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Isolation of Actinomycetes from the Soils of Menengai Crater in Kenya

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ABSTRACT

This study was carried out to isolate actinomycetes from the soils of Menengai crater. The study area was divided into regions A, B, C and D. Soil samples were collected from 8 sampling points from each region. The samples were separately mixed to form composite samples. Starch casein, Luria Bertani and starch nitrate agar were used in isolating actinomycetes using spread plate technique. Prior to isolation, the soil samples were heat for 1h at 121°C followed by serial dilution upto 10⁻⁶. The isolates were characterized using cultural, morphological and biochemical means. Region B and C produced many actinomycetes than A and D. Luria Bertani agar was better in recovering actinomycetes than starch casein and starch nitrate agar. There was significant difference in the number of actinomycetes isolated using the three media (F=3.315 P=0.04218). The number of actinomycetes isolated from region A, B C, and D also varied significantly (F= 27.50 P=0.000). A total of 152 actinomycetes were isolated. There is need to test the actinomycetes isolates for production of antimicrobials.

Keywords: actinomycetes, isolation, Kenya, Luria Bertani, Menengai, Starch casein.

INTRODUCTION

For long, actinomycetes have been thought to be fungi. However, current studies have shown that indeed they are bacteria (Prasannan et al., 2016). As a result, taxonomists have placed them between true bacteria and true fungi. This has been inspired by an in depth study of their cell wall coupled with other characteristics (Modi et al., 2016).

Actinomycetes inhabit a wide range of ecological zones (Chavan et al., 2013). Despite this, they are known to be ambiqutious free living, filamentous microorganisms with their main home being the soil. In addition, they are gram positive, saprophytic bacteria (Atsuko and Takahashi, 2017). The GC content in their DNA is more than 55% a feature that makes them unique compared with other microorganisms (Siddique et al., 2014).

In the soil, they are involved in a symbiotic relationship with plants besides playing a key role in nutrient cycling (Zhang, et al., 2014). Actinomycetes are also involved in degradation of polythenes in damping sites. The organisms are also abundant in water bodies where they help in biodegradation of industrial effluents dumped from industries (Zakalyukina et al., 2017). By doing this, they help in preservation of aquatic flora and fauna (Prashith et al., 2015).

According to Ashokvardhan and Satyaprasad (2016), when found in any other environment away from soil, actinomycetes must have been taken there by various agents such as floods. In their natural habitat actinomycetes are affected by soil pH, temperature, soil texture, ploughing, organic matter content, amount of oxygen in the soil and the cation exchange capacity of the soil (Varalakshmi et al., 2014).

Actinomycetes are specifically unique in their wide range of metabolites production (Manojkumar and Subbaiya, 2016). They produce vitamins, herbicides, anthelmintes, immunosuppressants, enzymes, nutritional supplements and most significantly, antibiotics. Production of these products varies depending on their habitat (Janaki et al., 2016). Currently, over 5000 antibiotics have been screened from actinomycetes for use in human, animal and plant disease therapy. Over 60% of the known antibiotics have been produced from Streptomycetes while 15% come from rare actinomycetes such as Micromonospora, Actinomadura, Streptovercillum and Thermoactinomycetes that have varying morphological characteristics (Wadetwar and Patil, 2013). The aim of this study was to isolate and partially characterize actinomycetes from soils of Menengai crater.
MATERIALS AND METHODS

The study area

The current study was carried out in Menengai crater which is located to the North of Nakuru town in Kenya. The crater is a product of a volcanic eruption which took place 200,000 years ago. The eruptions lead to formation of a caldera that rises 2,278m above the sea level. The flour of the crater is characterized by steams which come from underground geothermal vents. However, the crater is dormant but speculations maintain that it may turn active after every 50 years. Though these are mare speculations, the shaky grounds around the crater attest to this fact.

The caldera extends over an area of 90 km² with a diameter of 12 km. In Africa, Menengai comes second after Ngorongoro of Tanzania. In terms of location, the crater is located in Rongai and Nakuru North Sub-counties at 35° 28', 35° 36'E, and 0° 13', 1° 10'S (Omenda et al., 2000).

Soil sample collection

The study area was divided into four regions viz A, B, D and D and 8 sampling points were randomly identified from each region. From each of the four regions, 8 two hundred grams sub-samples were collected from the top 5 cm. The samples from each of the regions were mixed to form a composite sample. The samples were separately packed in sterile polythene bags and transported to Egerton University, Department of Biological Sciences laboratories. In the laboratory the samples were separately air dried on the benches for one week to help reduce the population of gram negative bacteria (Khasabuli and Kibera, 2014). The samples were separately sieved through 250 µm pore size sieve (United Kingdom). Heat treatment was carried out by separately placing the samples in a hot air oven at 121°C for 1h to prevent growth of other bacterial flora.

Preparation of culture media

Actinomycetes were cultured on starch casein agar (SCA) (starch: 10 g, KH2PO4: 2 g, KNO3: 2 g, casein: 0.3 g, MgSO4.7H2O: 0.05 g, CaCO3: 0.02 g, FeSO4.7H2O: 0.01 g, agar: 15 g, filtered sea water: 1000 ml and pH: 7.0±0.1), Luria Bertani (M1) medium (Soluble starch: 10g, Peptone: 2.0g, Yeast Extract: 4.0g, Agar: 18.0g, Distilled water: 1000ml, pH; 7.0±0.1) and starch nitrate agar (soluble starch: 20.0g, KH2PO4: 1.0g, KNO3: 2.0g, MgSO4: 0.5G, CaCO3: 3.0g, NaCl: 100g, FeSO4: 0.1g, MnCl2: 0.1g, ZnSO4: 0.1g, Distilled water 100 ml, pH; 7.0±0.1). The media were dissolved in distilled water as per the manufacturer’s instructions before autoclaving at 121°C for 15 min. Following this, the media were supplemented with 25 µg ml⁻¹ nystatin and 10µg ml⁻¹ nalidixic acid to minimize contamination with fungi and bacteria species respectively.

Isolation of actinomycetes on culture media

From each of the composite samples, 1 g of soil sample was separately added to a test tube containing 9 ml distilled water and shaken vigorously at room temperature (25 ± 2°C), using an orbital shaker at 200 rpm for 10 min. The test tubes were considered as stock culture for the soil sample. Aseptically, 1 ml aliquots from the stock solutions were separately transferred to a test tube containing 9 ml of sterile distilled water and mixed well. From these test tubes, 1 ml of aliquots were transferred and mixed with another 9 ml of distilled water to make 10⁻² dilution factor. Similarly, dilutions up to 10⁻⁶ were made using serial dilution technique for all soil samples (Saravana et al., 2014).

After serial dilution, 0.1ml of each sample was separately plated in the three isolation media using spread plate technique. The plates were incubated at 28°C, and observed from 5th day onwards for 25 days. After incubation, actinomycete isolates were distinguished from other microbial colonies by characteristics such as tough, leathery colonies which are partially submerged into the agar (Wang et al., 2016). Colonies with suspected actinomycetes morphology were be sub-cultured on yeast extract malt extract agar medium and incubated at 28°C for 5 to 25 days. Pure cultures were inoculated in 10 ml of yeast extract malt extract broth (YMB) and incubated at room temperature (25 ± 2°C) for 24 to 48 h in a rotary shaker (200 rpm). The pure cultures were maintained in slant culture on yeast extract malt extract agar (ISP2) as well as in glycerol broth at 4°C for further studies (Rajan et al., 2014).

Morphological characterization of actinomycetes

Identification of the actinomycetes was carried out using cover slip method. The morphological characteristics of the isolates were observed under the microscope. M1 agar was placed on sterile glass slides. The isolated
actinomycetes were streaked on the slide prior to incubation at 37°C for 7 days. Two drops of methylene blue were added followed by observation of the slides using the microscope (Ajunwa et al., 2016).

**Biochemical characterization of actinomycetes**

**Gram’s staining**

Crystal violet, gram’s iodine, 95% ethyl alcohol and safranin were used in Gram staining. Briefly, Actinomycetes were separately placed on glass slides using a wire loop. Crystal violet was added and allowed to stand for 1 min. Excess stain was removed using running water. Gram’s iodine was added and the preparation allowed to stand for another 1 min. The excess Gram’s iodine was removed using running water. Ethyl alcohol was added dropwise followed by washing with water. Safranin was used as a counter stain for 45s prior to microscopic examination of the culture (Cassir et al., 2014).

**Use of API strips**

Large volumes of the actinomycetes colony were separately inoculated into 0.85% Nacl solution. McFarland barium sulfate was used in standardizing the inocula which were then inoculated into the wells of API strips. The strips were incubated at 30°C for up to 7 d (Humera et al., 2016).

**Carbon source utilization**

Tests were carried out to determine the ability of the isolates to utilize carbon as recommended by International Streptomyces Project (ISP) (Duddu et al., 2016). The carbon sources (1% w/v) such as D-Glucose, D-Xylose, L-Arabinose, D-Fructose, D-Galactose, Raffinose, D-Mannitol, Sucrose, Maltose, Lactose, Cellulose were mixed (1% w/v) with the basal medium. The actinomycetes isolates were streaked on the medium and incubated at 30°C for 7 d.

**Coding of the isolates**

Isolates bearing the same morphological, cultural and biochemical characteristics were grouped together and coded PAN followed by a number.

**Data analysis**

All data analyses procedures were carried out using statistical package for social sciences (SSPS) version 17.0 software. Comparisons of the means between regions A, B, C and D and between the isolation media were carried out using one way ANOVA.

**RESULTS**

In region A, the actinomycetes isolates varied from 2±3x10^8 to 9±2x10^3 in SC, M1 (5±2x10^8-14±3x10^3) and SN (4±3x10^8-14±2x10^3) (Table 1). On the other hand, the variation of actinomycetes isolates in region B were Sc (15±2x10^8-25±3x10^3), M1 (20±3x10^8-30±2x10^3) and SN (14±2x10^8-27±3x10^3). In addition, actinomycetes isolated from region C ranged from (16±2x10^8-24±2x10^3) in SC, M1 (20±2x10^8-31±3x10^3) and SN (17±3x10^8-28±2x10^3). Besides, Actinomycetes ranges in region D were SC (5±2x10^8-9±2x10^3), M1 (7±2x10^8-15±3x10^3) and SN (4±3x10^8-13±3x10^3). There was a significant difference in the number of actinomycetes isolated using the three media (F=3.315 P=0.04218). Likewise, the number of actinomycetes isolated from region A, B C, and D varied significantly (F= 27.50 P=0.000).

A total of 152 actinomycetes having varying morphological and cultural characteristics were isolates from soils of Menengai crater (Table 2). PAN 1-20 showed green aerial mycelium and yellow substrate mycelium. The isolates did not produce diffusible or melanin pigments in addition to having a flat elevation and a rough surface. However PAN 21-34 had grey aerial mycelium and cream substrate mycelium. The isolates didn’t produce any diffusible or melanin pigments but the surface was flat and rough. PAN 35-50 had a dark grey surface mycelium with a cream substrate mycelium. The isolates produced brown diffusible pigments but no melanin pigments. Their surfaces were raised and rough. PAN 51-81 were white with a cream substrate mycelium. They produced neither diffusible nor melanin pigments. Their surface was raised and rough.
Conversely, PAN81-100 were violet in both aerial and substrate mycelium. They had violet diffusible pigments with no melanin pigments while their surface was raised. Additionally, PAN 101-105 had a purple surface with a black subsurface mycelium. The isolates had brown diffusible pigments but no melanin pigments. Their surface was flat and rough. PAN 106-116 were light brown with brown substrate mycelia. Their diffusible pigments were brown with no melanin pigments. The surface was flat and smooth. PAN117-120 had dark brown aerial and brown subsurface mycelium with brown diffusible pigments but no melanin pigments. The isolates had a flat surface that was rough. In PAN121-126, the aerial mycelia were blue while the substrate mycelia were black. The isolates did not produce either diffusible or melanin pigments. They had a surface that was raised and rough. The isolates PAN127-130 produced dark blue aerial mycelium and black substrate mycelium. Additionally, they produced brown diffusible and melanin pigments. Their surface was raised and rough. In PAN131-133, their aerial and substrate mycelia were black. They produced no diffusible and melanin pigments. Their surface was raised and rough. PAN134-138 had an orange surface mycelium with yellow subsurface mycelium. Their surface was raised and rough. In PAN139-152, the aerial mycelia were blue while the substrate mycelia were black. The isolates did not produce either diffusible or melanin pigments. They had a surface that was raised and rough. On the other hand, PAN 139-152 had peach surface mycelium with no diffusible and melanin pigments production. Their surface was flat and rough.

Biochemical characteristics of actinomycetes isolated from Menengai crater

All the isolates tested positive for Gram stain, catalase, oxidase, urea hydrolysis and gelatin liquefication (Table 3). They were all negative for deaminase and Indole production. PAN 101-105, PAN121-126 and PAN 139-152 were positive for beta-galactosidase while PAN 1-20, PAN 21-34, PAN 35-50, PAN 51-81, PAN 106-116, PAN 117-120, PAN 127-130, PAN 131-133 and PAN 134-138 were negative. PAN 1-20, PAN 51-81, PAN 117-120 and PAN 134-138 were positive for lysine decarboxylase. PAN 21-34, PAN 35-50, PAN 81-100, PAN 101-105, PAN 106-116, PAN 121-126, PAN 131-133 and PAN 139-152 were negative for lysine decarboxylase. The isolates PAN 21-34, PAN 106-116, PAN 117-120, PAN 131-133, PAN 134-138, PAN 134-138, PAN 139-152 were positive while PAN 1-20, PAN 35-50, PAN 51-81, PAN 81-100, PAN101-105, PAN 121-126, PAN 127-130 were negative for ornithine decarboxylase. In addition, PAN 1-20, PAN 21-24, PAN81-100, PAN 101-105, PAN 105-116, PAN 117-120, PAN 121-126, PAN 127-130, PAN 131-133, PAN 134-138 and PAN 139-152 were positive for citrate utilization. Only PAN 35-50 and PAN 51-81 tested negative for citrate utilization. When tested for H$_2$S production, PAN 35-50, PAN 51-81, PAN 117-120, PAN 121-126, PAN 131-133 were positive while PAN 1-20, PAN 21-34, PAN 81-100, PAN 101-105, PAN 106-116, PAN 127-130, PAN 134-138, PAN 139-152 tested negative.

Carbon source utilization of actinomycetes isolated from Menengai Crater

All the isolates utilized D-glucose, D-galactose, sucrose, D-fructose and L-arabinose. In addition, all of them could not utilize cellulose, D-mannitol, salicin, raffinose and meso-inositol (Table 4). However, all the isolates apart from PAN 35-50 could utilize sucrose. PAN 21-34, PAN 51-80, PAN 81-100, PAN 101-105, PAN 121-126, PAN 127-130, PAN 131-133, PAN 134-138, PAN 139-152 were positive while PAN1-20, PAN35-50, PAN106-116, PAN117-120 were negative for lactose utilization. All the isolates utilized maltose apart from PAN 35-50 and PAN51-80.

DISCUSSION

The current study indicates that Menengai crater is a fertile ground for isolation of actinomycetes (Table 1). Region B and C produced the highest number of actinomycetes compared to regions A and D. This can be attributed to the topography of the crater where region B and C are located on the lower side while regions A and D are on the upper side. This agreed with a previous study carried out in semi-arid regions of North Africa (José et al., 2017). As a result most of the actinomycetes could have been transported to the lower regions through soil erosion (Surabhi, and Srividya, 2016).
### Table 1: Number of actinomycetes isolated from soils of Menengai crater using different media

| DF       | Region A | Region B | Region C | Region D |
|----------|----------|----------|----------|----------|
| SC       | M1       | SN       | SC       | M1       | SN       | SC       | M1       | SN       |
| $10^{-3}$| 9±2      | 14±3     | 25±3     | 30±2     | 27±3     | 24±2     | 31±3     | 28±2     | 9±2      | 15±3     | 13±3     |
| $10^{-4}$| 6±2      | 12±2     | 10±2     | 21±3     | 30±2     | 24±3     | 22±2     | 31±2     | 25±2     | 7±3      | 11±3     | 10±3     |
| $10^{-5}$| 4±3      | 10±2     | 7±2      | 19±2     | 28±3     | 21±3     | 22±2     | 30±3     | 22±3     | 5±2      | 10±3     | 11±2     |
| $10^{-6}$| 3±2      | 8±3      | 8±3      | 18±2     | 26±2     | 18±3     | 17±3     | 24±2     | 21±3     | 6±2      | 10±2     | 5±2      |
| $10^{-7}$| 3±3      | 7±2      | 5±2      | 15±3     | 27±3     | 15±2     | 18±3     | 22±2     | 19±2     | 5±2      | 9±2      | 3±2      |
| $10^{0}$ | 2±3      | 5±3      | 4±3      | 15±2     | 20±3     | 14±2     | 16±2     | 20±2     | 17±3     | 5±2      | 7±2      | 4±3      |
| **Mean** | **4.5±3**| **9.3±2**| **8.0±1**| **27.3±3**| **19.8±3**| **27.0±2**| **22.0±3**| **6.2±2**| **10.3±3**| **7.67±3**|

DF; dilution factor, SC; starch casein, M1; Luria Bertani, SN; starch nitrate.

### Table 2: Morphological and cultural characteristics of actinomycetes isolated from Menengai crater

| Code     | Aerial mycelium | Substrate mycelium | Diffusible pigments | Melanin pigments | Elevation | Surface |
|----------|-----------------|--------------------|---------------------|------------------|-----------|---------|
| PAN 1-20 | Green           | Yellow             | -                   | -                | flat      | rough   |
| PAN 21-34| Grey            | Cream              | -                   | -                | flat      | rough   |
| PAN 35-50| Dark grey       | cream              | +                   | -                | Raised    | rough   |
| PAN          | Color       | Description   | Texture  | Height | Surface |
|--------------|-------------|---------------|----------|--------|---------|
| PAN 51-80    | White       | cream         | -        | -      | raised  | rough   |
| PAN81-100    | Violet      | violet        | +        | -      | raised  | rough   |
| PAN 101-105  | purple      | Black         | +        | -      | flat    | rough   |
| PAN 106-116  | Light brown | Brown         | +        | -      | flat    | smooth  |
| PAN117-120   | Dark brown  | Brown         | +        | -      | flat    | rough   |
| PAN121-126   | Blue        | black         | -        | -      | raised  | rough   |
| PAN127-130 | Dark Blue | black | + | + | raised | rough |
|------------|-----------|-------|---|---|--------|------|
| PAN131-133 | Black     | black | - | - | raised | rough |
| PAN134-138 | Orange    | yellow| - | - | raised | smooth|
| PAN 139-152| Peach     | cream | - | - | flat   | rough |
Table 3: Biochemical characteristics of actinomycetes isolated from Menengai Crater

| Test | PAN 1-20 | PAN 21-34 | PAN 35-50 | PAN 51-81 | PAN 101-105 | PAN 106-116 | PAN 117-120 | PAN 121-126 | PAN 127-130 | PAN 131-133 | PAN 134-138 | PAN 139-152 |
|------|----------|----------|----------|----------|------------|------------|------------|------------|------------|------------|------------|------------|
| GS   | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          |
| ONPG | -        | -        | -        | -        | +          | -          | -          | -          | -          | -          | +          | +          |
| CAT  | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          |
| GLU  | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          |
| LDC  | +        | -        | -        | +        | -          | -          | +          | -          | -          | +          | +          | +          |
| ODC  | -        | +        | -        | -        | -          | -          | +          | -          | -          | +          | +          | +          |
| CIT  | +        | +        | -        | -        | +          | +          | +          | +          | +          | +          | +          | +          |
| H₂S  | -        | -        | +        | -        | -          | +          | -          | -          | -          | -          | -          | -          |
| URE  | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          |
| TDA  | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          |
| IND  | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          |
| GL   | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          |

GS: Gram stain, ONPG: beta-galactosidase, CAT: catalase test, GLU: oxidase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, CIT: citrate utilization, H₂S: Hydrogen sulphide production, URE: urea hydrolysis, TDA: deaminase, IND: Indole production, GL: Gelatin liquefication.

Table 4. Carbon source utilization of actinomycetes isolated from Menengai Crater

| Test | Strain | PAN 1-20 | PAN 21-34 | PAN 35-50 | PAN 51-80 | PAN 81-100 | PAN 101-105 | PAN 106-116 | PAN 117-120 | PAN 121-126 | PAN 127-130 | PAN 131-133 | PAN 134-138 | PAN 139-152 |
|------|--------|----------|----------|----------|----------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| DG   | +      | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          | +          |
| DGal | +      | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          | +          |
| Cellulose | -    | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          | -          |
| Su   | +      | +        | -        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          | +          |
| DM   | -      | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          | -          |
| Lactose | -    | -        | +        | +        | +        | -          | -          | +          | +          | +          | +          | +          | +          | +          |
| Mal  | +      | +        | -        | -        | -        | +          | +          | +          | +          | +          | +          | +          | +          | +          |
| Salicin | -     | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          | -          |
| DF   | +      | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          | +          |
| Raffinose | -   | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          | -          |
| Meso | -      | -        | -        | -        | -        | -          | -          | -          | -          | -          | -          | -          | -          | -          |
| LA   | +      | +        | +        | +        | +        | +          | +          | +          | +          | +          | +          | +          | +          | +          |

DG; D-Glucose, DGal; D-Galactose, Su; sucrose, DM; D-Mannitol, Mal; Maltose, DF; D-Fructose, Meso; Meso-Inositol, LA; L-Arabinose.
M1 agar was able to recover higher number of actinomycetes than either starch casein and starch nitrate agar (Table 1). This differs with earlier studies carried out in other parts of the world (Atta, 2015; Qais et al., 2017). The hostility of the crater in terms of growth conditions of actinomycetes may be a contributing factor. Slam et al. (2014) asserts that the growth conditions that actinomycetes are subjected to may results in change in nutrient requirements.

The isolates obtained had varying cultural and morphological characteristics (Table 2). This agrees with a previous study carried out in Kericho (Rotich et al., 2017). However, the isolates differed in some details such as the colony colour, elevation and surface morphology (Kumari et al., 2016). This could have resulted from differences in physico-chemical characteristics of the soil from which the actinomycetes were isolated (Yabe et al., 2017).

Biochemical characteristics of actinomycetes are used in their identification. The microorganisms isolated in the current study produced biochemical characteristics that are typical of actinomycetes (Table 3). The results concurred with a previous study that was carried out in Tamilnadu, India (Cholarajan and Vijayakumar, 2016). This may have resulted from the isolated actinomycetes strains been the same (Basha et al., 2017).

On carbon source utilization, the results slightly differed with a previous study (Tebo et al., 2015). The difference could have originated from differences in the study area. According to Memon et al. (2016) the environment in which actinomycetes are growing in determine their carbon source requirements. In addition Arya and Singh (2016) affirm that different actinomycetes have different carbon source requirements based on their metabolic pathways.

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