Screening of O-7 Isolate Actinomycete Producing Antimicrobials in Different Growth Conditions against Selected Pathogens

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ABSTRACT

Actinomycetes are the main microbial population in soil generating active secondary metabolites. However, the objectives of this research were to isolate, select a promising strain and improve the antibiotic production of actinomycetes. A prospective analytical study of several actinomycetes was performed from soil samples collected from different locations in Khartoum, Sudan. The crowded plate technique was used to isolate actinomycetes using different media. The strains were evaluated for their antimicrobial activity against certain Gram-positive, Gram-negative bacteria, yeasts, and fungi. Varied fermentation conditions such as temperature, pH, and light, agitation, aeration, and fermentation period have also been configured for the maximum production of antibiotics. From 62 isolates, 18% were active against at least one of the test organisms: *Bacillus subtilis* (ATCC 10400), *Staphylococcus aureus* (ATCC 289213), *Escherichia coli* (ATCC 13706), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (ATCC 630), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella typhimurium* (ATCC 13311), *Aspergillus niger* (ATCC 16404), and *Candida albicans* (ATCC 10231). The strain (O-7) showed a very broader spectrum than other isolates. For the maximum production of antibiotics, suitable fermentation conditions were found to be as follows: Temperature 28°C, pH 7.0, Agitation 180 rpm, and fermentation duration 96 hours. It can be concluded that antimicrobial compounds formed by (O-7) isolated from the soil in Khartoum were efficient. The antibiotic generated exhibited the highest activity against various pathogens.

Key Words: *Actinomycetes, Antimicrobial activity, Isolation, Soil*

eIJPPR 2021; 11(2):13-23

HOW TO CITE THIS ARTICLE: Ibnouf EO. Screening of O-7 Isolate Actinomycete Producing Antimicrobials in Different Growth Conditions against Selected Pathogens. Int J Pharm Phytopharmacol Res. 2021;11(2):13-23. https://doi.org/10.51847/WaOnDShxEP

INTRODUCTION

Actinomycetes are Gram-positive bacteria found in soil, industrially suitable as sources of a wide variety of secondary metabolites, including several antibiotics of medical and commercial significance [1]. Antibiotic-resistant pathogens such as methicillin and vancomycin-resistant strains of Staphylococcus aureus (S. aureus) and others pose a significant risk to the treatment of severe infections [2, 3]. To prevent this, an urgent replacement of the existing antibiotic is required, and the development of new drugs against drug-resistant pathogens is considerable for today [4].

A significant number of commonly used antibiotics, like erythromycin, streptomycin, rapamycin, and gentamycin, are all substances isolated from soil Actinomycetes. Streptomyces and Micromonospora are the two major classes of soil actinomycetes that act as the primary producers of antibiotics. It was reported that Streptomyces responsible for around 80% of the total antibiotic products, whereas Micromonospora closely follows Streptomyces constituted below than one-tenth of the total antibiotic products [5].

Various strains of actinomycetes normally produce many products that assist to improve the isolation and screening of new strains to discover new products [6]. Several studies are currently underway on antibacterial products produced from actinomycetes that are efficient against several types of antimicrobial-resistant bacteria [7].

The purpose of research is to profit from the availability of different types of soil in Sudan, enabling the growth of several species of antibiotic-producing actinomycetes. The

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Received: 03 February 2021; **Revised:** 06 April 2021; **Accepted:** 07 April 2021

development of antibiotics from isolated microbes locally adds to the cost of importing these medications from abroad and helps in the development and promotion of the local industry and export market. This research goal to isolate, select the promising strain, and improving antibiotic production of actinomycetes isolated from Khartoum state soil.

MATERIALS AND METHODS

This was a prospective analytical analysis of many actinomycetes. Samples were isolated from soils collected from different locations in Khartoum state. The crowded plate method was utilized to separate actinomycetes using a variety of media. The isolates were tested for antimicrobial effectiveness against multiple Gram-positive, Gram-negative, yeast, and fungi bacteria. Numerous fermentation factors like temperature, pH, light, agitation, aeration, and fermentation period have also been enhanced for optimal antimicrobial production.

Soil samples collection

Soil samples were taken from multiple places in Khartoum, using clean, dry, and sterile polythene bags, bands, pens, and other accessories. These samples were air-dried for one week, and used for isolation of Actinomycetes [8].

Isolation of actinomycetes

One gram of soil was dissolved in 9 ml of sterile distilled water. The dilution was completed up to 10-5 dilutions. Aliquots (0.1 ml) of 10-2, 10-3, 10-4, and 10-5 were distributed to starch casein agar (SCA). Based on colonial morphology, the culture of actinomycetes was chosen and purified by the International Streptomyces Project [9].

The actinomycete colonies isolated from the crowded plate were selected for further study **(Figure 1)**, which were named as B (30 strains), D (8 strains), K (15 strains), NS (2 strains), and O (7 strains).

Standard strains

The following reference bacterial cultures have been taken from the Department of Pharmaceutics, College of Pharmacy, University of Khartoum, Sudan: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 10400), *Escherichia coli* (ATCC 13706), *Klebsiella pneumoniae* (ATCC 10031), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella typhimurium* (ATCC 13311), *Aspergillus niger* (ATCC 16404), and *Candida albicans* (ATCC 10231).

Screening for antibiotic production

The antimicrobial activity of soil actinomycete isolates was assessed using the agar streak method [10].

Characterization of the isolates

Biochemical characteristics such as melanin pigment, nitrate reduction, H_2S production, citrate utilization, milk coagulation, and the use of carbon sources have been evaluated using standard methods. Cultural characteristics such as aerial color and mycelium substrate have been studied in different media following the guidelines established by the ISP [11].

Morphological characterization

Cultural characterization

Selected actinomycete isolates were studied by inoculation in different sterile media: carbon utilization agar (ISP-9), Czapek's sucrose agar, glycerol-arginine medium (ISP-5), glucose yeast extract agar, glucose agar, starch agar, and starch casein medium.

Microscopic characterization

The direct method, slide culture method, and Gram-staining method have been used [12]. The isolates showed the properties of actinomycetes. Morphological characteristics were determined as proposed in Bergey's manual of determinative bacteriology and biochemical tests were performed [13].

Fermentation process for antibiotic production

It is well established that glucose and other favorable carbon sources suppress morphological and chemical differentiation of Streptomyces, and similar observations have been made concerning nitrogen [14]. The isolates were cultivated in a variety of media. The culture filtrates were harvested, and the antibacterial activity was evaluated by agar well diffusion assay [6].

Medium complexity

Various sources of nitrogen, such as yeast extract, ammonium chloride, and peptone, have been tested for maximum antibiotic productivity [15]. Different concentrations of glucose and lactose as a carbon source have been used in the basal medium.

Suitable parameters for fermentation

The pH values of the medium containing 4% glucose, 1% peptone, 0.3% yeast extract, 0.35% $CaCO_3$, and 0.5% Na Cl have been adjusted by (NaOH) and (HCl). The optimal temperature for the productivity and growth of the strains was determined by keeping the inoculated fermentation media at 27°C, 28°C, 29°C, 30°C, 31°C, 37°C, and 38°C for 4 days in a shaker incubator [16]. Subsequently, the growth of microorganisms and productivity of an antimicrobial activity was evaluated using the cup-plate method [17, 18]. Two groups of conical flasks containing media inoculated with the selected strain and incubated in a rotary shaker into two ways for studying light effectivity.

The first group was covered with black foil to prevent light, and the second group was left uncovered. In the end, antimicrobial activity was determined. Three different sizes of conical flasks were used (250, 500, and 750 ml). Each contained 150 ml of fermentation medium and was inoculated with the selected isolate. The antimicrobial activity was determined after the incubation period. The incubation of the seeded flasks took place in two ways. The first group was incubated in a shaker, and the second group was left at room temperature on the bench. The antimicrobial activity was then determined.

The duration of the fermentation process has been optimized to obtain the maximum antibiotic production time for harvesting [17, 19]. Using the above-mentioned media formulation and fermentation parameter conditions, the media were inoculated with the selected strain and incubated for up to 144 hours. The harvesting time of the maximum antibiotic productivity was determined and the antimicrobial activity was also determined using the cup-plate method [20, 21].

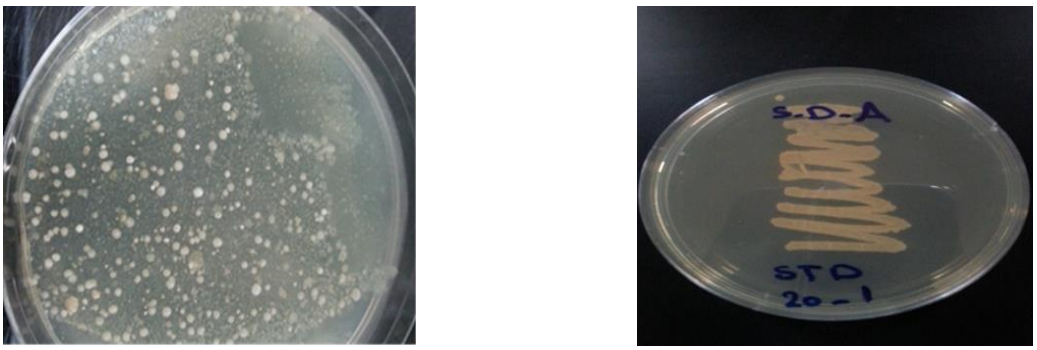
Statistical analysis

The statistical methods used in this study include Descriptive statistics (mean, standard deviation, maximum, minimum, Range, and graphs), a T-test for independent samples, and Two Way (ANOVA) to compare the significant difference between levels. Statistical software (SPSS, version 20) has been used in this study.

RESULTS AND DISCUSSION

Cultural and microscopic characterization of the isolates

The cultural **(Figures 1a and 1b)**, and microscopic **(Figure 2)** characteristics of the isolates confirmed that the isolates were belonging to the genus Actinomycetes.



a) b)

Figure 1. a) Crowded plate technique, and b) cultural characterization

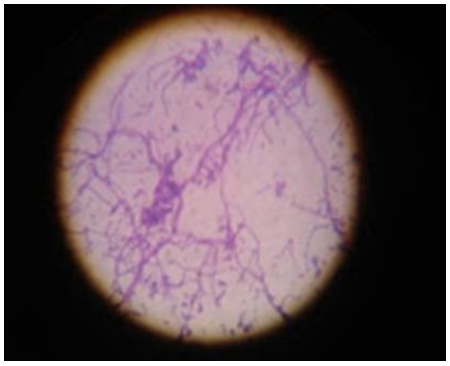


Figure 2. Microscopic characterization

Antimicrobial activity of Actinomycetes

Isolated actinomycete strains were tested for anti-microbial activity against seven bacteria, one fungal, and one yeast strain by the agar streak method. Out of sixty-two actinomycetes screened, eleven strains showed significant antimicrobial activity against Gram-positive and/or Gram-negative organisms **(Table 1)**. Out of the eleven strains, six actinomycetes were selected for taxonomic characterization using an antimicrobial activity test. However, the strain (O-7) showed a very broad spectrum with higher scores than all other strains.

Table 1. Activity of isolates against different organisms

Soil isolates	*S.aureus* ATCC 29213	*B.subtilis*ATCC 10400	*E.coli* ATCC 13706	*K.pneumoniae* ATCC 10031	*P.aeruginosa*ATCC 15442	*P.vulgaris* ATCC 6380	*S.typhimurium* ATCC 13311	*A.niger* ATCC 16404	*C.albicans* ATCC10231
B-5	++	+	+	-	-	+	-	++	+
B-8	+++	+	+	++	-	++	+	+	+
B-29	-	++	-	+	+	+	-	++	-

D-1	++	+	+	-	-	+	-	+++	+
D-3	+++	++	++	+	+	+	-	++	++
D-5	-	-	+	+	++	-	+	+	+
D-6	++	+	++	++	-	-	++	+	+++
K-3	+	+	-	-	-	++	-	++	+
K-12	-	-	+	+	-	-	+	++	+
K-15	+	+	-	-	+	+	+	+++	++
O-7	+++	+++	-	++	+	++	-	+++	+++

(+++) = wide Inhibition, (++) = Medium Inhibition, (+) = Low Inhibition, (-) = No Inhibition

Taxonomic characterization

Biochemical tests were carried out for the six selected isolates **(Table 2)**. The strain (O-7) showed positive results in gelatin liquefaction, nitrate reduction, pigment production, acid production, indole, proteolytic activity, methyl red, and starch hydrolysis tests.

Table 2. Biochemical tests of soil isolates

Soil Isolates	Starch Hydrolysis	Proteolytic Activity	Melanoid Formation	Acid Production	Nitrate Reduction	Gelatin Liquefaction	Carbohydrate Assimilation	H2S Production	Citrate Utilization	MR	Indo-le
B-5	-	+	-	+	+	+	-	-	-	+	+
B-8	-	-	+	+	+	+	+	-	-	+	+
D-1	+	-	-	+	+	+	-	-	-	+	-
D-3	+	+	+	+	+	+	-	-	-	+	+
D-6	+	+	+	+	+	+	-	+	-	-	+
O-7	+	+	+	+	+	+	+	-	-	+	+

(+) Positive Reaction, (-) Negative Reaction

Morphological and cultural characterization of (O-7) strain

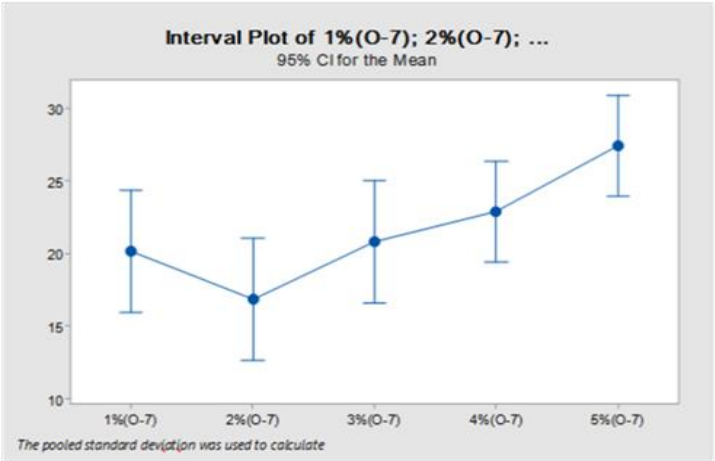
The cultural and morphological characteristics of the strain (O-7) were studied in different media suitable for actinomycetes. Microscopically, the observations revealed that the strain (O-7) is a filamentous Gram-positive microorganism. By studying morphology, cultural and taxonomic characteristics, it has been confirmed that the strain (O-7) belongs to the genus actinomycete.

Fermentation process for antibiotic production

Medium formulation

Carbon source

Based on the results presented in **Figures 3a and 3b**, the results showed that monosaccharide, glucose, was the best among the different sugars. It has been observed that 5% glucose and 3% lactose are optimal for the production of antibiotics for the (O-7) strain.



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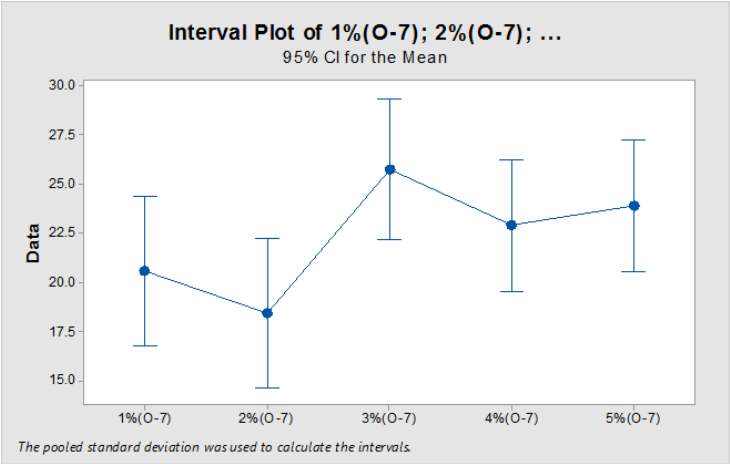


b)

Figure 3. Influence of Glucose (a), and Lactose concentration (b) on antibiotic production

Nitrogen source

By determining the influence of nitrogen sources on the inhibition zone, peptone at a concentration of 1% was found to be a good source of nitrogen for the (O-7) isolate **(Table 3)**.

Table 3. Mean, standard deviation, and Two Way ANOVA for nitrogen source (1% each)

	Factor	Zone of inhibition (mm)		Statistical significance					
		Variables (1%)	Mean±Sd (min-max)	Source	Sum of squares	Df	Mean squares	F	P-value
Lactose concentration (%)	(O-7) strain	Ammonium chloride	22.3±5.3 (12-30)	Between the group	52.6	2	26.30	0.94	0.40
		Peptone	24.8±5.7 (17-32)						
		Yeast-extract	23.9±4.8 (18-30)	Within the group	321.5	47	28.12		
Diameter of zone of inhibition (mm)	(O-7) strain	*S.aureus*	28.3±1.5 (27-30)	Between the group	570.0	8	71.25	6.29	0.00**
		B.subtilis	27.0±3.5 (23-29)						
		E.coli	23.3±5.7 (17-28)						
		K.pneumoniae	17.3±4.7 (12-21)						
		P.aeruginosa	16.5±0.7 (16-17)	Within the group	169.8	15	11.32		
		P.vulgaris	18.0±1.4 (17-19)						
		Sal.typhimurium	19.0±0.0 (19-19)						
		A.niger	28.3±3.8 (24-31)						
		C.albicans	28.7±0.6 (28-29)						

* P ≤ 0.05 ** P ≤ 0.01

Fermentation parameters

The potential of Hydrogen (pH)

Optimum pH for maximum growth and productivity of (O-7) isolate was measured. From the obtained results, pH 7.0 was found to be the most suitable for the selected isolate **(Table 4)**.

Table 4. Mean, standard deviation, and Two Way ANOVA for (pH)

Factor		Zone of inhibition (mm)		Statistical significance					
		pH	Mean±Sd (min-max)	Source	Sum of squares	Df	Mean squares	F	P-value
pH	(O-7) strain	4.0	14.0±1.8 (12-16)	Between the group	503.5	7	71.93	4.61	0.00**
		4.5	17.0±1.6 (15-19)						
		5.0	18.0±1.8 (15-20)	Within the group	686.2	44	15.60		
		5.5	18.6±2.8 (16-24)						
		6.0	20.3±2.9 (16-25)						
		6.5	22.6±4.7 (17-29)						
		7.0	24.8±6.4 (15-32)						
		7.5	17.4±4.3 (13-26)						
		8.0	15.0±1.0 (14-16)						
		8.5	13.0±0.0 (13-13)						
Diameter of zone of inhibition (mm)	(O-7) strain	*S.aureus*	19.1±6.0 (12-27)	Between the group	185.3	8	23.16	0.96	0.84
		B.subtilis	20.0±4.6 (16-29)						
		E.coli	20.2±4.7 (14-28)						
		K.pneumoniae	16.8±2.8 (13-20)						
		P.aeruginosa	16.5±3.1 (13-20)	Within the group	1184.5	49	24.17		
		P.vulgaris	16.8±2.6 (13-19)						
		Sal.typhimurium	17.0±1.2 (15-18)						
		A.niger	20.4±6.3 (13-32)						
		C.albicans	21.6±6.2 (15-30)						

* P ≤ 0.05 ** P ≤ 0.01

Temperature

The temperature optimization study found that (O-7) showed good growth and maximum antibiotic production at 28°C. **(Table 5)**.

Table 5. Mean, standard deviation, and Two Way ANOVA for Temperature (in ^{0}C)

Factor		Zone of inhibition (mm)		Statistical significance					
		Temperature (in ^{0}C)	Mean±Sd (min-max)	Source	Sum of squares	Df	Mean squares	F	P-value
Temperature (in ^{0}C)	(O-7) strain	20	18.8±2.3 (15-22)	Between the group	291.3	4	72.83	3.90	0.01**
		25	18.9±1.9 (15-20)						

		28	26.3±6.3 (15-33)	Within the group	594.9	31	19.19	
		33	23.5±5.6 (17-31)					
		37	22.4±3.1 (18-27)					
Diameter of zone of inhibition (mm)	(O-7) strain	*S.aureus*	24.2±4.7 (19-30)	Between the group	339.7	8	42.47	2.04	0.08
		B.subtilis	26.2±5.3 (20-33)					
		E.coli	20.8±3.4 (18-25)					
		K.pneumoniae	19.6±2.7 (17-24)					
		P.aeruginosa	17.5±3.5 (15-20)	Within the group	584.2	28	20.86	
		P.vulgaris	20.0±1.2 (19-21)					
		Sal.typhimurium	15.5±0.7 (15-16)					
		A.niger	25.2±6.6 (18-33)					
		C.albicans	22.2±5.7 (15-29)					

* $P \leq 0.05$ ** $P \leq 0.01$

Light

The results indicated that the culture flasks incubated in the light had a high level of activity against the test organisms.

The effect of light on the productivity of (O-7) has been determined **(Figure 4)**.

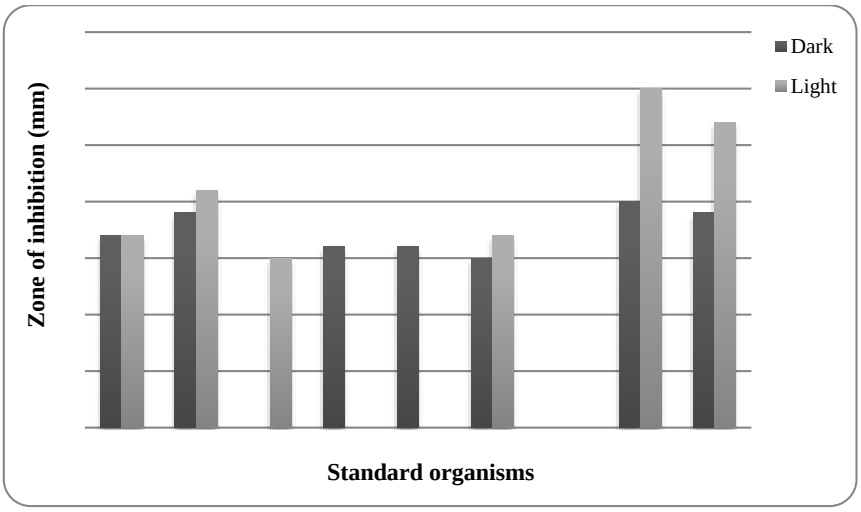
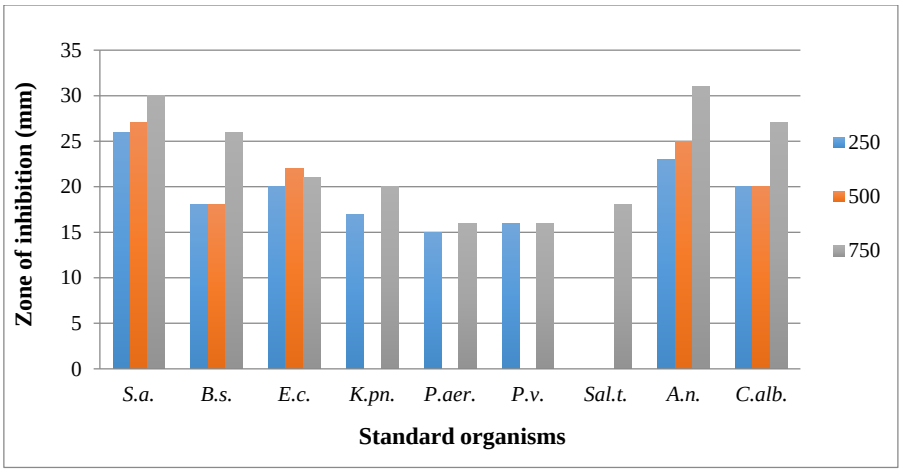


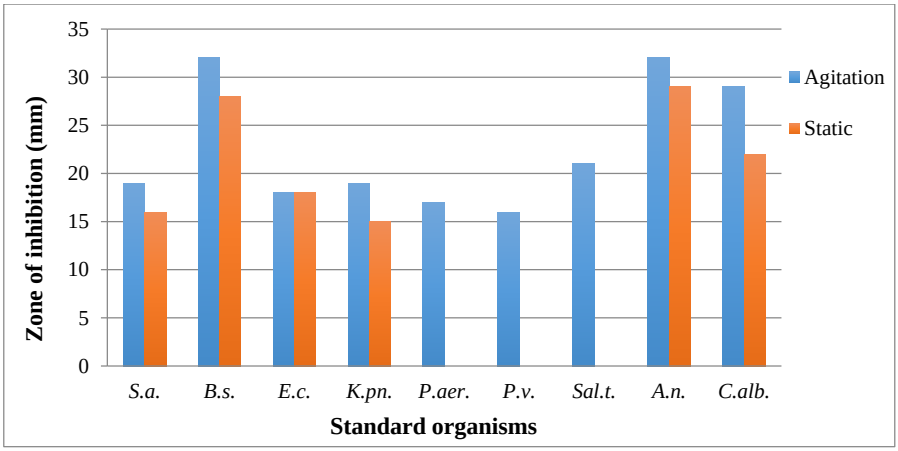
Figure 4. Effect of light on antimicrobial productivity

Aeration and agitation

The effect of aeration and agitation on the productivity of (O-7) was determined in **Figures 5a and 5b**.



a)



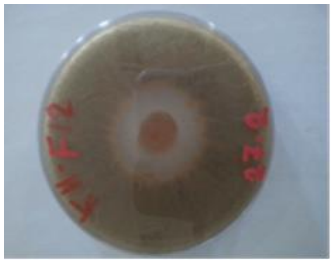
b)

Figure 5. a) Effect of aeration on antimicrobial productivity. b) Effect of agitation on antimicrobial productivity

Duration of fermentation

Processed fermentation batch containing optimized medium formulation and fermentation conditions was studied to determine the maximum duration of fermentation **(Figure 6)**.

a)



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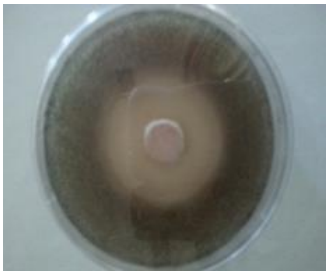

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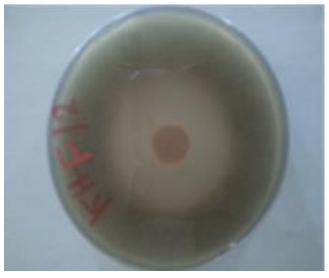

d)

Figure 6. Antimicrobial activity in different fermentation durations (24, 48, 72, and 96 hours) respectively

Based on the above-consolidated results, the fermentation medium and fermentation conditions have been optimized

for the maximum production of antibiotics and are given as follows:

- Condition of fermentation: temperature (28°C), pH (7.0), agitation (180 rpm).
- Duration of fermentation (96 hours).
- Light and air condition.

Actinomycetes are known to be a producer of antibiotics, the most famous of which is Streptomycetes, to which this type (O-7) belongs, which has not been isolated in Sudan before, and antibiotics have not been extracted from it. The extracts of these isolated bacteria were tested on several pathogens after they were grown under different growth conditions and showed high efficacy.

Nutritional sources such as carbon, nitrogen, and minerals, as well as environmental factors, are known to have a profound effect on the production of antibiotics by actinomycetes [22, 23]. In the present study, the effect of different carbon sources on the production of antibiotics by (O-7) strain is presented. Among the carbon sources, glucose has proved to be the best carbon source for both cell growth and strain-producing antimicrobial metabolites. The strain was found to produce high levels of antimicrobial metabolites in the medium supplemented with 5% glucose as the sole carbon source. Actinomycetes strains produced the highest biomass when glucose was added to the medium [21, 24]. In another strain that produces high levels of antimicrobial metabolites in the medium supplemented with glucose (2%) and reported that in the case of Streptomyces species, with regards to carbon sources species-specific variation may occur in cell growth and production of secondary metabolite [25, 26]. It has been reported that the concentration of 3% of glucose was the optimum for maximum metabolite production [13]. On the other hand, this result disagreed with a previous study, which reported the chitin, starch was the appropriate carbon source to increase antimicrobial production [27, 28]. In the present study, there were significant differences between the (O-7) strain and the different glucose concentrations.

The effect of nitrogen sources on metabolite production was detected by fermentation media with the selected isolate (O-7). The results obtained showed that the optimal source of nitrogen for antibiotic production was a peptone for (O-7) isolate. This result was agreed with a previous study, which reported that the highest inhibition zone was recorded after peptone was used as a nitrogen source [29]. In other research, peptone is optimal for the production of bioactive agents [24]. It has been reported that, an inhibition zone as wide as 32 mm and 29 mm against *E. coli* FBFC-1407 and *S. aureus* ATCC-259233, in a medium supplemented with sodium nitrate [30]. In the current study, although the peptone is the best for the (O-7) strain, there were no significant differences in the statistical analysis using different nitrogen sources, but there were very significant differences in the diameter of the inhibition zone.

The role of pH in the production of antibiotics by (O-7) strain showed a high level of activity at neutral pH (7.0). This finding was an agreed study that reported an optimum pH of 7.0 [31, 32]. In another study, the optimum pH of the medium was adjusted to the basic scale of 9.0 for high activity of *Streptomyces albolongus* against *S.* aureus [33]. The environmental requirements and cultural conditions for the growth and production of antimicrobial agents have been studied by (O-7) isolate. The temperature of 28^0C was found to be optimal for the highest growth as well as for the maximum production of the antimicrobial agent by the isolate. There was a very significant difference in the use of different temperatures for (O-7) strain, but there were no significant differences in the diameter of the inhibition zone. The same result with the strain *S. sannanensis* SU 118 has been reported [32]. The optimum temperature for antibiotic production of *Streptomyces sp* was found in other previous studies 45^0C and 35^0C [6, 30, 34].

The use of a wide-bottomed flask and agitated deep culture at a speed of 180 rpm increased the production of antibiotics by (O-7) isolate, particularly with a flask capacity of 750 ml. This result was agreed with a study that found the inhibitory rate reached a peak of about 90% when the speed was 180 rpm [35, 36]. In a previous study, the agitation has shown a direct effect on the growth of Streptomyces sp. KOD1028. There were no significant differences in the use of different sizes of flasks in this study. In other research, agitation at 150 rpm was found to be the most appropriate for Streptomyces spp [37].

In the present research, an incubation period of up to 4 days was found to be optimal for maximum growth as well as for maximum production of antimicrobial agents. Similar results were reported in a previous study [11]. It has been reported that the seven days [32] and 12 days [13] were optimal for maximum growth and antimicrobial production. The results of the present study disagreed with the findings in a previous study, which reported a maximum antibiotic production in 30 days [6, 38].

CONCLUSION

A promising strain was selected and carefully studied for maximum antimicrobial production, the suitability factors were optimized experimentally, carbon source (5% glucose), pH (7.0), temperature (28^0C), agitation (180 rpm), and incubation period (96 hours).

Acknowledgments: This publication was supported by the Deanship of scientific research at Prince Sattam bin Abdulaziz University.

Conflict of interest: None

Financial support: None

Ethics statement: None

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