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Original Research Article

Biochemical characterization of antibiotic producing actinomycetes isolated from tobacco contaminated soil

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Abstract

Isolation of antibiotic producing actinomycetes is useful in identifying the compounds responsible for antibacterial activity. The aim of this study was to explore the biochemical characterization of antibiotic producing actinomycetes and to evaluate the antibacterial activity of the actinomycetes against *Staphylococcus aureus*. In this particular study starch casein agar was used to isolate actinomycetes from tobacco contaminated soil in Zimbabwe. Four different colonies were identified based on morphology. The different colored colonies observed were pink, white, off-white and green. The colonies were further classified under microscope using iodine crystal. White colonies showed rod shaped cells with no external features, off-white colonies had non-branched sporulating mycelium whereas green colonies exhibited sporulating mycelium with vegetative features and pink colonies were spherical shaped cells with no external features. All of the four colonies were gram positive and were positive for the catalase test. The four different colonies were also positive for the lipase test whereas only the white, off-white and green colonies were positive for the methyl red test. Zones of inhibition were observed in well diffusion assays for antibacterial tests of these actinomycetes (green, white, off-white and pink colonies) against *S. aureus*. Thus, this study shows that some actinomycetes have antibacterial activity and may therefore be used as sources of antibacterial agents.

Keywords: Actinomycetes, antibacterial activity, *Staphylococcus aureus*, lipase test, catalase test, gram stain

INTRODUCTION

Over 5,000 antibiotics have been identified from the cultures of filamentous fungi and gram-positive and gram-negative organisms, but only about 100 antibiotics have been commercially used to treat human, animal and plant diseases (Sujatha and Swethalatha, 2017).. The *Streptomyces* genus is responsible for the formation of more than 60 % of known antibiotics while a further 15 % are made by a number of related *Actinomycetes, Micromonospora, Actinomadura, Streptover; ticillium and Thermoactinomycetes* (Sujatha and Swethalatha, 2017).

Actinomycetes are aerobic, able to form spore, gram-positive bacteria, and belong to the order Actinomycetales which is characterized by possessing both substrate and aerial mycelium growth at their entire growth (Rotich *et al*., 2017). Actinomycetes are differentiated by the forming of branching threads or rods at normal state. The hyphae are generally found to be non- septated; under definite particular conditions, septa may be seen in some different forms. The sporulating mycelium may be branched or none branched, straight or spiral shaped (Chaudhary *et al*., 2013)

The by-products (secondary metabolites) of actinomycetes are organic compounds that are thought to be indirectly involved in normal growth curve, reproduction and development of the producing microorganism (Ismail *et al*., 2015). Microbial secondary metabolites have potential applications in many fields such as antimicrobial agents, enzyme technology, pigment production, antitumor agents against cancer cells and production of toxins. Approximately, 7000

compounds were reported in the glossary of natural products from actinomycetes secondary metabolites (Ismail *et al.*, 2015). Actinomycetes are well known as an inexhaustible source for antibiotics. Many of the known antimicrobials are originally isolated from actinomycetes. The produced substances include all important drug classes used in clinics today, such as β-lactams, tetracyclines, macrolides, aminoglycosides, and glycopeptides. The concentration on the actinomycetes in the biotechnological applications is as a result of their naturally great metabolic variety of these organisms and their long involvement with the environment. Actinomycetes are a unique collection of prokaryotes microorganisms having diverse morphological, biochemical, cultural and physiological characters (Masoumeh *et al.*, 2017). Therefore, this study focuses on the biochemical characterization of antibiotic producing actinomycetes isolated from tobacco contaminated soil and the evaluation of the antibacterial activity of the actinomycetes against *Staphylococcus aureus* in Zimbabwe.

Methodology

Sample collection

The soil sample was collected from Tobacco Research Board (TRB) farm within the depth of 10 – 12.5 cm. The sample was collected in sterile polythene bags, dried in a hot air oven at 37 ^{o}C for 1 hour and then left to cool at room temperature.

Preparation of stock culture

A mass of 1 g of soil sample was placed in a conical flask containing 100 ml of sterile water and few drops of tween-80 were added. The flask was placed in an orbital shaker incubator at 27 ^{o}C for 30 minutes. The obtained mixture was stored as a stock culture.

Crowded-plate technique

About 1 ml of stock culture of the soil sample was diluted from 10^{-1} up to 10^{-6} using a sterile pipette. A volume of 0.1 ml of each of serial dilution was transferred into sterile starch-casein digest agar plates, aseptically using the spread plate method. The plates were then incubated at 27 ^{o}C for 84 hours.

Isolation and Identification of the actinomycetes

The different colonies that were obtained after 84 hours were sub-cultured in casein broth then grown on agar plates at 28 °C for 7 days. Identification of the actinomycetes was done on the basis of macroscopic and microscopic examination and physiological tests as suggested by Bergey's Manual of Systematic Bacteriology.

Macroscopic Characterization

The isolated actinomycetes were observed for aerial mycelium, submerged mycelium, color, and diffusible pigments.

Microscopic Observation

Microscopic examination was performed by a gram-staining method. A smear from the selected strain was prepared on a clean glass slide. It was allowed to air-dry and then heat fixed. The heat-fixed smear was flooded with crystal violet. After one minute it was washed with water and then flooded with mordant gram iodine. The smear was decolorized with 95 % ethyl alcohol, washed with water and then it was countered with safranin for 45 seconds. The smear was dried with tissue paper and examined under oil immersion at 100x magnificent.

Biochemical Tests

For all the isolates, a colony of pure culture of about 7 days of incubation was placed in starch casein broth and incubated at 28 °C for 4 days. After the appearance of turbidity, the culture suspension was used for catalase, lipase and methyl red tests.

Catalase test

A volume of 0.2 ml of the 3 % of H_2O_2 solution was added to clear the glass slide. Different isolates were rubbed on the slides. Effervescence within 30 seconds would indicate a positive reaction.

Lipase test

Tween-80 was dissolved in the starch casein medium and poured into the plates. The cultures were inoculated and then incubated for 7 days. Opaque halo seen around the growth would indicate a positive reaction.

Methyl red test (mixed acid fermentation)

The test was used to differentiate enteric bacteria. Cultures were inoculated in broth (peptone, k_2HPO4, glucose) and grown for 4 days. Few drops of methyl red reagent (0.25g methyl red dissolved in 100 ml of ethanol) were then added. A red color would indicate a positive test whereas a weakly positive test would be indicated by red-orange and a negative result would be indicated by yellow or orange color.

Agar Well Diffusion Method

Test microorganisms *Staphylococcus aureus* (ATCC 25923) was obtained from the University Of Zimbabwe department of Biotechnology and Biochemistry. Overnight cultures of bacterial tester strains were prepared by mixing 1 ml of tester strain and 10 ml of lysogeny broth and incubated at 37 ^{o}C. The following day optical density (OD) of the cultures was measured using a spectrophotometer and standardized to 1 OD by diluting with starch casein broth. A volume of 100 µl of 1 OD bacterial tester strain culture was then spread on four nutrient agar plates. Plates were then left for 1 hour and then a hole on each plate was created using the back of 200 µl pipette tips. Into the four agar plates a volume of 50 µl white, off white, green, and pink colonies were added respectively. Plates were placed in the 37 ^{o}C incubator and observed after 24, 48 and 96 hours. The measurements of the diameter of the zone of inhibition were recorded after 4 days.

RESULTS

Isolation of actinomycetes

Four different actinomycetes were observed and these were isolated based on their colour. The obtained colonies were pink, white, green and off -white.

Iodine crystal staining

The iodine crystal staining was used to determine if the four different actinobacteria were gram positive or gram negative. The purified green, white, off-white and pink colonies stained purple as shown in Figure 1, hence all were gram positive.

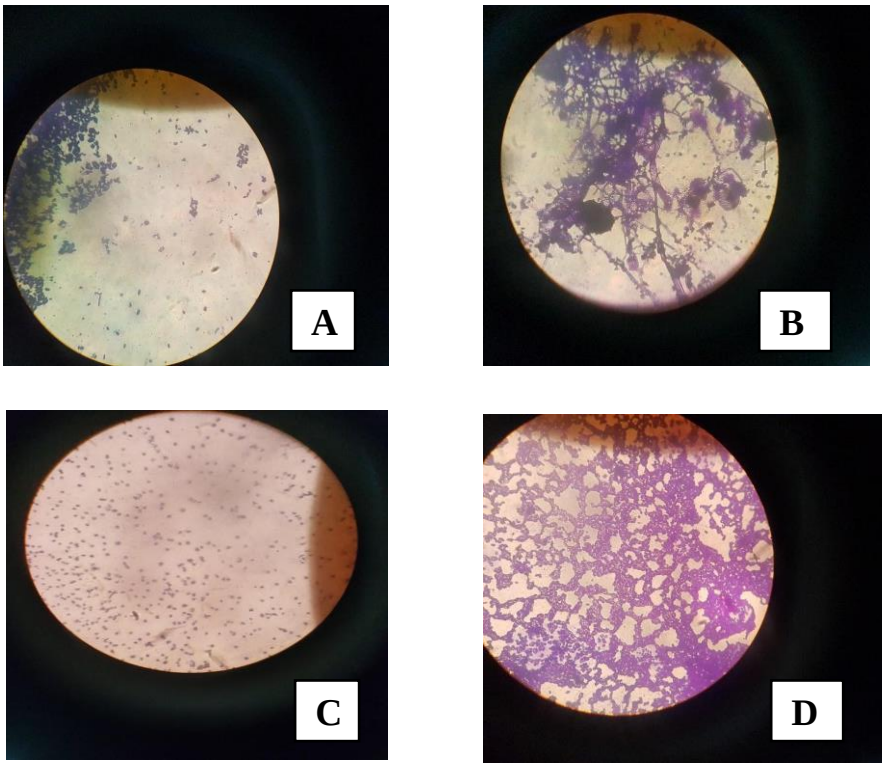

Figure 1: Colonies after iodine crystal staining a: white colonies, rod shaped and non-branched filamentous, b: off white colonies, rod shaped with branched filamentous and mycelium, c: green colonies, spherical shape with branched aerial mycelium and vegetative features and d: pink colonies, spherical shape and are single cells with no external features.

Catalase test

Green and white colonies showed high activity of catalase as indicated by many oxygen bubbles produced within 2 minutes. Off white and pink colonies slides produced effervescence; however, the activity was low compared to that of white and green colonies as shown in Figure 2.

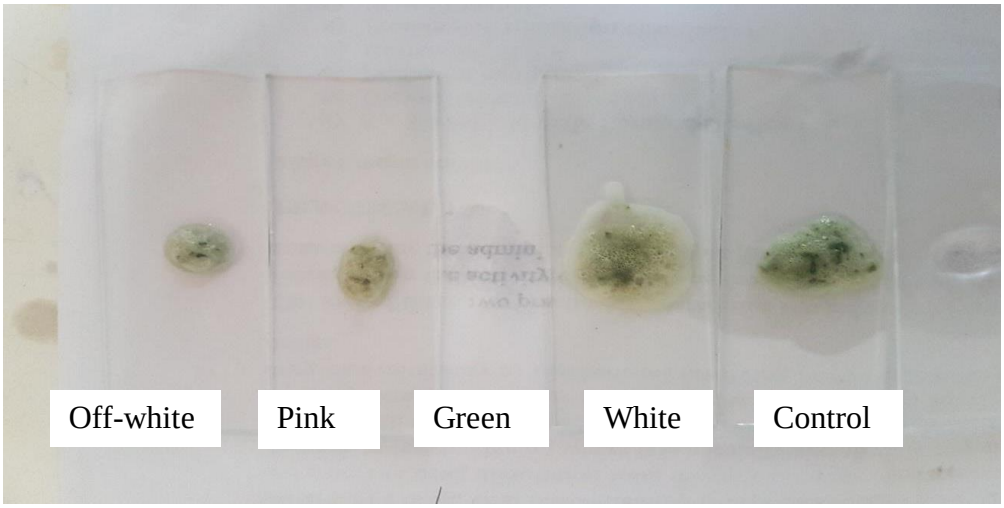

Figure 2: Catalase test activity showing effervescence from four different isolates

Lipase test

Lipolytic organisms split off the fatty acid, and the calcium salts of the fatty acids produce opaque zones around the colonies. Opaque zones are cloudy and misty coloured. All four colonies tested positive for lipase test therefore all actinomycetes isolated had lipase activity.

Methyl red test

Green, white and off-white actinomycetes showed a red colour which is a positive test for methyl red test whereas pink actinomycetes showed an orange colour which is a negative test for methyl red as shown in Figure 3.

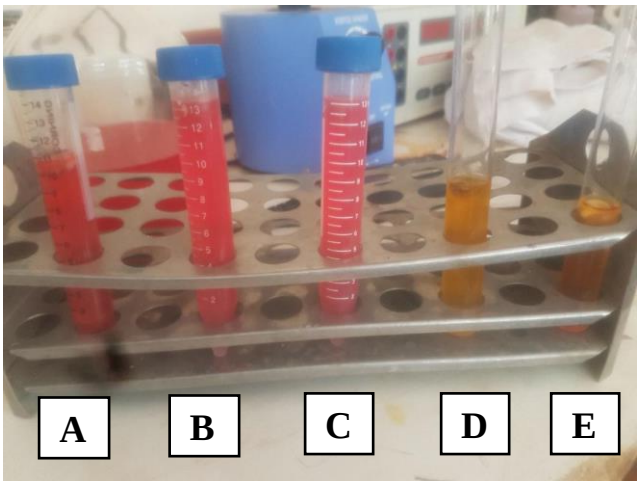

Figure 3: Test tubes of four different actinomycetes exhibiting different colors of methyl red indicator.(a) are white colonies, (b) are off-white colonies, (c) are green colonies, (d) are pink colonies and e is the control.

Antibacterial test

The isolated actinomycetes from tobacco contaminated soil exhibited antibacterial activity against *Staphylococcus aureus*. The zones of inhibition were observed and measured using a 30 cm ruler for each of the different colored actinomycetes. Ciprofloxacin was used as the positive control. Results show that all the isolates have antibacterial activity.

Table 1: Zones of inhibition obtained for each isolate tested against *S. aureus.*

Colour of colonies	Diameter of zone of inhibition (mm)
Pink	8
Off-white	22
White	24
Green	30
Ciprofloxacin	28

n = 2

DISCUSSION

Actinomycetes have been reported to have antibacterial activity (Sarika *et al*., 2021), and this study focused on the investigation of the antibacterial activity of actinomycetes against *Staphyloccocus aureus*. In this particular study the actinomycetes were isolated from tobacco dust contaminated soil and after the preparation of a stock solution from the sample serial dilution was done on the stock solution of soil to reduce the number of microbacteria which made it easier to control the growth of microorganisms on the agar plates.

Starch casein agar was used in isolating actinomycetes as starch casein contains excess salts which make it difficult for other microorganisms to grow on it (Anadal *et al.*, 2016). Four different colonies were identified as pink, white, off-white and green on the agar plates. The colonies were further classified under microscope using iodine crystal. White colonies showed rod shaped cells with no external features, off-white colonies had non-branched sporulating mycelium, green colonies had branched sporulating mycelium with vegetative features and pink colonies had spherical shaped cells with no external features. The morphological difference in these cells was an indication that they are different actinomycetes with different properties. Actinomycetes are gram-positive bacteria and all colonies isolated in this study were proven to be gram positive since they all stained purple after gram staining.

Actinomycetes have a broad range of activities that helps them to adapt in different environments which explains their survivability in a wide range of habitats (Barka *et al*., 2016). Biochemical tests were carried out to distinguish their capabilities. Catalase catalyzes the disproportion reaction of H_2O_2 into H_2O and O_2. The four different actinomycetes had catalase activity but with different activity. Off white and pink colonies had the least activity as they took more time to produce the oxygen bubbles whereas the white and green colonies had the greatest activity of catalase. White and green colonies translate high levels of catalase enzymes and are likely to be more adaptable in a high oxidative environment. All of the four colonies tested positive for lipase test therefore all colonies had lipase activity. The four colonies isolated in this study are able to split off the fatty acid and the calcium salts of the fatty acids. The combination of acids in the mixed acid fermentation usually lowers the pH. Enzymes which involve fermentation include hexokinase, isomerase and pyruvate kinase (Chaudhry and Varacallo, 2021), hence a positive test for methyl red test for white, off-white and green colonies indicated that the 3 actinomycetes in this study have the combination of these enzymes.

In this study actinomycetes were tested *against S. aureas*. Antibacterial activity was observed from all actinomycetes. However antibacterial activities were different for the four actinomycetes. Inhibition zones were measured using a ruler to determine the extent of antibacterial activity. Pink actinomycetes had the least antibacterial activity shown by its small zone of inhibition with a diameter of 8 mm, followed off by the off-white actinomycetes with zone of inhibition with a diameter of 22 mm, followed by the white actinomycetes with zone of inhibition with a diameter of 24 mm and green actinomycetes had the greatest antibacterial activity with inhibition zone with a diameter of 30 mm.

CONCLUSION

Based on the results obtained in this study, it can be concluded that the actinomycetes isolated from the tobacco dust contaminated soil can be used in the production of antibiotics as they showed antibacterial activity against the *S. aureus*.

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