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Research Article

**MICROBIOLOGY**

# Screening and identification of actinomycetes from animals' dung for antimicrobial activity

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**KEY WORDS**

Antimicrobial activity, Animals' dung, Optimization, *Streptomyces hypolithicus*.

## ABSTRACT

The present study was searching for actinomycetes isolates with antimicrobial activity from animals' dung. Twenty-two actinomycetes isolates were purified and nominated consistent with their culture features on starch nitrate agar medium. These purified isolates were surveyed for antimicrobial activity against different bacterial and fungal strains. Among all tested actinomycetes isolates, the isolate A12 had the highest antimicrobial activity. The A12 isolate's morphological, physiological, and biochemical data all pointed to it being a member of the *Streptomyces* genus. The 16S rRNA gene sequence and phylogenetic connection of strain A12 revealed that it belongs to the *Streptomyces hypolithicus* HSM#10. The optimum conditions of growth and antimicrobial activity were submerged cultivation, temperature 30 $^{\circ}$C, pH 7 for 7 days. Starch and potassium nitrate as best carbon and nitrogen source respectively and1% (w/v) NaCl. Ethyl acetate was used to extract antimicrobial metabolites from the isolate A12, which were then examined using thin layer chromatography (TLC) which showed single spot. Ethyl acetate extract of the selected isolate was analyzed by UV, IR, HPLC and GC/MS. 1,2-Benzenedicarboxylic acid and diisooctyl ester was found to be the major component. MIC of purified extract was found to be 3.1, 3.1, 3.1, 6.3 and 25 mg/mL against *B. cereus*, *S. aureus* ATCC 29213, *B. subtilis*, *S. epedermadides* and *K. pneumonia* ATCC53637 respectively. MBC of *S. hypolithicus* strain HSM#10 A12 extract against *B. cereus*, *S. aureus* ATCC 29213, *B. subtilis*, *S. epedermadides* and *K. pneumonia* ATCC53637 were 6.3, 3.1, 12.5, 6.3 and 25 mg/mL, respectively. *S. hypolithicus* HSM#10A12, was proved to have a broad-spectrum action against bacteria.

## Introduction

The emergence of drug/multi-drug resistance in most pathogenic microbes has necessitated the search for bioactive metabolites produced by microorganisms, such as antibiotic compounds, for possible use in agriculture, pharmaceuticals, and industrial applications. Researchers are scouring the globe for new antimicrobial compounds that are strong, long-lasting, and have broad-spectrum effects, including those derived from microorganisms **(Singh and Tripathi, 2011).** In addition, Infections caused by resistant microbes have arisen and reemerged as a result of antimicrobial overuse (**Kaaria *et al*., 2015).** Bacterial resistance creates both substantial morbidity and mortality, as well as high treatment costs (**Sharif *et al*., 2013)**. World Health Organization data indicates infectious diseases are the second most important cause of death **(WHO, 2014; Ravnikar *et al*., 2015)**. There have been suggestions that, new microorganisms and their products are being found in areas that are poorly studied, including soil, water, and marine ecosystems, as well as Jordan, Antarctica, and a specific species of Manipur (**Singh *et al*., 2014).** We have easy access to cheap and easily available bio resources on our planet in the form of animal dung. Dung may provide an unlimited source of microbial diversity that hasn't been fully tapped **(Hozzein *et al*., 2011)**.

So far, only a handful of researchers have found evidence that dung microflora has antimicrobial properties **(Gupta and Rana, 2016). Naskar *et al*. (2003)** found that, cow dung inhibited yam postharvest rot pathogens. Additionally, cow dung microflora has also been reported to be antagonistic to *Fusarium oxysporum*, possibly because of the creation of antifungal metabolites **(Swain and Ray, 2009)**. Studies have shown that, actinomycetes may serve as a marker for compost maturity **(Steger *et al*., 2007)**, as they suppress pathogens in the curing process. Actinomycetes create a diverse spectrum of natural chemicals with diverse biological functions including antibacterial, antifungal, anti-protozoan, antiviral, insecticide, and herbicide action **(Rashad *et al*., 2015)**. They also serve a key part in the natural recycling of organic materials **(Akond *et al*., 2016)**. Extracellular enzymes produced by actinomycetes include amylases, cellulases, chitinases, lipases, proteases, ureases, and keratinases, which are used in industry, agriculture, and wastewater treatment **(Dahdah *et al*., 2021)**. Actinomycetes are considered to account for around a third of all antibiotics found in nature **(Moghannem, 2018)**.

*Streptomyces* is the most common actinomycetes **(Okami and Okazaki, 1972)**. *Streptomyces* is well-known for manufacturing a variety of industrial and medically beneficial compounds (antibiotics, fungicides, chemotherapeutics, immune suppressants, and herbicides) **(Genilloud, 2017)**. Thousands of bioactive natural chemicals can be found in them with economic value as antibiotics, antifungal, and antibacterial agents for a variety of diseases **(Barka *et al*., 2016; Ullah *et al*., 2022).**

The current study being undertaken to identify potential actinomycetes isolated from animals' dung that could produce antimicrobial materials.

## Material and methods

### Samples collection

Animals dung samples were collected aseptically from three different locations of Beheira governorate – Egypt. The samples were collected from (cow, horse, rabbit, goat, buffalo, donkey, and sheep dung) in May 2019. The samples were all tagged and delivered to the microbiological lab, Department of Botany and Microbiology, Faculty of Science, Tanta University for additional dispensation. The dung samples were processed on the same day.

## Isolation, purification, and maintenance of actinomycetes

The following media are used for the isolation, starch-nitrate agar medium, yeast extract-malt extract agar medium and nutrient agar medium. Actinomycetes isolates were recovered from dung samples using a serial dilution approach **(Gupta and Rana, 2016)**. For each sample three plates were used and incubated at 30 °C for 7 to 14 days. The plates were observed periodically for the growth of actinomycetes. The colonies that developed were selected and purified based on colour, dryness, roughness, and convexity. **(Moghannem, 2018)**. The pure colonies were chosen, isolated, sub cultured, purified, and kept at 4 °C in starch nitrate agar slants for further research. The selected isolates were maintained by suspended in 50% of glycerol and kept at -80 °C **(Cohen and Johnston, 1964)**.

## Screening the actinomycetes isolates for antimicrobial activity

Test microorganisms were used to examine the potency of the actinomycetes isolates for antimicrobial activity. Nine test bacterial strains (*E. coli* ATTC8739, *Salmonella typhi* ATTC14028, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATTC35639, *Klebsiella pneumonia* ATCC53637, *Proteus* sp ATTC35659, *Staphylococcus epedermadides*, *Bacillus cereus* and *B. subtilis*) and five test fungal strains (*Fusarium equiseiti*, *Fusarium sublutinase*, *Fusarium proliferatum*, *Aspergillus niger* and *Candida albicans* ATTC90028) were used for screening the actinomycetes isolates for antimicrobial activity. Preliminary testing of antimicrobial activity of pure actinomycete isolates using an agar plug assay (Cork borer method). The isolates that display antimicrobial activity were submitted to secondary screening using starch nitrate broth by Agar well method based on the results obtained from the agar plug assay **(Moghannem, 2018)**. The most effective isolates were selected for the further experiments.

## Antibiotic sensitivity profile of tested bacteria

The tested bacterial strains were kindly supplied by Assoc. Prof. Dr. Lamiaa Al-Madboly at the microbiology department, Faculty of pharmacy, Tanta University. According to Clinical and Laboratory Standard Institute (CLSI) guidelines, an antibiotic susceptibility profile of the tested bacteria was performed to confirm the resistant percentage **(CLSI, 2017)**. Antibiotic susceptibility test for the tested bacteria was carried out according to **(Bauer *et al.*, 1966; NCCLS, 2003)**. Transferring 500-1000µL culture broth onto Mueller Hinton agar plates, followed by surface streaking the whole agar surface with a sterile wire loop, resulted in seeded Mueller Hinton agar plates. The antibiotic discs were aseptically deposited onto the agar surfaces after the seeded agar plates had been left for around 15 minutes. After that, the plates were incubated for 18-24 hours at 37°C. Inhibition zones were measured and documented in mm. after 18 to 24 hours incubation at 37ºC.

## Identification of the most active actinomycetes isolates

For complete and useful identification of our isolate, several physical, morphological, and chemical properties were examined. The conditions outlined in the identification keys were followed Bergey's manual of systematic bacteriology **(Williams *et al.*, 1989)** and Bergey's manual of determinative bacteriology **(Holt *et al.*, 1994)** were followed for identification. Morphological characteristics of the actinomycete isolates were studied using inorganic-salts starch agar medium according to the ISP methods **(Shirling and Gottlieb, 1966)** in addition to cover slip culture technique **(Kawato and Shinobu, 1959)**. Cell wall analysis was carried out using the methods described by **Becker *et al*. (1964); Lechevalier and Lechevalier, (1970).** At the Faculty of Science, Alexandrea University, microscopic examinations were carried out using a light microscope (Optika, Italy) by cover slip technique and a scanning electron microscope (JEOL Technics JSM-IT200, Japan) and molecular identification was carried out in Sigma company, Cairo-Egypt **(Williams, 1989; Abd-Elnaby *et al*., 2016).**

## Optimization of cultural conditions on growth and antimicrobial activities of the selected actinomycetes isolate

Several factors were investigated, including effect of culture method **(Ababutain *et al*., 2013)**, different incubation periods, different pH values, different incubation temperatures, different nitrogen sources **(Mangamuri *et al*., 2014)**, different carbon sources **(Jonsbu *et al*., 2002)**, and different NaCl concentrations **(Akond *et al*., 2016)** on the growth and antimicrobial activity of the isolate A12. After each incubation period of all parameters, each culture was centrifuged at 3000 rpm for 20 minutes then the supernatant of each parameter was taken to evaluate the antimicrobial activity of the selected isolate against the most sensitive tested microorganisms by using well diffusion agar method as mentioned before. Using a clean spatula, the biomass of the chosen isolate was transferred to a pre-weighed dry filter paper, which was then placed in an oven at 50°C overnight to achieve a constant weight. Mycelial dry weight was determined and expressed as g/50 mL for each parameter **(Singh *et al*., 2014)**. Three replicates were used for each parameter.

## Extraction of active antimicrobial compounds

Based on the primary and secondary screening, the high activity of an actinomycetes isolate was chosen for detection of antimicrobial metabolites using a liquid-liquid extraction method with various solvents. **(Rajivgandhi *et al*., 2019).** Production of antimicrobial compounds was performed by submerged fermentation according to **Egorov, (1985)**. Briefly, under sterile conditions, the most powerful antimicrobial generating actinomycetes were grown in 50 mL of starch–nitrate broth in a 250 mL capacity conical flask and incubated at 30 °C for 7 days at 150 rpm rotation **(Romankova *et al*., 1971)**. Afterwards, to separate cell debris, the media was centrifuged at 10,000 rpm. After centrifugation, the fermented culture filtrate was mixed with an equal amount of butanol, diethyl ether, ethyl acetate, petroleum ether, chloroform, hexane, acetone, methanol, and ethanol separately (all solvents were purchased from Sigma Company in Cairo, Egypt) and shaken vigorously for 30 minutes. The antimicrobial activity of organic phase or the precipitate of the culture tested using the agar well diffusion method against tested microorganisms. The antimicrobial activities of all solvents were detected. The optimum solvent for subsequent extraction was chosen based on the maximum inhibition, and the organic phase was evaporated using a rotary evaporator, (SENECO Technology Co., Ltd., Taiwan). The completely dried crude extract was collected and used for further studies.

## Purification and characterization of antimicrobial active compounds in crude extract

### TLC analysis

Thin layer chromatography (TLC) using silica gel plate was applied to screen and investigate the purity degree of the crude ethyl acetate extract of the active actinomycetes strain **(Kumar *et al*., 2018)**.

By a capillary tube, the sample was spotted in three different TLC plates. Then, the plates were investigated in three distinct solvent systems, *i.e.*, water: methanol (4:6, v/v), chloroform: methanol (4:6, v/v), and chloroform: methanol (9:1, v/v) applying the ascending development. Afterwards, the plates were visualized after drying by UV lamp Model Spectro line (highest ultraviolet intensity, U.S.A) at 254 nm and 366 nm.

**HPLC analysis**

Waters Alliance 2695 with Waters PDA detector 2998 (Waters, Milford, MA, USA) was used for HPLC analysis, as described by **Ludwig *et al*. (2015)** with certain modifications. In brief, the crude ethyl acetate residue was re-suspended in HPLC-grade methanol and filtered through a 0.45 µm PTFE disc filter (VWR International, Germany). Following that, the filtrate was chromatographed with a Discovery HS $C_{18}$ (5 µm, 250 mm x 4.6 mm) column combined with a guard column (Phenomenex®) at a constant temperature of 16 °C. At a flow rate of 1 mL/minute, an isocratic elution with methanol: 0.1% formic acid (92:8 v/v) was carried out. The injection volume was set at 20 µL and a PDA detector adjusted at 205 nm was used.

## UV analysis

The UV analysis of the separated tested extract was recorded by using quartz cuvette containing the extract in methanol. The UV/Vis spectrum was achieved using (pg. instruments, T80 spectrophotometer, United Kingdom) at the Faculty of Science, Tanta University in the range of 250 to 500 nm **(Rajivgandhi *et al*., 2018)**.

## FTIR analysis

The functional groups present in the partially purified crude extract were characterized in the range of 600-4000 $cm^{-1}$ in KBr disc using Fourier-transform infrared spectroscopy (FTIR), Model (Tensor 27 Bruker) at the Central lab, Tanta University **(Balachandar *et al*., 2018)**.

## GC-MS analysis

The selected isolate's extract was tested using the GC-MS technique. A chromatograph mass spectrometer (Perkin Elmer model, Clarus 580/560S) at the Central lab, Tanta University was used to perform the GC-MS procedure. The following chromatographic conditions were used, where carrier gas, helium; flow rate 1 mL/min; sample input temperature 280 °C; initial temperature 60 °C maintained for 7 minutes, then programmed to 170 °C for 5 minutes by 10 °C/minute, then programmed to 280 °C for 10 minutes by 10 °C/minute; capillary column, HP-5MS, length 30 m, diameter 0.25 mm. The antimicrobial compounds were identified in GC-MS and their retention times were compared to those of standards **(Alqahtani *et al.*, 2022)**.

## Determination of the minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was assayed for the purified active ethyl acetate extract of the isolate A12 using microtiter plate technique **(Zgoda and Porter, 2001)**. A serial dilution (100, 50, 25, 12.5, 6.5, 3, 1.5 and 0.75 mg/mL) of ethyl acetate extract was tested against tested bacteria: *S. aureus* ATCC 29213, *K. pneumonia* ATCC53637, *S. epedermadides*, *B. cereus* and *B. subtilis*. From each dilute o.1 mL was added to 5 mL of Nutrient broth containing 0.05% phenol red and added with 10% glucose (NBPG medium) **(El-Shouny *et al*., 2017)**. One hundred microliters of each concentration were added in a well (96-wells micro plate) containing 95 µL of NBPG and 5 µL of the test bacterial suspension containing $10^6$ CFU/mL. The negative control well contained the same mixture without adding the active compound of ethyl acetate extract. Covered plates were incubated for 24 hours at 37 °C. The experiment was carried out twice more. The change in color in the wells was used to determine microbial growth (red when there is no growth and yellow when there is growth). The MIC was considered as the lowest concentration of extract that caused no color change **(Zgoda and Porter, 2001).**

### Determination of minimum bactericidal concentration (MBC).

(MBC) determined by microtiter broth dilution method: After a 24-hour incubation period at 37 °C, the minimum bactericidal concentration (MBC) was determined as the lowest extract concentration that killed 99.9% of the bacterial inoculum. The approach of **Ozturk and Ercisli, (2006)** was used to determine MBC. MBC was carried out on a purified active ethyl acetate extract of a chosen isolate. Ten microliters were taken from the MIC experiment's (MIC value) well and two wells above it and spread on MHA plates. After 18–24 hours of incubation at 37 °C, the colonies were counted. The MBC value was defined as the concentration of a sample that produces < 10 colonies. Each experiment was carried out three times.

## Results and discussion

### Isolation and screening of antimicrobial producing actinomycetes.

Twenty-two different actinomycetes isolates were isolated from animals' dung samples. Each isolate was tested against each of the test microorganisms for its antimicrobial activity. Twelve isolates showed antimicrobial activity against one or more test organism. A secondary screening is performed on actinomycetes isolates which show antimicrobial activity in primary screening against the tested bacterial and fungal species. The isolates No. A12 had the greatest antimicrobial activity Table (1). This made it desirable to identify and study it further.

**Table (1)**: Secondary screening for antimicrobial activities of the isolates actinomycetes against the tested bacterial and fungal species showed antimicrobial activity against one or more test organism. (Inhibition zone diameter mm).

| Tested organism / Isolates | *B. cereus* | *B. subtilis* | *S. aureus* ATCC 29213 | *S. Epedermadides* | *K. pneumonia* ATCC 53637 | *S. typhi* ATTC14028 | *Proteus* sp ATTC35659 | *C. albicans* ATTC 90028 | *A. niger* | *F. equiseiti* | *F. proliferatum* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A12 | 24±0.3 | 18±0.25 | 21±0.6 | 15±0.25 | 27±0.4 | 0±0 | 0±0 | 16±0.5 | 15 ±0.5 | 0±0 | 14±0.2 |
| A13 | 16±0.25 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 20±0.3 | 30±0.3 | 22±0.1 | 25±0.2 |
| A16 | 18±0.25 | 14±0.3 | 16±0.3 | 0±0 | 13±0.4 | 12±0.25 | 14±0.2 | 0±0 | 0±0 | 0±0 | 12±0.2 |
| A5 | 13±0.3 | 0±0 | 0±0 | 0±0 | 12±0.25 | 15±0.25 | 0±0 | 0±0 | 18±0.25 | 0±0 | 0±0 |
| A18 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 19±0.3 | 0±0 | 10±0.6 |
| A9 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 15±0.2 | 0±0 | 0±0 | 0±0 |
| A23 | 0±0 | 0±0 | 13±0.25 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 13±0 |
| A21 | 17±0.4 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| A10 | 15±0.4 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 |
| A19 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 16±0.3 | 0±0 | 11±0.3 |
| A22 | 0±0 | 0±0 | 12±0.25 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 13±0.1 |
| A11 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | 20±0.4 |

**Values are the mean of three replicates, ± stander error of means**

The results showed that the isolate A12 exhibiting antibacterial activity more than antifungal activity, something that was also reported by **Al-Mahdi *et al*. (2011); Patel *et al*. (2014)**.

## Antibiotic sensitivity profile of tested bacteria.

Antibiotic sensitivity profile of tested bacteria was performed to appoint their resistant percentage to different antibiotics. Twenty-four antibiotics represented different classes of antibiotics were used in the development of the antibiotic sensitivity profile in the form of paper disks, The results showed that *K. pneumonia* was resistant to 47% of tested antibiotics, *S. aureus* was resistant to 47% of tested antibiotics, *B. cereus* was resistant to 42% of tested antibiotics, *S. epedermadides* was resistant to 31% of tested antibiotics. *B. subtilis* showed a highest susceptibility rate (21%) to tested antibiotics.

## Identification of the most active actinomycetes isolate.

According to ISP methods **(Shirling and Gottlieb, 1966; Lebeda *et al*., 2012)** the chosen isolate was characterized and identified. The data recorded showed that, on ISP 2 the isolate A12 shows good development, but after 14 days there is no sporulation. Very good growth is observed on ISP 4 with cream substrate and off-white aerial mycelia. On ISP 5, no diffusible pigments are produced, although there is strong growth on a beige substrate with sparse white aerial mycelia. After 14 days of growth, there is no melanin production on ISP 6 or ISP 7. ISP 7 has a strong growth rate, while ISP 1 has a slow growth rate. Light and scanning electron microscopy were used to examine the isolates' aerial hyphae arrangement, spore chain ornamentation, and spore surface as shown in Fig. (1). The data recorded showed that, the isolate A12 was a Gram-positive, non-motile actinomycete with a straight, off-white aerial mycelium and more than 20 cylindrical, non-flagellated spores with smooth surfaces. The selected isolate was subjected to a variety of physiological and biochemical tests.

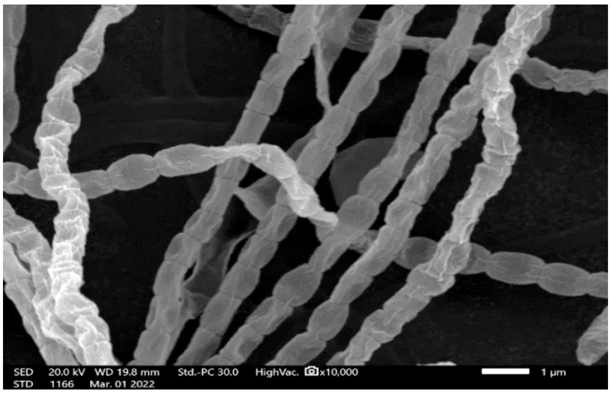



**Fig. (1)**: Scanning electron micrograph (X 10000) showing straight shaped mycelia and smooth spore surface of the isolate A12.

According to the data recorded the isolate A12 has the ability to utilize wide range of carbon and nitrogen sources. No growth was observed in the presence NaCl concentrations greater than 10%. Temperature range for growth was 15– 40 °C, with optimal growth at 30 °C. The pH range for growth was pH 4–10 with optimal growth on pH 7. The biochemical properties of the selected isolate explain its behavior toward various substrates, including its ability to hydrolyze starch and casein, lipids and gelatin but not cellulose, and its inability to reduce nitrate. Catalase test, methyl red and Voges-Proskauer test are negative. The isolate A12 were characterized among the actinomycetes due to the presence of LL-DAP in their cell wall **(Lechevalier and Lechevalier, 1976**). The selected organism was suggested to be belonging to family Streptomycetaceae for the following reasons, the presence of LL-DAP in their cell wall **(Palla *et al*., 2018),** the inability of vegetative mycelia to be fragmented into bacillary or coccoid forms **(Grantcharova *et al*., 2005),** the presence of large spore chains **(Manteca *et al*., 2010)**, excessive branching and aerial mycelia **(Claessen *et al*., 2006).**

## Molecular identification of the selected isolate A12.

Molecular identification of the most potent antimicrobial producer using 16S rRNA sequencing was carried out. The results indicated the appearance of single band indicating the purity of isolated RNA. The pure band was partially sequenced and then compared to the public data base of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) to *Streptomyces* sp. The partial 16s rRNA sequence of the isolates were determined and deposited in GenBank under accession number (NR-044431.1) for isolate A12. The selected isolate A12 showed similarity level 82.21% with *Streptomyces hypolithicus* strain HSM#10 as shown in Fig. (2).

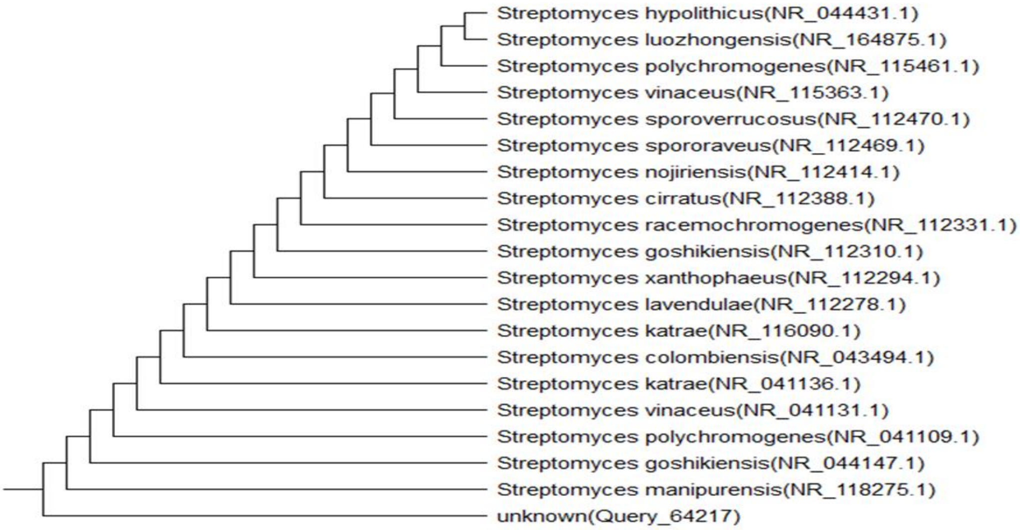



**Fig. (2):** Phylogenetic tree of isolate *Streptomyces* A12 strain and related *Streptomyces* sp. based on the 16S rRNA gene sequences.

## Optimization of different environmental and nutritional conditions for growth and antimicrobial production of *Streptomyces hypolithicus* strain HSM#10 A12.

Optimization of culture conditions was performed to achieve higher growth rate and antimicrobial production by *S. hypolithicus* strain HSM#10 A12 against the most sensitive tested microorganisms.

Table (2) showed that, submerged cultivation resulted in a considerable increase in antibacterial activity and growth (dry weight) of the *S. hypolithicus* strain HSM#10 A12 as compared to the static condition. Many other researchers' findings corroborated our findings. Streptomycetes are obligate aerobic creatures, which explains why this is the case. **(Hassan *et al*., 2001; Venkateswarlu *et al*., 2004; Oskay, 2011; Moghannem, 2018).**

**Table (2):** Effect of different cultivation methods on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 on tested bacteria.

| **Cultivation method** | **Shaking conditions** | **Static conditions** | **P value** |
|---|---|---|---|
| **Dry weight (g/50mL)** | 0.52± 0.01[a] | 0.49± 0.01[b] | 0.0061** |
| **Tested bacterial species** | Inhibition zones diameter (mm) | | |
| ***B. cereus*** | 26.0± 0.6[a] | 21.0± 0.5[b] | 0.0004*** |
| ***S. aureus* ATCC 29213** | 23.0± 0.3[a] | 21.0± 0.3[b] | 0.0006*** |
| ***B. subtilis*** | 18.0± 0.3[a] | 14.0± 0.5[b] | 0.0002*** |
| ***S. epedermadides*** | 15.0± 0.3[a] | 12.0± 0.5[b] | 0.0007*** |
| ***K. pneumonia* ATCC53637** | 27.0± 0.3[a] | 22.0± 0.3[b] | 0.0000*** |

Values are the mean of three replicates ± SD, values with different superscript letters are significant at $p < 0.05$.

Incubation period was an effective factor on the growth rate and antimicrobial production of *S. hypolithicus* strain HSM#10 A12 which was incubated at different periods in days (1-9). There was an increase in the growth as well as antimicrobial activity with the increase of incubation period from 1st to 7th day. However further increase in incubation period resulted in the decrease of growth and antimicrobial activity. The highest growth rate of the selected strain and its antibacterial activities on tested bacteria were obtained at 7th day of incubation, Fig. (3). This result agreed with **Singh *et al.* (2009) ; Shazia *et al*. (2013).** In contrast **Fahmy, (2020)** who found that, the maximum antimicrobial activity of *Streptomyces* sp. NMF76 obtained at 14 days.

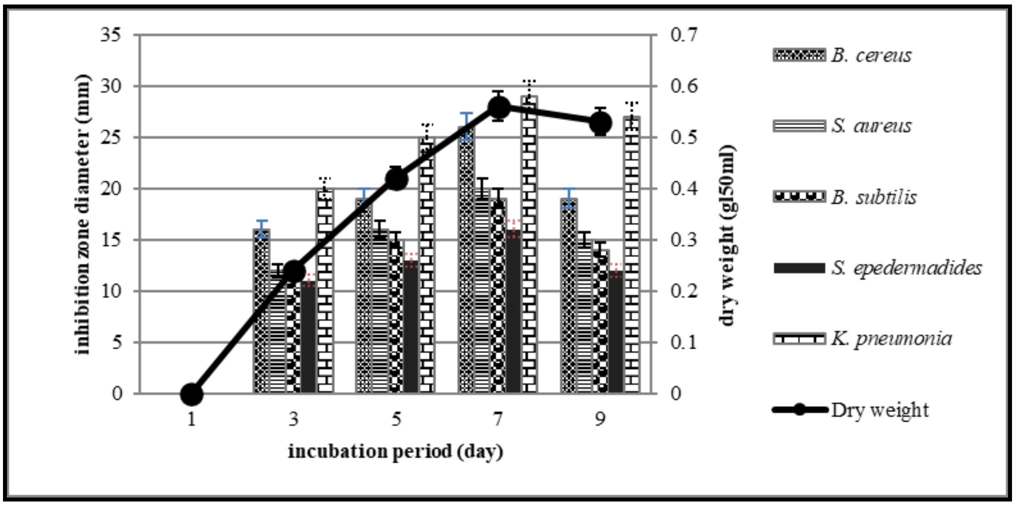



**Fig. 3:** Effect of different incubation periods on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 on tested bacteria

The results represented in Fig. (4) indicated that, the acidic pH 4 unsuitable for growth or antimicrobial activity for *S. hypolithicus* strain HSM#10 A12. On the other hand, the highest growth and antibacterial activities of *Streptomyces hypolithicus* strain HSM#10 A12 were recorded at pH value 7 followed by alkaline pH 9 suggesting its inclusion in the neutrophilic actinomycetes group. Low growth and activity were recorded at pH 5 and pH 10. This result is comparable to that published by **Moghannem, (2018) ; Akond *et al*. (2016).** In contrast **Ripa *et al*. (2009)** recovered a new *Streptomyces* strain from Bangladesh soil with its activity maxima at alkaline pH.

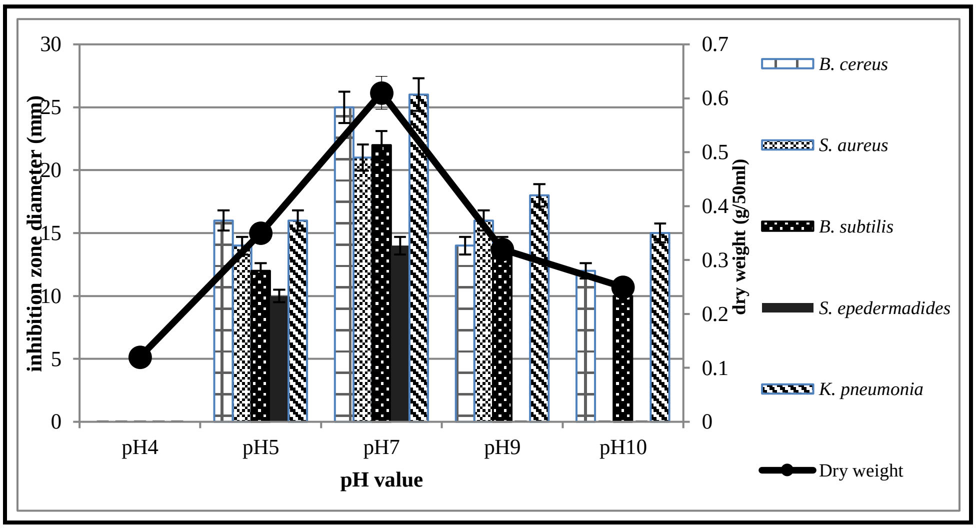



**Fig. 4:** Effect of different incubation pH values on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 against the tested bacteria.

Normally actinomycetes are sensitive to temperature. All metabolic processes including enzyme activity and the synthesis of active metabolites are affected by temperature **(Vijayakumar *et al*., 2012).** As shown in Fig. (5) When the incubation temperature was raised from 20°C to 30°C, there was a rise in both growth and antibacterial activity. But further increase in temperature (above 30ºC) resulted in the decline of growth and antimicrobial activity. The *S. hypolithicus* strain appeared to be mesophilic in nature when it came to its optimal temperature for growth. This result agreed with **Akond *et al*. (2016); Moghannem, (2018) ; Fahmy, (2020). In contrast Rakesh *et al*. (2014); Krishnan and Kumar, (2015)** who reported that, the optimum temperature for growth and production of bioactive metabolites by *Streptomyces* species was 45 °C and 40 °C respectively.

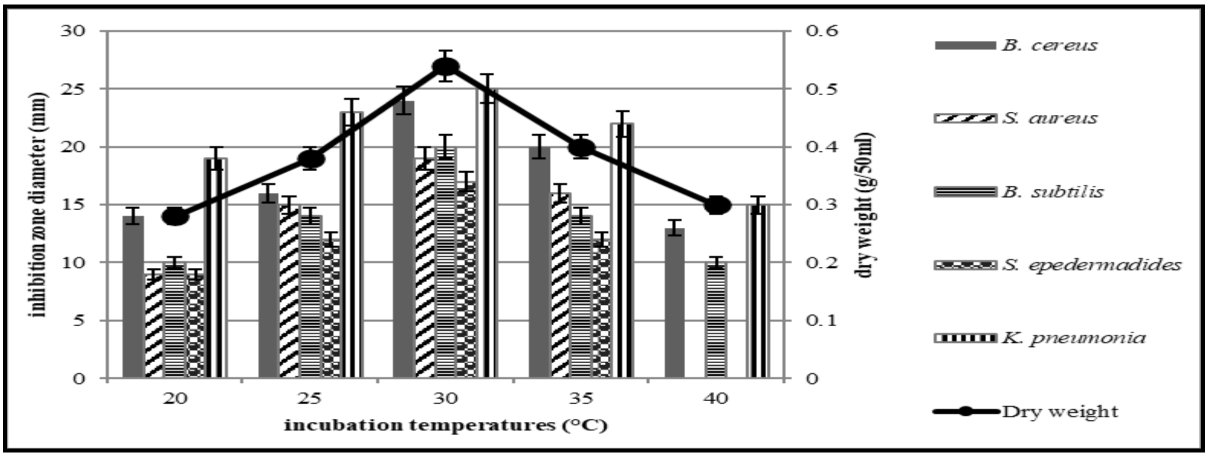



**Fig. 5:** Effect of different incubation temperatures on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 against the tested bacteria.

Actinomycetes' antibiotic production is known to be influenced by their dietary supplies of carbon and nitrogen. **(Devi *et al*., 2015)**. Potassium nitrate produced the highest mycelial dry weight and antimicrobial activity of *S. hypolithicus* strain HSM#10 A12 against all of the tested bacterial species, among the various organic and inorganic nitrogen sources tested indicating that, the nature and type of nitrogen source used in the culture medium can have a significant impact on antibiotic production. Inorganic nitrogen sources had stronger antibacterial activity than organic nitrogen sources by *S. hypolithicus* strain HSM#10 A12 when compared to organic nitrogen sources as shown in Fig. (6). This result agreed with **Vijayakumar *et al.* (2012) ; Awadalla *et al*. (2018). In contrast Fahmy, (2020)** found that, the maximum antimicrobial activity of *Streptomyces* sp. NMF76 obtained with L-asparagine as nitrogen source.

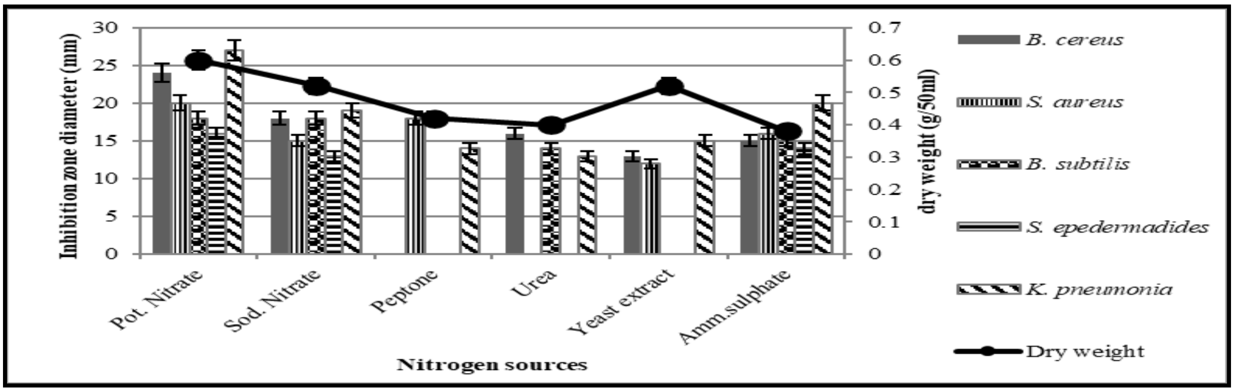



**Fig. 6:** Effect of different nitrogen sources on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 against the tested bacteria.

The ability of the selected isolate to utilize different carbon sources can greatly affect its growth rate and antimicrobial compound productivity. The best carbon source for growth and antimicrobial activity of *S. hypolithicus* strain HSM#10 A12 against all tested bacteria was starch, Fig. (7). Slowly absorbed complex carbon sources like polysaccharides have been found to stimulate secondary metabolite synthesis **(Bertasso *et al*., 2004)**. Similar result was obtained by **Vijayakumar *et al*. (2012) ; Awadalla *et al*. (2018).** In contrast **Ripa *et al*. (2009)** revealed that, providing new *Streptomyces* species (RUPA-08PR) isolated from Bangladeshi soil with glucose (2%) as the sole carbon source resulted in significant levels of antibiotic metabolites.

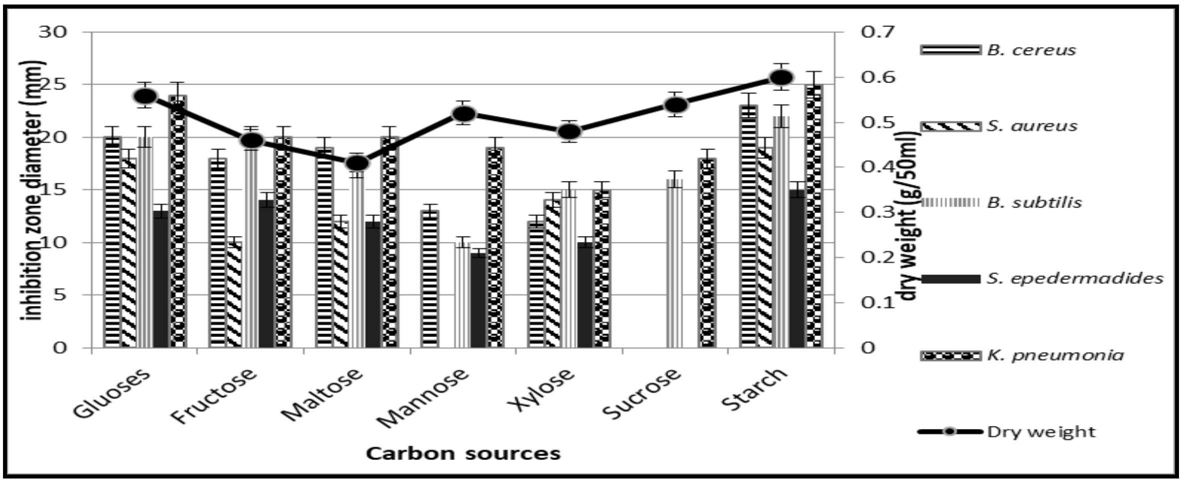



**Fig. 7:** Effect of different carbon sources on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 against the tested bacteria.

Concentration of NaCl has significant effect on the production of antibiotic from microorganism because of its effect on the osmotic pressure to the medium **(Akond *et al*., 2016).** Effect of different concentrations of NaCl was studied at (1-15%) giving variable growth rates and antibacterial activities by *S. hypolithicus* strain HSM#10 A12. The isolate was evaluated for its ability to withstand salt stress by growing at NaCl concentrations of 1, 3, 5, 7 and 10%. The highest growth rate and antibacterial activities were obtained at 1%, and further increase in salt concentration reduced both growth rate and the antimicrobial activities as shown in Fig. (8), this result is similar to that obtained by **Song *et al*. (2012); Rakesh *et al*. (2014)**; **Krishnan and Kumar, (2015).** In contrast three species of *Streptomyces* from Malaysia have been found to preferred NaCl concentration of 3.0% by all three isolates **(Hamid *et al*., 2015)** and 5% for *Streptomyces* VITSVK9 **(Saurav and Kannabiran, 2010).**

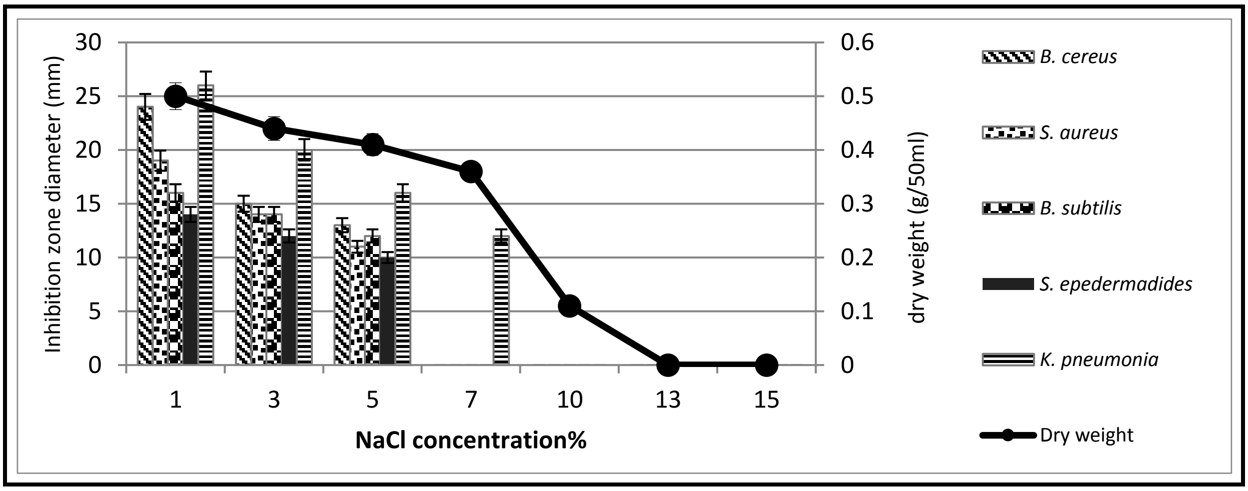



**Fig. 8:** Effect of different NaCl concentrations on mycelial dry weight and antibacterial activities of *S. hypolithicus* strain HSM#10A12 against the tested bacteria.

## Extraction of active antimicrobial compounds

When it comes to extracting chemicals for antibacterial activity, organic solvents have always been found to be more efficient than water-based approaches **(Lima-Filho *et al*., 2002).** Notably, ethyl acetate was primarily used as an extraction solvent in several prior reports to obtain crude extracts of bioactive chemicals from actinomycetes **(Franco and Coutinho, 1991; Kavitha *et al*., 2010; Balachandar *et al*., 2018)**. In the present study, the active antimicrobial materials were extracted using different solvents, The best solvent for maximal antibiotic extraction was ethyl acetate, this result is similar to that obtained by **El-Naggar *et al*. (2017); Srivastava and Shanmugaia, (2019)** who found that, the best solvent for maximal antibiotic synthesis by *Streptomyces* was ethyl acetate.

## Purification and characterization of antimicrobial active compounds in crude extract

### TLC analysis

Screening of the chemical composition was performed using TLC for the active antimicrobial crude extract. TLC plates showed one band with all different solvent systems. TLC analysis of the selected isolate *S. hypolithicus* strain HSM#10 A12 showed that, the rate of flow ($R_f$) 0.8, 0.9 and 0.6 with different solvent systems: water-methanol (4:6 v/v), chloroform-methanol (4:6, v/v) and chloroform-methanol (9:1, v/v) respectively. Similar results were obtained by **Ramani and Kumar, (2012)** who found that, the antibacterial compounds produced by *Streptomyces* sp. Sh7, extracted using butanol and purified by TLC had $R_f$ 0.6 and also by **El-Naggar *et al*. (2017)** who reported that, the antimicrobial materials produced by *Streptomyces anulatus* NEAE-94 had $R_f$ 0.8.

## HPLC

Ethyl acetate extract of the selected isolate was further analyzed by HPLC. The analytical HPLC result revealed a single sharp peak with a retention time of 18.8 min, indicating that, the active fractions are highly pure with no impurities as shown in Fig. (9).

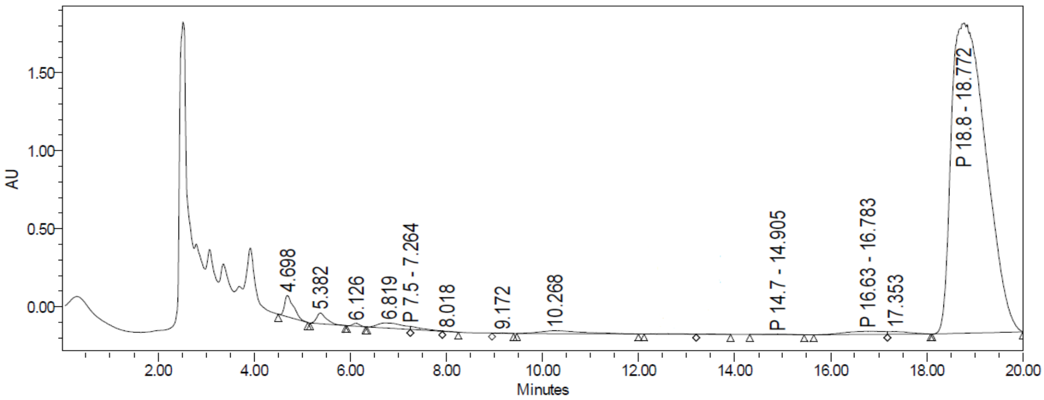



**Fig. 9:** The HPLC chromatogram of the antimicrobial materials produced by *S. hypolithicus* strain HSM#10 A12

## Ultraviolet spectroscopy

The UV spectrum of the antimicrobial materials of *S. hypolithicus* strain HSM#10 A12 was found to have a maximum absorption ($\lambda_{max}$) at 275 nm as shown in Fig. (10). This result agrees with **Rajivgandhi *et al*. (2018)** who reported that, the UV spectrum of bioactive compound from endophytic actinomycetes (EA) *Nocardiopsis* sp. GRG 2 (KT 235641) was observed at 274 nm.

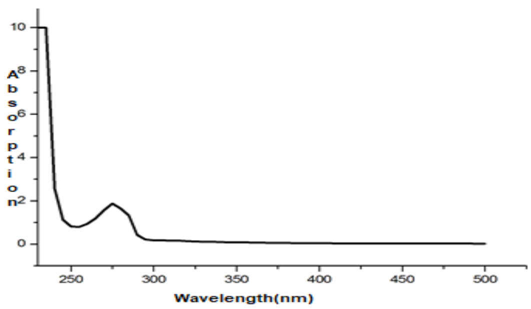



**Fig. 10:** UV Spectrum of antimicrobial materials produced by *S. hypolithicus* strain HSM#10 A12.

## IR analysis

The FT-IR finding was confirmed as a useful method for identifying functional groups (chemical bonds) in an unknown mixture or crude extract **(Balachandar *et al*., 2018)**. Because of the lack of the NH stretching at 3400-3200cm$^{-1}$, the FT-IR data clearly demonstrated that, the antimicrobial compound produced by *S. hypolithicus* strain HSM#10A12 was free of protein or nucleic acid impurities. The presence of aromatic groups of CH bending and -C-C alkanes, respectively, was suggested by peaks at 685 and 1019 cm$^{-1}$, as well as an ether bond at 1095 cm$^{-1}$. C-H stretching and CH2 were also observed rationally at 1655 and 2358 cm$^{-1}$, respectively. Also, the C=O of carboxyl stretching, alkane groups, and–O-H stretching were all seen at 1454, 2924, and 3344 cm$^{-1}$. The appearance of a strong band at 1755cm$^{-1}$ proved the carbonyl group's presence. As a result, our findings revealed that, the purified fraction contains a carbonyl group. The functional groups described above were the most likely to have an antibacterial impact, according to previous research **(Kim *et al*., 1997; Sun *et al*., 2015; Balachandar *et al*., 2018)**. Interestingly, the toxic cyano (C≡N; 2220-2260 $cm^{-1}$) and acetylenic (C≡C; 2100-2260 $cm^{-1}$) groups are absent as an indicator of the safety of *S. hypolithicus* strain HSM#10 A12 extract as shown in Fig. (11).

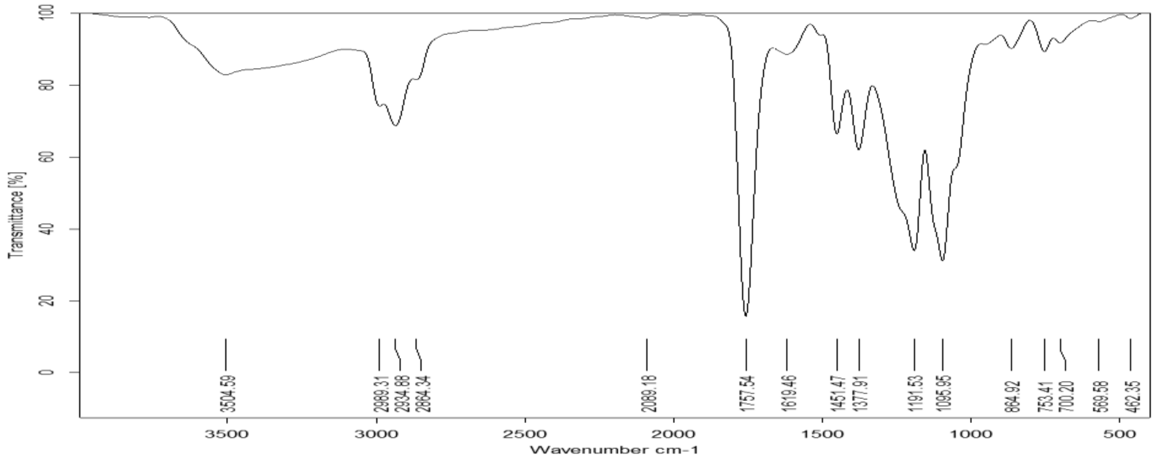
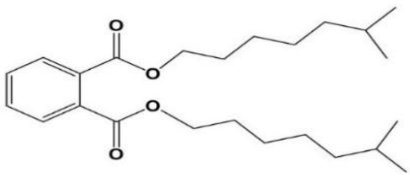



**Fig. 11:** The infrared spectrum (IR) of the antimicrobial compound produced by *S. hypolithicus* strain HSM#10 A12.

## GC-MS analysis

GC-MS analysis of *S. hypolithicus* strain HSM#10A12 extract was carried out and the profiles of the fractions revealed the existence of a single abundant peak, as well as a number of minor compounds with varying retention time. The chromatogram of the components of *S. hypolithicus* strain HSM#10A12 is illustrated in Fig. (12). The most abundant peak was obtained at retention time 32.87 minutes for 1,2-benzenedicarboxylic acid, diisooctyl ester, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, Diisooctylphthalate and Di-n-octyl phthalate. This result agrees with the results obtained by IR analysis for the extract. 1,2-Benzenedicarboxylic acid, diisooctyl ester was found to be major component in extract. Similar result was obtained by **Rajeswari *et al*., (2012)** who reported that, 1,2-benzenedicarboxylic acid, diisooctyl ester was the major component in the bioactive components of leaves of *Hugonia mystax* L. was analyzed by GC-MS. It's important to shed light on that, the extraction of 1,2-benzenedicarboxylic acid, diisooctyl ester from *S. hypolithicus* strain HSM#10 is detected for the first time in our study.

The extract of the selective isolate has broad spectrum antimicrobial activity against Gram-positive bacteria, *such as S. epedermadides, B. cereus, B. subtilis and S. aureus* and Gram-negative bacteria *such as K. pneumonia* ATCC53637. The higher activities of the extract have been attributed to the presence of 1,2-benzenedicarboxylic acid, diisooctyl ester that is known to have antimicrobial activity **(Agoramoorthy *et al*., 2007; Salem *et al*., 2016)**. this result agrees with **Waheed *et al*., (2019)** who reported that, extract which contain 1,2-benzenedicarboxylic acid diisooctyl ester have antimicrobial activity against *S. epedermadides*, *B. subtilis*, *S. aureus*, *S. typhi* and *K. pneumonia*. Also, **Salem *et al*., (2016)** reported that, 1,2-benzenedicarboxylic acid, diisooctyl ester has antimicrobial and antifouling effect. Besides that, this compound has some industrial applications, such as being employed as industrial chemicals in polymers to impart flexibility in polyvinyl chloride (PVC) resins. They are also employed as synthetic base stocks for lubricating oils, and they're commonly used for building wire insulation. Plasticizers for vinyl, cellulosic and acrylate resins, and synthetic rubber are among the other applications mentioned. 1,2-benzenedicarboxylic acid, diisooctyl ester is also used in the fabrication of building wire jackets, conveyor belts, bottle cap liners, floorings, tarps, pool liners, garden hoses, and automotive hoses and parts. It was also identified in some children toys and in commercial milk products **(Huang *et al*., 2021).**



1, 2-Benzenedicarboxylic acid, diisooctyl ester

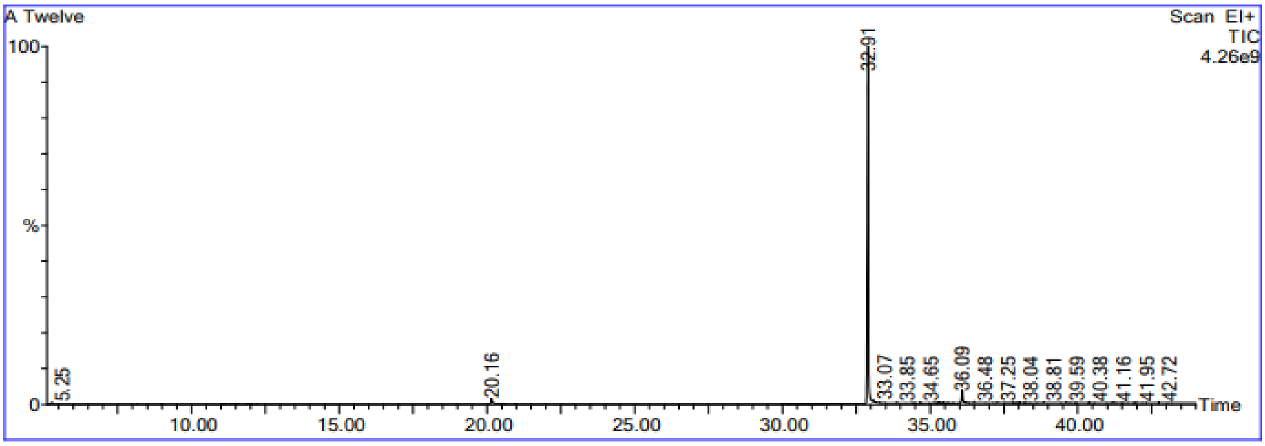



**Fig. 12:** GC-Mass spectrum of the antimicrobial materials produced by *S. hypolithicus* strain HSM#10A12 showing a single main peak at 32.91 min.

**Minimum inhibition concentration (MIC) and Minimum bactericidal concentration (MBC) of purified extract.**

The lowest concentration of a chemical that suppresses observable bacterial growth is known as the minimum inhibitory concentration (MIC). While the minimum bactericidal concentration (MBC) was determined to be the lowest extract concentration capable of killing 99.9% of the bacterial inoculum during a 24-hour incubation period at 37 °C. In our study, the result revealed that, the minimal inhibitory concentrations of the bioactive antimicrobial materials of *S. hypolithicus* strain HSM#10 A12 against *B. cereus*, *S. aureus* ATCC 29213, *B. subtilis, S. epedermadides* and *K. pneumonia* ATCC53637 were 3.1,3.1,3.1,6.3 and 25 mg/mL, respectively. While the minimum bactericidal concentration (MBC) of extract of *S. hypolithicus* strain HSM#10 A12 against *B. cereus, S. aureus* ATCC 29213, *B. subtilis, S. epedermadides* and *K. pneumonia* ATCC53637 were 6.3,3.1,12.5,6.3 and 25 mg/mL, respectively as shown in Table (3).

Similar antimicrobial activity had been also reported against *S. aureus*, *B.*

*subtilis* and *S. typhi* by ethyl acetate crude extracts of actinomycetes with MIC value ranging between 0.5-5 mg/mL with *B. subtilis* and *S. typhi* and MIC value ranging between 0.05-5 mg/mL with *S. aureus* **(Sengupta *et al*., 2015).**

**Kurnianto *et al*., (2020)** reported that, Disk-diffusion assay of the metabolite-crude-extract from the *Streptomyces* isolates had broad-spectrum inhibitory effects against *S. aureus* ATCC 25923, *B. cereus* ATCC 10876, *E. coli* ATCC 25922, and *P. aeruginosa* InaCC B52 with MIC and MBC ranging from 2.5–10 mg/mL and 5–10 mg/mL, respectively.

The variation in antibacterial activity amongst the test bacteria was most likely attributable to differences in cell wall structure and composition. Peptidoglycan polymers are relatively close to the cell surface in Gram-positive bacteria, allowing antibiotic chemicals to easily penetrate cells. It differs from Gram-negative bacteria in that it has a lipopolysaccharide based outer membrane that acts as a barrier to hydrophobic and hydrophilic substances with certain molecular weights **(Soares *et al*., 2012; Kurnianto *et al*., 2020).**

**Table 3:** Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of extract of *S. hypolithicus* strain HSM#10A12 against tested bacteria**.**

| **Tested bacterial species** | **MIC (mg/mL)** | **MBC (mg/mL)** |
|---|---|---|
| ***B. cereus*** | 3.1 | 6.3 |
| ***S. aureus* ATCC 29213** | 3.1 | 3.1 |
| ***B. subtilis*** | 3.1 | 12.5 |
| ***S. epedermadides*** | 6.3 | 6.3 |
| ***K. pneumonia* ATCC53637** | 25 | 25 |

## Conclusion

Because of the threat of the rise of drug/multidrug resistance bacteria, new antibiotics are urgently needed. To solve this challenge, we need to find new antibiotic classes from previously unexplored sources. Actinomycetes found in animal faeces are an alternative source for these problems, and they have a wide range of pharmacological bio-potential for inhibiting a variety of infections-producing microbes. *S. hypolithicus* HSM#10A12 which was isolated from animal dung was found to have broad-spectrum action against Gram positive and Gram-negative bacteria in the current investigation. These findings suggest that, this extract could be a viable treatment option for some bacterial diseases. The findings of this study highlight the importance of actinomycetes found in the dung of the animals studied as a potential source of novel bioactive chemicals. As a result, intense efforts must be made to screen animal faeces, as this underexplored source has a lot of promise for producing novel bioactive chemicals, which could lead to the development of new drugs. Antimicrobial agents can be researched for potential uses in the treatment of human diseases. The mechanism for this bioactive compound's antimicrobial activity has to be investigated further.

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## فحص وتحديد الأكتينوميسيتات من روث الحيوانات بحثًا عن نشاط مضاد للميكروبات

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هدفت هذة الدراسة الي البحث عن اكتبنوميسيتات ذات نشاط مضاد للميكروبات من روث الحيوانات. تم عزل و تنقية ٢٢ عزلة من الاكتينوميسيتات وذلك وفقاً لخصائص زراعتها على وسط نشاء نترات أجار المغذي وقد تم فحص هذه العزلات المنقاة من حيث الفعالية المضادة للميكروبات ضد السلالات البكتريه والفطرية المختلفه ومن بين جميع العزلات المختبرة كان للعزلة رقم ١٢ أعلى نشاط مضاد للميكروبات وقد أشارت جميع النتائج المورفولوجية والفسيولوجية والكيميائية الحيويه لهذه العزله إلى أنها تنتمي إلى جنس استربتوميسيس وقد أظهر التعريف الجيني بأستخدام تقنية 16S rRNA أن العزله رقم ١٢ سلالة استربتوميسيس هيبوليثيكس وقد كانت الظروف المثلى للنمو والنشاط المضاد للميكروبات هي الزراعة المغمورة عند درجة حرارة ٣٠ درجة مئوية ودرجة حموضة ٧ ولمدة ٧ أيام وقد كان النشا هو أفضل مصدر للكربون ونترات البوتاسيوم أفضل مصدر للنيتروجين مع تركيز ١٪ (جم / مللي) من الصوديوم كلوريد. وقد تم استخلاص المواد المضاده للميكروبات من راشح النمو للعزله بواسطة مذيب خلات الايثيل وتم تحليلها بواسطة الطبقة رقيقة اللوني والتي أظهرت بقعة واحدة مع جميع أنظمة المذيبات المختلفه وبعد ذلك تم تحليل مستخلص الايثيل اسيتات للعزلة المختارة بواسطة الأشعة فوق البنفسجية والأشعة تحت الحمراء وجهاز فاصل اللون للسائل عالي الكفائه HPLC ، و الكروماتوجرافيا الغازيه GC/MS ووجد أن المركب ٢,١داي ايزو اوكتيل استر- بنزين داي كربوكسيلك اسيد هو المكون الرئيسي في المستخلص كما أوضحت النتائج أن أقل تركيزمثبط للمستخلص المنقى ضد البكتريا الباسيلس سيرس ، ستافيلوكوكس اوريس ، باسيلس ساتلس ، ستافيلوكوكس ابيديرماديدس، كلبسيلا نيومونيا كانت ٣.١ ، ٣.١ ، ٣.١ ، ٦.٣ ، ٢٥ مجم / مل ، على التوالي. وكان أقل تركيز قاتل للبكتريا الباسيلس سيرس ,ستافيلوكوكس اوريس، باسيلس ساتلس، ستافيلوكوكس ابيديرماديدس، كلبسيلا نيومونيا هو٦.٣، ٣.١، ١٢.٥، ٦.٣، ٢٥مجم/مل على التوالي.