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Effect of cultural conditions and physical factors on production of antibiotic metabolites by selected actinomycetes

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Abstract

Antibiotic resistance is one of the biggest global problem of our times. The leading producers of antibiotics that can be used to curb this problem are actinomycetes. This study was conceived to isolate antibiotic producing actinomycetes from the soils of Menengai crater and identify the cultural and physical factors that favoured production of antibiotics by selected actinomycetes. Soil samples were collected from 32 randomly selected sampling points within Menengai crater. The actinomycetes were isolated using serial dilution technique. The actinomycetes were tested for antagonism against selected bacterial and fungal pathogens using primary and secondary screening bioassays. Based on broad spectrum of activity and the size of zone of inhibition, four potent actinomycetes were selected for further studies. The effect of growth media, pH, temperature, incubation period, aeration, inoculum concentration, carbon source, nitrogen source and salt concentration on growth and production of antibiotic metabolites was determined. The actinomycetes isolated presented varying morphological characteristics. There was a significant difference in the diameters of zones of inhibition produced by the test pathogens when subjected to the antibiotic metabolites from the selected actinomycetes ($F = 6.6046$ $P = 0.001338$). The growth and production of antibiotics by the selected actinomycetes was favoured by use of Luria Bertani as the culture medium, a pH of 6, incubation temperature of 28°C, incubation period of 7d, aeration rate of 200rpm, inoculum concentration of 1%, glycerol as carbon source, oat meal as nitrogen source and a salt concentration of 1.5%. The growth and production of antibiotics by the selected actinomycetes is affected by culture medium, pH, incubation temperature, incubation period, aeration rate, inoculum concentration, carbon source, nitrogen source and salt concentration. There is need to curry out structure elucidation of the antibiotics from the selected actinomycetes.

1. Introduction

The increase of antibiotic resistance is attributed to a number of factors such as their overuse in agriculture, misuse by human beings and unnecessary prescription by physicians **(Mohsen *et al.*, 2019)**. As a result, the search of novel antibiotics is a continuous process **(Bi *et al.*, 2017)**. Actinomycetes produce varied biologically active secondary metabolites **(Jami *et al.*, 2015)**. Harsh environments harbor actinomycetes that produce unique antibiotics **(Waithaka *et al.*, 2019b)**. It is estimated that 2/3 of the renowned antibiotics are produced by actinomycetes **(Bo *et al.*, 2019)**. About 75% of the useful antibiotics in the market today are produced by *Streptomyces* spp **(Hamed *et al.*, 2021)**. Currently, there are about 23,000 known secondary metabolites from actinomycetes 80% of which are produced by *Streptomyces* spp **(Al-Rubaye *et al.*, 2018)**.

World health organization's working document on stability testing of active substances and pharmaceutical products asserted that the purpose is to provide evidence of how the quality of an active substance varies under the influence of a number of factors **(Mukesh, 2014)**. Product related factors such as chemical and physical factors, pharmaceutical excipients, dosage, form and composition and manufacturing process are considered **(Jodi *et al.*, 2017)**. In addition, other factors that can lead to reactive degradation of the active substance should also be tested **(Ashkani *et al.*, 2015)**. **Awla *et al.* (2016)** observed that stability of the active substance should go through a series of re-testing. The shelf life of the compound is then indicated which helps in recommending the storage conditions of the substance **(Gopalakrishnan *et al.*, 2017)**.

New antibiotics are urgently required. The most promising sources are from harsh habitats. Volcanic areas are largely unexploited source for new antibiotics **(Al-Ghazali *et al.*, 2017)**. However, the ability of actinomycetes to form these bioactive products is not a fixed. It can be greatly increased or completely lost under different conditions of nutrition and cultivation **(Bundale *et al.*, 2015)**. This is because antibiotic biosynthesis is a specific property of microorganisms which depends greatly on culture conditions. Improvement in the growth and antibiotic production can be varied by manipulating the nutritional and physical parameters of the culture conditions. As a result, media composition plays an important role in the growth and efficiency of antibiotic production in actinomycetes. **Padma *et al.* (2018)** asserts that designing an appropriate fermentation medium is of

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critical importance in the production of secondary metabolites **(Tuli *et al*., 2014)**. In addition, changes in the form and type of carbon and nitrogen sources affect antibiotic biosynthesis in actinomycetes. Moreover, several growth factors play a significant role in growth and production of antibiotics by actinomycetes **(Anuprita *et al*.,2015)**.

2. Material and methods

2.1 The study area

Soil sampling was carried out in Menengai crater. The crater has a height of 2,278m above sea level. It is a product of a volcanic eruption that occurred about 200,000 years ago **(Omenda *et al*., 2014)**. The crater is dormant but has a high temperature geothermal resource represented by a steaming ground at a temperature of 88^{o}C **(Omenda *et al*., 2014)**. Menengai caldera is located in Rongai and Nakuru North Sub-counties at 35^{o} 28', 35^{o} 36'E, and 0^{o} 13', 1^{o} 10'S **(Waithaka *et al*., 2015)**.

2.2 Collection of soil samples

Thirty-two soil sampling points were randomly selected. From each sampling point, 100g of soil was collected using a soil auger from the top 5cm. The soil samples were mixed to make a composite sample. The composite sample was packed in new polythene bags and immediately carried to Egerton University, Department of Biological Sciences laboratories. The sample was placed in sterile khaki bags and stored at 4^{o}C awaiting further processing and isolation of microorganisms **(Wekesa *et al*., 2016)**.

2.3 Pretreatment of soil for isolation of actinomycetes

The soil sample was air dried on the laboratory benches for one week to reduce the population of Gram negative bacteria **(Floros *et al*., 2016)**. The sample was sieved through 250µm pore size sieve. The soil sample was placed on aluminium foil and heat treated at 120^{o}C in a hot air oven (Heidolph Laorota, 4001, Buchi Vacuum Conroller V-805) for 1 h to reduce the number of vegetative bacterial flora **(Waithaka *et al*., 2019a)**.

2.4 Isolation of actinomycetes

Actinomycetes were isolated using serial dilution technique. From the composite soil sample, 1g of soil was added to a test tube containing 9mL distilled water and shaken vigorously at room temperature (23±2^{o}C), using a vortex mixer (PV-1, V-32, rpm 200) for 10 min. Aseptically, 1mL aliquot from the stock solution was transferred to a test tube containing 9mL of sterile physiological saline and vortexed. From these test tube, 1mL of aliquot was again transferred and mixed with another 9mL of distilled water to make 10^{-2} dilution factor. Similarly, dilutions up to 10^{-6} were made using serial dilution technique **(Sujatha and Swethalatha, 2017)**.
After serial dilution, 0.1mL aliquots of the soil suspensions were separately placed on sterile media plates using a micropipette. The samples were spread on the surface of culture media Luria Bertani (M1) using an L-shaped glass rod. The plates were incubated at 28±2°C and observed after 7d. Actinomycete isolates were distinguished from other microbial colonies by characteristics such as tough, leathery colonies partially submerged in the agar medium **(Yang *et al*., 2018)**. Colonies with suspected actinomycetes morphology were sub-cultured on M1 agar medium and incubated at 28±2^{o}C for seven days. The pure cultures were maintained as slant culture on M1 agar medium as well as in glycerol at -4°C for further studies. Characterization of the actinomycetes was carried out using morphological and biochemical characteristics **(Orooba *et al*., 2017).**

2.5 Test pathogenic microorganisms

Methicillin resistant *Staphylococcus aureus* (ATCC 25923), *Streptococcus pneumoniae* (ATCC 49617), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus vulgaris* (ATCC 49990) were obtained from Kenya Medical Research Institute (KEMRI). *Alternaria citri* (ATCC 1015), *Candida albicans* (ATCC 10231), *Fusarium oxysporum* (ATCC 16608) and *Ustilago maydis* (ATCC 14826) were acquired from Culture Collection Centre of the Department of Crop Science and Crop Protection, University of Nairobi.

2.6 Primary screening of actinomycetes for antagonism to test microorganisms

Antagonistic activity of the isolates to the test pathogenic microorganisms was determined by cross streak method **(Mohammadipanah and Wink, 2015)**. As positive control, vancomycin (30µg/mL) was steaked at the centre of Mueller-Hinton agar for bacterial pathogens and clotimazole (1% topical solution) for fungi. The pathogens were streaked perpendicularly to the positive control. Plane Petri dishes were used as negative controls. Antagonism was measured by determination of the size of the inhibition zone in millimeters following incubation of bacteria cultures at 37 ± 2^{o}C for 24h and fungal pathogens at 28 ± 2^{o}C for seven days **(Elbendary *et al*., 2018)**.

2.7 Secondary screening of actinomycete isolates for antibiotic production

About 250mL of M1 broth in conical flasks was prepared and separately inoculated with isolated actinomycete cultures using a sterile wire loop. The flasks were incubated at 28±2^{o}C for 7d in an orbital shaker. Ethyl acetate was added in 1:1 ration and centrifuged for 10 minutes at 600rpm. Antibiotic activity against the selected test pathogens was determined using Kirby Bauer disk diffusion technique **(Jayshree *et al.,* 2016)**.
Among the actinomycetes isolates, cultures of four promising strains coded PAN 25, PAN 41, PAN 75 and PAN 110 with the largest inhibition zones against Gram-positive bacteria, Gram-negative bacteria, filamentous fungi and yeast and recorded the largest diameter of clear zones of inhibition. These isolates were selected for species identification and antibiotic metabolites production studies **(Jodi *et al*., 2017)**.

2.8 Effect of cultural conditions and physical factors on growth and production of antibiotic metabolites by cultures of the selected actinomycetes

Starter cultures of isolates PAN 25, 41, 75 and 110 were grown in 50 M1 broth for 2 d at 28±2^{o} C in conical flasks. The selected actinomycete isolates were grown in: starch casein, Luria Bertani (M1) and starch nitrate; pH adjusted to 4, 5, 6, 7 and 8 using 1M HCL and IM NaOH; incubation temperature 25^{o}C, 28^{o}C, 31^{o}C, 34^{o}C, 37^{o}C and 40^{o}C at 100rpm for 7 days; incubation for 5, 6, 7, 8, 9 and 10 days; aeration at 100, 150, 200, 250, 300 and 350 rpm for seven days; inoculum concentration at varying cell concentrations (0.5, 1, 1.5, 2 and 2.5%); carbon source in the broth substituted with glucose, sucrose, maltose, glycerol and starch to a final concentration of 1% (w/v); nitrogen source substituted with malt extract, yeast extract, oatmeal, cornmeal, and peptone to a final concentration of 1% (w/v) in conical

flasks and salt concentration maintained at 0.5, 1.0, 1.5, 2.0 and 2.5 % of sodium chloride.
Unless where otherwise stated, incubation was carried out at 28±2°C for 7 d in an orbital shaker (Gallenkamp, Model 10X 400) at 100rpm. The metabolites were assayed for antibiotic activity using *S. aureus* (ATCC 25923) using Karby Bauer disk diffusion technique.

2.9 Data analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS) Version 17.0 software. Diameter of zones of inhibition were compared by performing One-way ANOVA. Statistical values with $p \leq 0.05$ were statistically significant.

3. Results

3.1 Morphological characteristics of actinomycete isolates

A total of 138 actinomycetes were isolated from the soils of Menengai crater. The actinomycetes isolates gave varying colours of the aerial and substrate mycelia (**Figure 1**).

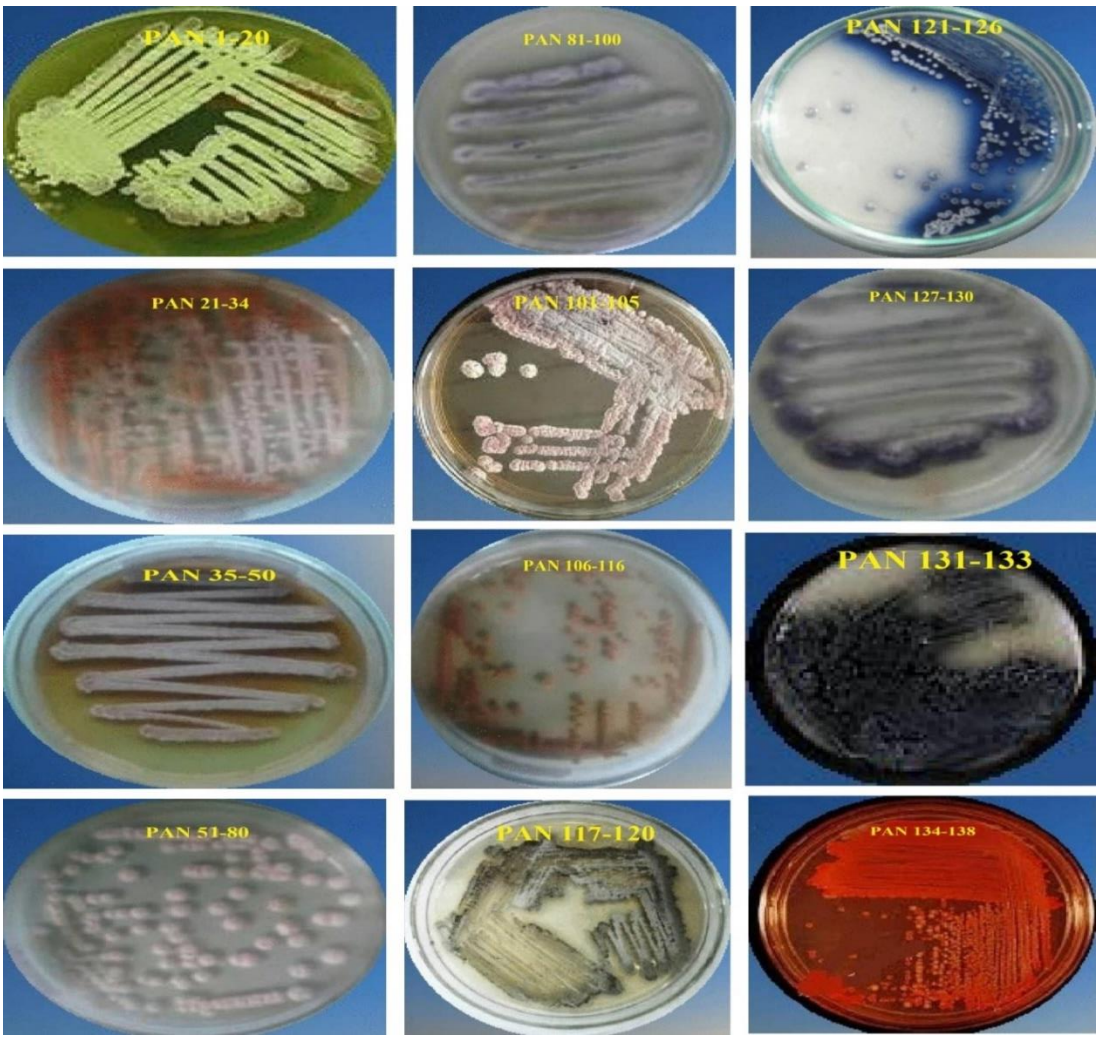

Figure 1 Cultural characteristics of actinomycetes isolated from soils of Menengai crater.

3.2 Antibiotic properties of the metabolites from the selected actinomycetes

There was a significant difference in the diameters of zones of inhibition produced by the test pathogens when subjected to the antibiotic metabolites from the selected actinomycetes ($F = 6.6046$ $P = 0.001338$). Among the bacteria, the mean diameter of zone of inhibition varied from 24.67±0.2mm in isolate PAN 25 to 41.83±0.2mm in isolate PAN 75 while in fungi, the zones of inhibition ranged from 29.00±0.2 in PAN 25 to 32.75±0.2 in PAN 41 (**Table 1**). The mean diameter of zone of inhibition decreased in the order metabolites from isolate PAN 75 (37.60±0.2 mm); PAN 41 (35.10±0.2mm); PAN 110 (34.30±0.2mm) and PAN 25 (26.40±0.2mm).

3.3 Effect of cultural conditions and physical factors

3.3.1 Culture media

There was no significant difference in the zones of inhibition produced by antibiotic metabolites extracted from isolates grown on starch casein, Luria Bertani and starch nitrate broth when tested against *S. aureus* (ATCC 25923) ($F = 2.5618$, $P = 0.128$). Antibiotic metabolites from cultures grown in Luria Bertani broth (M1) produced the largest zones of inhibition in metabolites from isolates PAN 75 (30±0.1mm) and the smallest from PAN 25 (23±0.2mm) (**Figure 2**). However, in starch casein broth, zones of inhibition ranged from PAN 25 (18 ± 0.1mm) to PAN 41 (25± 0.2mm). In starch nitrate broth, the zones of inhibition varied from metabolites from isolate PAN 25 (19±0.3mm) to PAN 75 (24±0.2mm). Among the three culture

media, Luria Bartani medium was the most ideal for growth and production of antibiotics by the four actinomycetes isolates.

Table 1 Mean diameters of zones (mm) of inhibition of test pathogenic microorganisms by extracts from the selected actinomycete isolates.

Pathogen	Isolate				
	PAN 25	PAN 41	PAN 75	PAN 110	Van/Clot
S. aureus	26±0.2	35±0.2	39±0.1	32±0.1	30±0.1
S. pneumoniae	25±0.3	28±0.2	35±0.2	25±0.2	27±0.2
E. faecalis	25±0.2	41±0.3	45±0.3	38±0.2	25±0.2
E. coli	24±0.2	47±0.3	49±0.3	47±0.1	18±0.1
P. aeruginosa	20±0.3	42±0.1	40±0.1	41±0.2	15±0.2
P. vulgaris	28±0.2	37±0.3	43±0.3	36±0.3	17±0.1
Mean	24.67± 0.2	38.33± 0.2	41.83± 0.2	36.50± 0.2	22.00±0.2
C.albicans	27±0.2	40±0.1	37±0.3	41±0.2	19±0.1
U. maydis	28±0.2	30±0.3	25±0.2	30±0.1	12±0.2
A. Citri	31±0.2	29±0.1	30±0.3	27±0.2	11±0.1
F. oxysporium	30±0.1	32±0.2	33±0.1	26±0.3	13±0.2
Mean	29.00± 0.2	32.75± 0.2	31.25± 0.2	31.00± 0.2	13.75±0.2

Each value represents the mean value (±SD) of five independent replicates. Van; vancomycin, clot; clotrimazole; *S. aureus; Staphylococcus aureus, S. pneumoniae; Streptococcus pneuminiae, E. faecalis; Enterococcus faecalis, E. coli; Escherichia coli, P.aeruginosa; Pseudomonas aeruginosa, P. vulguris; Proteus vulgaris, C. albicans; Candida albicans, U.maydis; Ustilago maydis, A. citri; Alternaria citri, F. oxysporum; Fusarium oxysporum.*

3.3.2 pH

The zones of inhibition produced by antibiotic metabolites from cultures of isolates PAN 25, PAN 41, PAN 75 and PAN 110 when tested against *S. aureus* (ATCC 25923) at different levels of pH varied significantly (F = 15.67, P = 0.001). At pH 4, the zones of inhibition varied from 21±0.1mm in metabolites from isolate PAN 25 to 33±0.2mm in PAN 75 (**Figure 3**). On the other hand, at pH 5 the range was from 24±0.2mm in metabolites from isolate PAN 25 to 36±0.1mm in PAN 75. In addition, the largest zone of inhibition at pH 6 was 39 ± 0.1mm in metabolites from isolate PAN 75 and the least was 26±0.2mm in PAN 25. However, at pH 7, the zones of inhibition ranged from 23±0.3mm in metabolites from isolate PAN 25 to 34 ± 0.3mm in PAN 75. The zones of inhibition at pH 8 varied from 20±0.1mm in metabolites from isolate PAN 25 to 31±0.3mm in PAN 75. The optimum pH for growth and production of antibiotics by the selected actinomycetes cultured on Luria Bertani medium was 6.

3.3.3 Incubation temperature

Temperature significantly (F = 14.23, P = 0.002) affected the production of antibiotic metabolites by isolate PAN 25, 41, 75 and 110. The zones of inhibition at 25^{o}C ranged from 20 ± 0.1mm for metabolites from isolate PAN 25 to 34 ± 0.2mm for PAN 75. For antibiotic metabolites produced at 28^{o}C the zones ranged from 22 ± 0.3mm for isolate PAN 25 to 36 ± 0.1mm for PAN 75 (**Figure 4**). In addition, at 31^{o}C, the zones of inhibition differed from 21 ± 0.2mm in metabolites from isolate PAN 25 to 33 ± 0.3mm in PAN 75. The zones of inhibition at 37^{o}C varied from 20 ± 0.1mm in metabolites from isolate PAN 25 to 29 ± 0.1mm in PAN 75. However, at 40^{o}C, metabolites from isolate PAN 75 produced the biggest (27 ± 0.2mm) while PAN 41 presented the smallest (19 ± 0.3mm) zone of inhibition. The optimum temperature for growth and production of antibiotics by the actinomycetes isolates cultured on Luria Bertani medium was 28^{o}C.

3.3.4 Incubation period

There were significant differences (F = 12.52, P= 0.001) in the zones of inhibition produced by antibiotic metabolites obtained from isolate PAN 25, 41, 75 and 110 when incubated for different time regimes. On day 5, the biggest zone of inhibition was produced by metabolites from isolate PAN 75 (33 ± 0.2mm) while the least was produced by PAN 25 (23 ± 0.1mm) (**Figure 5**). On day 6, the zones of inhibition varied from 25 ± 0.2mm (isolate PAN 25) to 35 ± 0.3mm (PAN 75). However, on day 7 the zones of inhibition ranged from 27 ± 0.2mm in metabolites from isolate PAN 25 to 37 ± 0.1mm in PAN 75. Conversely, on day 8, the biggest zone of inhibition was produced by metabolites from PAN 75 (35 ± 0.1mm) and the smallest by PAN 25 (26 ± 0.3mm). On the other hand, on day 9 metabolites from isolate PAN 75 presented the largest zone of inhibition (33 ± 0.2mm) compared to PAN 25 which gave the smallest (23 ± 0.3mm). In addition, on day 10 the zones of inhibition ranged from 20 ± 0.1mm in metabolites from isolate PAN 25 to 32 ± 0.3mm in PAN 75. Antibiotic production by the actinomycete isolates grown on Luria Bertani medium and incubated at 28^{o}C reached its peak on day 7 and declined with further incubation of the cultures.

3.3.5 Aeration rate

The aeration rate achieved by agitation of the cultures affected production of antibiotic metabolites significantly (F = 22.11, P = 0.005) among isolate PAN 25, 41, 75 and 110. At 100 rotations per minute (rpm), the zones of inhibition produced by the metabolites varied from 23 ± 0.1mm in metabolites from isolate PAN 25 to 32 ± 0.3mm in PAN 75 (**Figure 6**). When shaken at 150 rpm, the biggest zone of inhibition was 37 ± 0.2mm in metabolites from isolate PAN 75 while the smallest was 24 ± 0.2mm observed in PAN 25. Metabolites from isolate PAN 75 presented the biggest zone of inhibition (39 ± 0.1mm) as compared to 26 ± 0.2mm presented by PAN 25 at 200 rpm. However, at 250 rpm, the zones of inhibition ranged from 23 ± 0.3mm in metabolites from isolate PAN 25 to 37 ± 0.2mm in PAN 75. At 300 rpm, the zones of inhibition varied from 22 ± 0.2mm in metabolites from PAN 25 to 35 ± 0.2mm in PAN 75. In addition, at 350 rpm, the zones of inhibition ranged from 20 ± 0.1mm in metabolites from isolate PAN 25 to 31 ± 3mm in PAN 75. Antibiotic production by the actinomycete isolates grown on Luria Bertani medium and incubated at 28^{o}C increased in aeration rate up to 200 rpm after which it declined.

3.3.6 Inoculum concentration

Inoculum size affected production of antibiotic metabolites from isolate PAN 25, 41, and 110 significantly (F = 4.31, P = 0.02). The zones of inhibition produced by antibiotic metabolites from cultures inoculated with 0.5% starter inoculum ranged from 23 ± 0.1mm in metabolites from isolate PAN 25 to 33 ± 0.2mm in PAN 75 (**Figure 7**). At a concentration of 1.0% of starter inoculum, the zones of inhibition varied from 27 ± 0.2mm in metabolites from isolate PAN 25 to 36 ± 0.1mm in PAN 75. However, at 1.5% starter inoculum concentration, the zone of inhibition ranged from 25 ± 0.2mm (isolate PAN 25) to 32 ± 0.3mm (isolate PAN 75). At 2.0% starter inoculum concentration, the biggest zone of inhibition was given by metabolites from PAN 75 (30 ± 0.2mm) while the smallest was presented by PAN 25 (23 ± 0.1mm). However, at 2.5% starter inoculum, the zones of inhibition varied from 20 ± 0.3mm in metabolites from isolate PAN 25 to 27 ± 0.3mm in PAN 75. Antibiotic production by the actinomycete isolates grown on Luria Bertani medium and incubated at 28^{o}C reached its peak at a concentration of 1% and declined with increase in inoculum concentration.

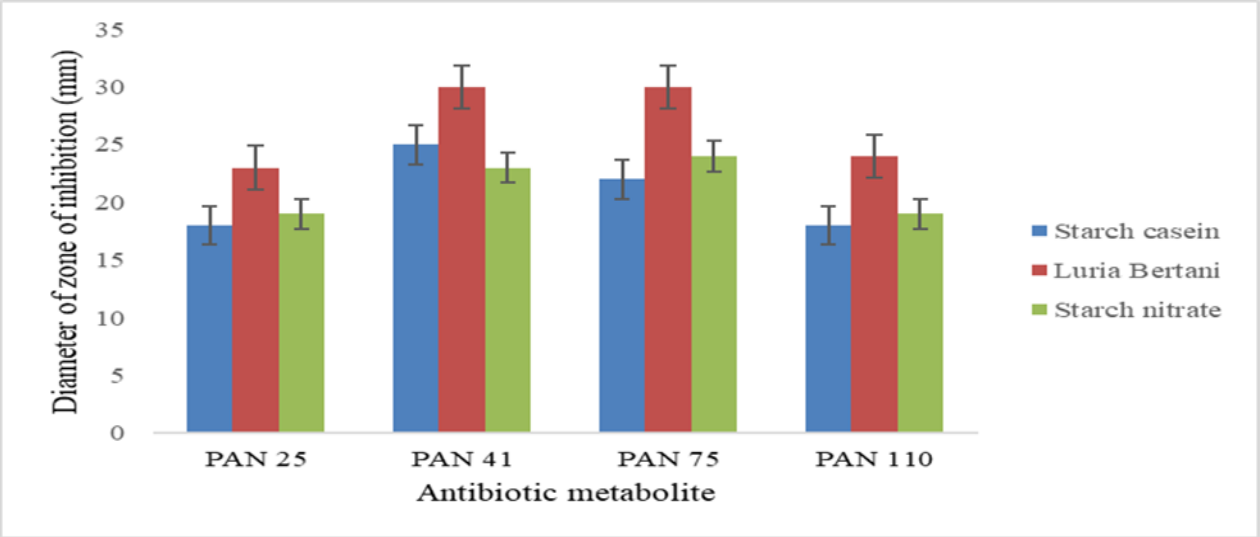

Figure 2 Effect of various culture media on the production of antibiotic metabolites by isolates PAN 25, 41, 75 and 110.

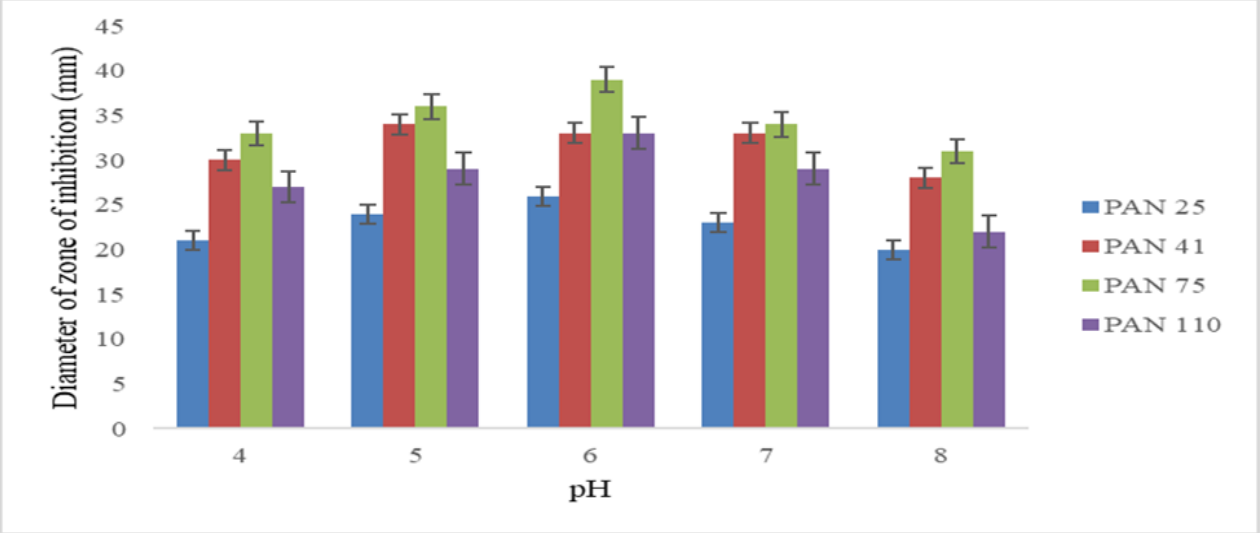

Figure 3 Effect of pH on the production of antibiotic metabolites by isolates PAN 25, 41, 75 and 110.

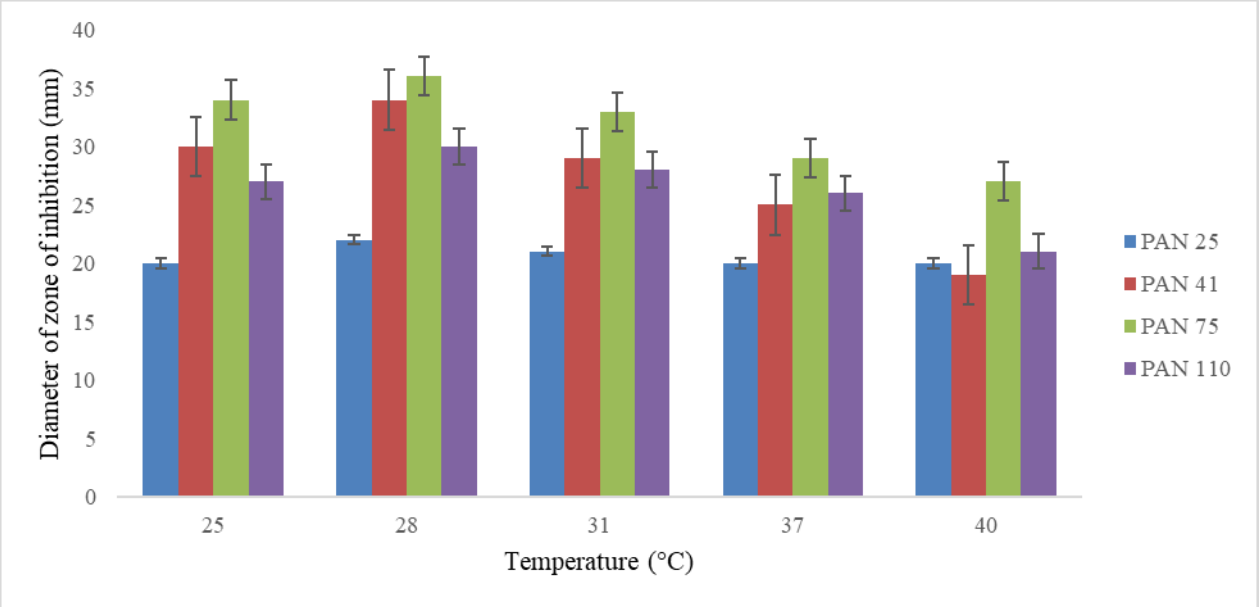

Figure 4 Effect of incubation temperature on the production of antibiotic metabolites by isolates PAN 25, 41, 75 and 110.

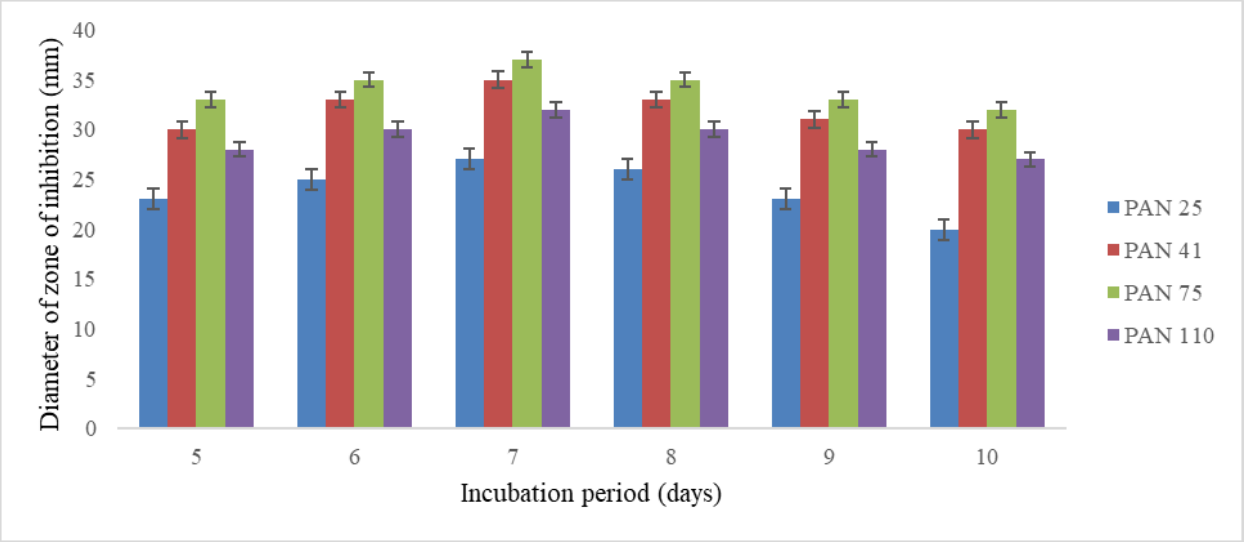

Figure 5: Effect of incubation period on the production of antibiotic metabolites by isolates PAN 25, 41, 75 and 110.

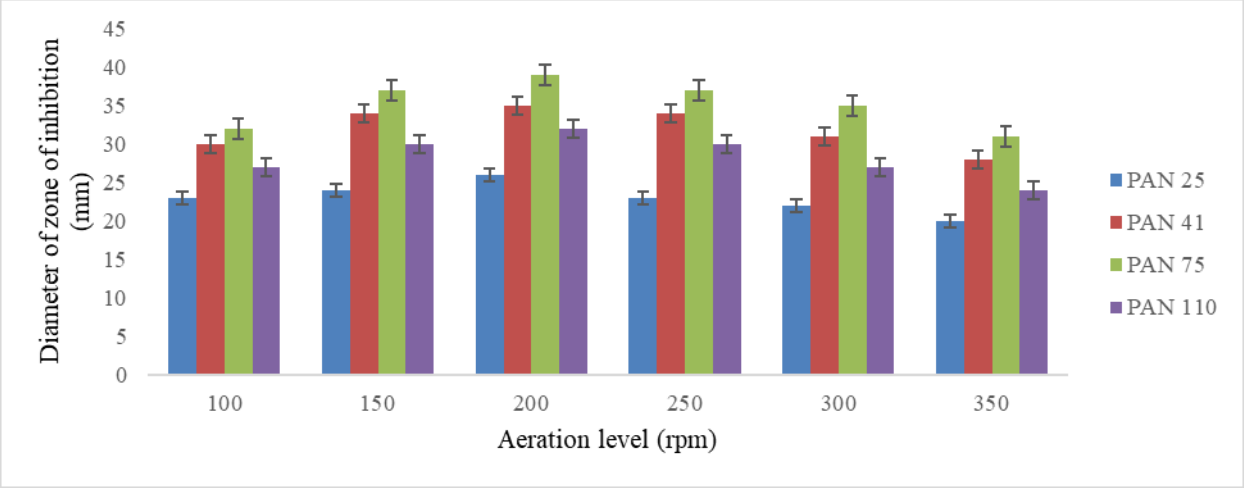

Figure 6: Effect of aeration rate on the production of antibiotic metabolites by isolates PAN 25, 41, 75 and 110.

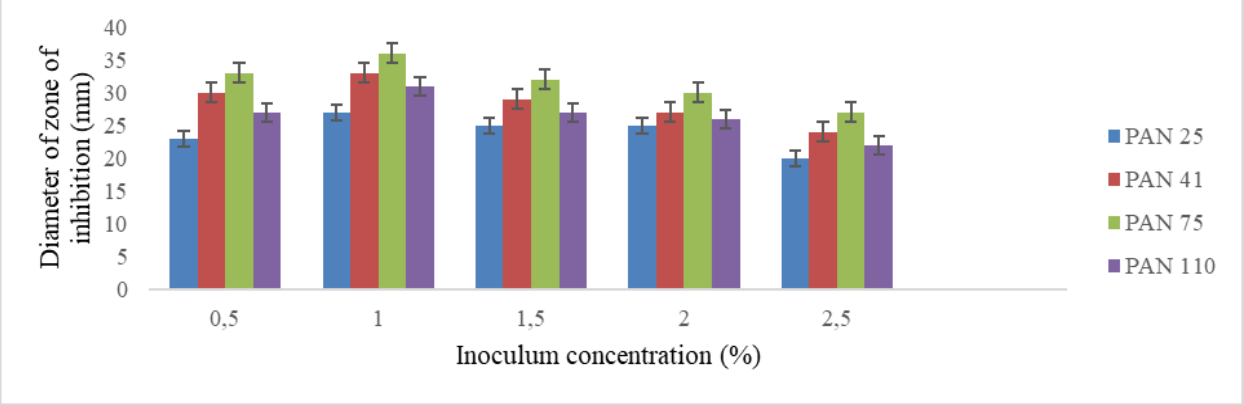

Figure 7: Effect of inoculum size on the production of antibiotic metabolites by isolates PAN 25, 41, 75 and 110.

3.3.7 Carbon source

The carbon source affected production of antibiotic metabolites significantly (F = 5.65, P = 0.0078). The zones of inhibition by the metabolites when glucose was used as the main carbon source in the culture medium varied from isolate PAN 25 (19 ± 0.1mm) to PAN 75 (30 ± 0.3mm) (**Table 2**). When sucrose was used as the main carbon source, the biggest zone of inhibition was given by metabolites from isolate PAN 41 (33 ± 0.2mm) while the smallest was presented by PAN 25 (21 ± 0.2mm). Conversely, when maltose was used, the biggest zone of inhibition was produced by metabolites from isolate PAN 41 (35 ± 0.1mm) and the smallest by PAN 25 (24 ± 0.3mm). In addition, the largest zone of inhibition when glycerol was used as the main carbon source was observed in metabolites from isolate PAN 75 (37 ± 0.1mm) while the smallest was presented by PAN 25 (26 ± 0.2mm). However, when starch was used as the main carbon source, the zones of inhibition ranged from metabolites from isolate PAN 25 (25 ± 0.2mm) to PAN 75 (35 ± 0.3mm).

Among the carbon sources, glycerol was the most ideal for growth and production of antibiotics by the four actinomycetes isolates.

Table 2 Effect of carbon source on the production of antibiotic metabolites by the selected actinomycetes when tested against *S. aureus* (ATCC 25923).

Carbon source	Diameter zone of inhibition (mm)			
	PAN 25	PAN 41	PAN 75	PAN 110
Glucose	19±0.1	20±0.2	30±0.3	23±0.3
Sucrose	21±0.2	33±0.2	32±0.2	26±0.2
Maltose	24±0.3	35±0.1	34±0.2	30±0.2
Glycerol	26±0.2	36±0.2	37±0.1	33±0.1
Starch	25±0.2	34±0.1	35±0.3	31±0.2

Each value represents the mean value (±SD) of five independent replicates.

3.3.8 Nitrogen source

Nitrogen source affected production of antibiotic metabolites by the actinomycete cultures significantly (F = 8.18, P = 0.0016). When malt extract was used as the main nitrogen source, the zones of inhibition varied from 24 ± 0.1mm in metabolites from isolate PAN 25 to 30 ± 0.1mm in PAN 41 (**Table 3**). In addition, when yeast extract was used, the zones of inhibition ranged from 25 ± 0.2mm in metabolites from isolate PAN 25 to 33 ± 0.2mm in PAN 41. However, when Oat meal was used, the zones of inhibition ranged from 27 ± 0.2mm in metabolites from isolate PAN 25 to 34 ± 0.2mm in PAN 41. When corn meal was used as the main nitrogen source, the biggest zone of inhibition was 32 ± 0.3mm in metabolites from isolate PAN 41 while the smallest was 26 ± 0.3mm in PAN 25. On the other hand, when peptone was used, the zones of inhibition varied from 23 ± 0.2mm in metabolites from PAN 25 to 30 ± 0.2mm in PAN 41. Among the nitrogen sources, oat meal was the most ideal for growth and production of antibiotics by the four actinomycetes isolates.

Table 3 Effect of nitrogen source on the production of antibiotic metabolites by the selected actinomycetes when tested against *S. aureus* (ATCC 25923).

Nitrogen source	Diameter of Zone of inhibition (mm)			
	PAN 25	PAN 41	PAN 75	PAN 110
Malt extract	24±0.1	30±0.1	29±0.2	28±0.2
Yeast Extract	25±0.2	33±0.2	32±0.1	31±0.2
Oat meal	27±0.2	34±0.2	33±0.1	33±0.1
Corn meal	26±0.3	32±0.3	30±0.3	30±0.2
Peptone	23±0.2	30±0.2	27±0.2	25±0.3

Each value represents the mean value (±SD) of five independent replicates.

3.3.9 Salt concentration

The levels of salt concentration affected antibiotic metabolite production significantly (F = 13.41, P = 0.00012). At a salt concentration of 0.5%, the zones of inhibition produced by the metabolites varied from (20 ± 0.2mm) in metabolites from isolate PAN 25 to PAN 75 (31 ± 0.3mm) (**Figure 8**). However, at a concentration of 1.0%, the zone of inhibition was highest in metabolites from isolate PAN 75 (35 ± 0.2mm) and lowest in PAN 25 (22 ± 0.1mm). At a concentration of 1.5%, the zones of inhibition varied from metabolites from isolate PAN (25 ± 0.2mm) to PAN 75 (37 ± 0.1mm). In addition, at 2.0%, the zones of inhibition ranged from metabolites from isolate PAN 25 (23 ± 0.3mm) to PAN 75 (35 ± 0.1mm). At a salt concentration of 2.5% the biggest zone of inhibition was observed in metabolites from isolate PAN 75 (30 ± 0.2mm) and the smallest in PAN 25 (21 ± 0.2mm). Antibiotic production by the actinomycete isolates grown on Luria Bertani medium and incubated at 28°C reached its peak at a salt concentration of 1.5% and declined with increase in inoculum concentration.

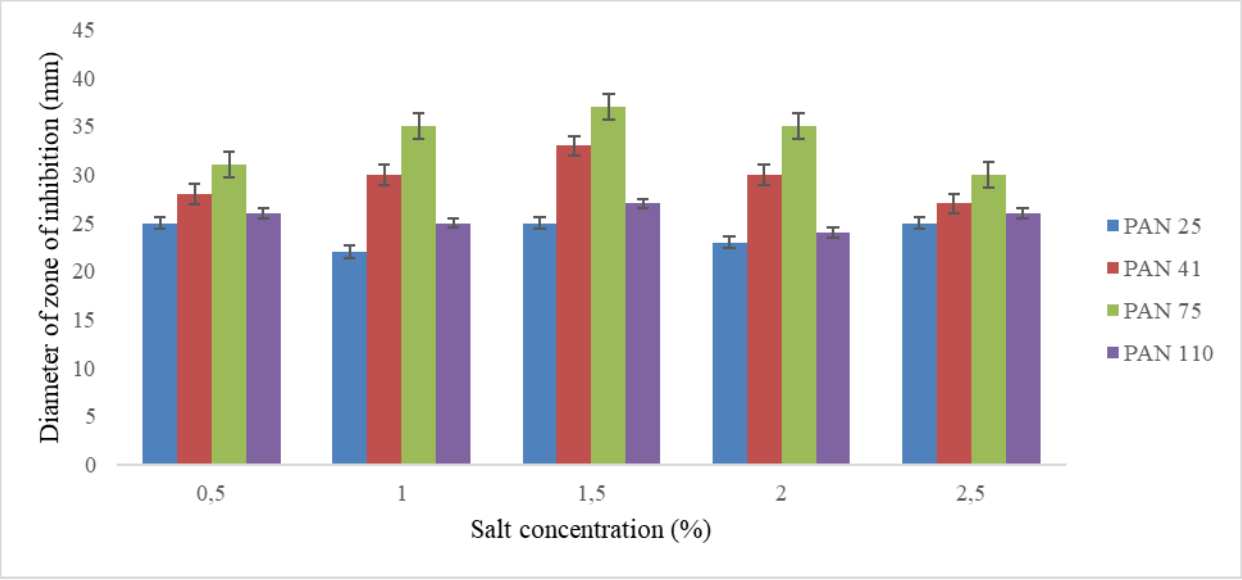

Figure 8 Effect of salt concentration on the production of antibiotic metabolites by the selected actinomycetes.

4. Discussion

In early stages of studying actinomycetes, morphological characteristics were considered adequate in characterizing actinomycetes **(Messaoudi *et al.*, 2015)**. Generally, actinomycetes have leathery or chalky appearance which is usually folded, branching, filamentous and bearing aerial mycelia **(Cockell *et al.*, 2016)**. In this study, a total of 138 actinomycete cultures were isolated from the soils of Menengai crater. The isolates had different morphological characteristics which differed with a study carried by **Fu *et al.* (2015)** on metagenomic analysis of bacterial community composition among the volcanic cave sediments of Indo-Burman biodiversity hotspot region. The variations in morphological characteristics of the isolates in the two studies may be attributed to the differences in diversity of actinomycetes recovered from the soils **(El-bondkly *et al.*, 2017)**.

In the current study, ethyl acetate was used in extraction of the antibiotic metabolites from the selected actinomycetes. The extracts produced zones of inhibition that varied from isolate PAN 25 (24.67 ± 0.2) to PAN 75 (41.83 ± 0.2) when tested against the test microorganisms. Ethyl acetate which was used as control

produced no zones of inhibition. These zones were bigger than those reported from other regions **(Kadiri and Yarla, 2015; Saker *et al*., 2015; Floros *et al*., 2016)**. According to **Meeta and Ekta (2015)**, different actinomycetes yield different types of antibiotics. Further, **Wink and Mohammadipanah (2015)** asserted that the environment in which actinomycetes are growing in to a great extent, influences the metabolic activities of the organisms thus influencing the types of antibiotics produced.

In the current study, several culture media were compared for their suitability in the production of antibiotic metabolites by the selected actinomycetes. Luria Bertani (M1) was the best medium for production of antibiotic metabolites by the selected actinomycetes. **Jami *et al.* (2015)** noted that growth media influences production of antimicrobial metabolites in actinomycetes. **Anuprita *et al.* (2015)** studied the use of different media in production of antibiotic metabolites by actinomycetes from hyper-saline soils of Vidarbha region in India. The study established that starch casein broth optimized production of antibiotic metabolites. The findings of this study differed with those of the present study. The possible reason may be variation in the composition of the medium used during fermentation coupled with differences in nutrient requirements among actinomycetes **(Bi *et al*., 2017)**.

In this study, production of antibiotic metabolites increased with increase in pH reaching a point of maximum production at a pH of 6. **Arocha-Garza *et al.* (2018)** studied the effect of pH on production of antibiotic metabolites by actinomycetes obtained from an extremely oligotrophic desert oasis using starch casein nitrate broth with pH maintained at 4, 5, 6, 7, 8 and 9 and obtained an optimum pH of 7. The variations in results may be attributed to differences in pH of the soils from which the actinomycetes were isolated **(Niu *et al*., 2016)**.

Luria Bertani (M1) was inoculated with the selected actinomycetes and incubated at 25^{o}C, 28^{o}C, 31^{o}C, 37^{o}C and 40^{o}C. The selected actinomycetes strains in the current study produced maximum levels of antibiotic metabolites at a temperature of 28^{o}C. Higher or lower temperatures led to decreased production of antibiotic metabolites. **Masad *et al*. (2018)** explained that low temperatures below the optimum or higher temperatures above the optimum leads to inactivation or denaturation of enzymes that are responsible of biosynthesis of antibiotic metabolites leading to reduced production of the metabolites. **Andam *et al*. (2016)** optimized the cultural conditions of *Streptomyces* isolated from super saline environments of Philippines in varying incubation temperatures such as 15^{o}C, 28^{o}C, 37^{o}C and 50^{o}C and obtained an optimum temperature for production of antibiotics as 28^{o}C. The similarity in temperature requirements between the previous and current study can be attributed to similarity in the soils physico-chemical conditions of the study sites which led to the actinomycetes adjusting their metabolic pathways to favour growth in the environment **(Gobalakrishnan *et al*., 2017)**. Further, **Orooba *et al*., (2017)** asserted that temperature has an effect on physiology, biochemistry and metabolites production by actinomycetes.

Highest antibiotic metabolites production occurred on the 7th day in the present study as indicated by the sizes of the inhibition zones. Further increase in incubation period reduced the zones of inhibition. **Fu *et al.* (2015)** indicated that actinomycetes isolates coded SMI-04 and SMI-10 required an incubation time of 4-5 days to optimally produce antibiotic metabolites. This differed with the findings of the current study. The difference in incubation time may have resulted from variations in nutrient requirements of the isolates. **Ravi and Vasantba (2017)** maintained that with increased incubation period beyond the point of optimum metabolites production, bioactivity of actinomycetes reduces due to exhaustion of nutrients availability in the culture medium and accumulation of toxic metabolites which inhibit growth of the cultures.

Aeration speed affects oxygen supply and contact time of actinomycetes with the media **(Suseela and Tabitha 2017)**. In this study, aeration by agitation directly affected the growth of actinomycetes and therefore production of antibiotic metabolites. The maximum production of antibiotic metabolites occurred at 200 rotations per minute (rpm). **Chen *et al.* (2016)** reported that an agitation of 150 rpm was most suitable for production of antibiotic metabolites when studying the effects of aeration by agitation on antibiotics production from actinomycetes isolated from South West Indian ridge. Differences in antibiotic metabolites production based on agitation are attributed to variations in oxygen and nutrient requirements among actinomycetes **(Trabelsi *et al*., 2016)**.

The effect of inoculum concentration on production of antibiotic metabolites was studied by inoculating M1 broth with varying concentration of the selected actinomycetes at 0.5%, 1.0%, 1.5%, 2.0% and 2.5%. An inoculum concentration of 1% was the best for optimum production of antibiotic metabolites. **Jami *et al.* (2015)** obtained an inoculum concentration of 2% for optimum production of antibiotic metabolites which differed with the results of this study. The concentration of the inoculum determines the duration of the actinomycetes get to lag phase before the cultures enters the log phase and thus in turn affects production of antibiotic **(Wurch *et al*., 2016)**. In addition, an inoculum concentration exceeding the optimum concentration level leads to reduced antibiotic production due to nutrients been directed towards formation of biomass rather than production of metabolites **(Qais *et al*., 2016)**.

In testing the effect of carbon source on production of antibiotic metabolites, different carbon sources such as glucose, sucrose, maltose, glycerol and starch were used in this study. The results demonstrated that glycerol was the best carbon source for production of antibiotics in the selected actinomycetes. These findings differed with those of a previous study carried out by **Priyambada and Roymon (2017)** where starch was found to be the most suitable carbon source. A possible reason for the difference may be variations in carbon source requirements between the isolates in the present and the previous study **(Waheeda and Shyam 2017)**. The preference of glycerol by the actinomycetes isolates studied may be explained by their ability to break the building blocks in glycerol **(Semra and Mustafa, 2017)**.

In this study, malt extract, yeast extract, oat meal, corn meal and and peptone were used to study nitrogen source requirements of the selected actinomycetes. The results showed that oat meal was the best nitrogen source for production of antibiotic metabolites. In a similar study carried out by **Ser *et al.* (2017)**, soybean was found to be the best nitrogen source for production of antibiotic metabolites while yeast and corn steep solids encouraged cellular growth of actinomycetes but not production of antibiotics. Further, **Sengupta *et al*. (2015)** showed that peptone was the best nitrogen source for production of antibiotic metabolites. This was contrary to the findings of this study. The possible reason for the difference could be variations in metabolic pathways followed by the actinomycetes when metabolizing the various nitrogen sources **(Singh *et al*., 2016)**. In actinomycetes, nitrogen affects their rate of growth and therefore indirectly determines production of antibiotic metabolites **(Tao *et al*., 2015)**.

In the current study, a salt concentration of 1.5% favoured optimum production of antibiotic metabolites by the selected actinomycetes. According to **Waheeda and Shyam (2017)**, salt concentration affects production of antibiotic metabolites due to its effects on osmotic pressure of the medium and the actinomycetes inoculum. **Usman *et al*. (2016)** found optimum concentration of NaCl to be 1%, which differed with the results

of the present study. The observed variations in the amount of NaCl required for optimal production of antibiotic metabolites between the previous and the current study may be attributed to differences in growth requirements in actinomycetes especially the ability to survive in saline environments **(Waithaka et al., 2017)**.

5. Conclusion

The soils of Menengai crater have actinomycetes some of which produce antibiotics. The growth and production of antibiotics by the selected actinomycetes is affected by culture medium, pH, incubation temperature, incubation period, aeration rate, inoculum concentration, carbon source, nitrogen source and a salt concentration. There is need to curry out structure elucidation of the antibiotics from the selected actinomycetes. In addition, similar studies from other harsh environments need to be carried out.

Conflict of Interest

The authors declare that they had no competing interests.

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