cure of certain human and animal diseases caused by bacteria, fungi, viruses etc. Antibiotics can be classified as broad spectrum, affecting a wide range of organisms or low spectrum, affecting few organisms. It can also be classified as 'cidal' e.g bactericidal (able to kill) or 'static' e.g bacteristatic (able to inhibit) the growth of organisms. Antibiotics characteristically act at precise sites in the cell wall, the cytoplasmic membrane, protein synthesizing machinery or it may be an enzyme involved in nucleic acid synthesis. The production of these compounds is expensive making their availability scarce. Some of the existing antibiotics failed to achieve the aim of their production. Emerging opportunistic pathogens need to be tackled and destroyed, to enable man enjoy his stay in this planet.

The aim of this research therefore is to use a cheap source (soil) to isolate an organism already known to produce an antibiotic and involve it in the laboratory to produce an antibiotic with broad spectrum. This aim is achieved as an actinomycete isolated from the soil, characterized and identified as *Streptomyces specie* was able to produce an antibiotic that acts against both bacteria and fungi. All the assays carried out on this organism confirm it to be producing antibiotic that can be purified and used as a wide spectrum antibiotic against large groups of bacteria and fungi. The antibiotic produced is effective against bacteria as well as fungi as shown in Tables 2 and 3. The antibiotic producing characters in this work is similar to that observed by Lyons and Pridham (1973). The antibiotic activity is more for fungi than bacteria and the product can be considered as antifungal antibiotic (Beheeptham and Tomita, 1999). A similar observation was reported by Miller and Hamilton (1973), in which resistaphylin, a polyene macrolide antibiotic was found to inhibit the growth of fungi and gram-positive bacteria.

Two culture media was effective for the antibiotic production, namely JAS Horikoshi Medium and Yeast Extract Peptone Medium. They are also the more effective media for testing the antibiotic activity. Antibiotic production was rapid at the growth phase where growth was exponential. B'ulock (1961) and Okafor (2007) pointed out that that the appearance of a secondary metabolite in parallel with growth is exceptional. More so, the synthesis of a secondary metabolite is suppressed while the cells are actively multiplying and while the culture enters the stationary phase. Thus, if one considers only the sequence of events in the defined medium, the antibiotic production by *Streptomyces* sp may not be a characteristic of secondary metabolite associated with iodophase, although no clear cut separation with iodophase and trophophase was noticed and this may be due to the heterogeneous population existing in such filamentous organisms. In conclusion, fresh soil can serve as a good source of raw material for the production of useful braod spectrum antibiotics. According to Okafor (2007), some existing antibiotics are effective but not permeable through the cell wall of the test organisms or the pathogens, therefore, newer ones should be sought from nature. Research is still in progress to identify the *Streptomyces* isolated to specie level and improve on the production of the novel antibiotic and perform other tests for its purification.

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