ISOLATION AND ANTIMICROBIAL ACTIVITY ASSESSMENT OF *STREPTOMYCES MUTABILIS* AZHD22 RECOVERED FROM THE MEDITERRANEAN SEA: A POTENTIAL SOURCE OF PROMISING COMPOUNDS .

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Abstract

A total of twenty-six diverse actinomycetes were obtained from various regions of the Mediterranean Sea in Alexandria, Egypt. These isolates underwent primary screening against multiple Gram-positive, Gram-negative, yeast, and fungal strains. In the secondary screening, nine isolates exhibited varying degrees of activity against the tested microorganisms. Among them, isolate A7 demonstrated potent antimicrobial properties and was chosen for further investigation. Through morphological, biochemical, microscopic, and molecular identification techniques, isolate A7 was identified as *Streptomyces mutabilis*. The study also explored the influence of nutritional and physical factors on the antimicrobial activity of the agent generated by isolate A7. The optimal conditions for antimicrobial activity were found to be a pH value of 6, an incubation temperature of 37°C, a NaCl concentration of 4 g/L, 1.5% starch concentration, 0.4% sodium nitrate concentration, and 2.5 g/L of $K_2HPO_4.3H_2O$. Additionally, 0.5 g/L of $MgSO_4$ was incorporated into the broth media. The incubation was performed in a shaking incubator for 7 days at 150 rpm. Ethyl acetate was used in combination with thin-layer chromatography to extract and purify the antimicrobial agent. Fraction No. 6 exhibited the highest activity against the tested microorganisms. The purified compound was identified as Phenol, 2-Methoxy-4-(2-Propenyl). It displayed antimicrobial activity against various microorganisms and demonstrated significant antiviral activity without any observed cytotoxic effects on the tested cell line.

Keywords: *Streptomyces mutabilis,* antimicrobial activity, 16S rRNA gene, GC-MS analysis

Introduction

Marine microbes are now widely used in the hunt for new microbiological products. Antimicrobial and antiviral compounds are found in a wide variety of marine microorganisms. Several of these materials are chemically distinct from any chemicals seen in terrestrial organisms **(Zhao *et al.*, 2011)**. According to **Zhao *et al.*, (2011),** extreme habitats' chemical and physical characteristics are markedly different from those of settings supporting a greater variety and abundance of living forms. Actinomycetes, among other microbes, have been shown to exist in a variety of hypersaline settings, including seawater and salty soils. However, these hypersaline habitats are still unexplored as a potential source of new actinomycetes for tropical and subtropical locations worldwide **(Xiang *et al.*, 2008).** Novel antibiotics and actinomycetes may be found in the marine environment. The three most prevalent genera within the maritime environment are Streptomyces, Micromonospora, and Nocardia. A few unique bioactive compounds have been identified from marine strains of actinomycetes and streptomycetes throughout the last ten years. **Canedo *et al.*, (2000)** with a wealth of biodiversity, Egypt's Mediterranean Sea is one of the country's most breathtaking coastal and marine habitats. The water is home to a variety of distinct microbial communities and marine ecosystems. As far as we know, few investigations have been done to separate actinomycetes from Mediterranean saltwater **(El-Gendy *et al.*, 2008).** The Sea is distinguished by its increased temperatures, strong sunshine, and high salinity. Actinomycetes like *Streptomyces* have been identified in this area. These actinomycetes have special metabolic properties and generate diverse secondary metabolites, some of which have potential uses in medicine and biotechnology. The objective of this research is to contribute to the exploration and advancement of novel antimicrobial agents derived from marine actinomycetes. These microorganisms, found in marine environments, exhibit distinct adaptations that are not found in their counterparts on land. The unique metabolic and genetic characteristics of marine actinomycetes make them an intriguing subject for future studies. Considering their long history of being utilized as a source of bioactive compounds, particularly antibiotics, it is essential to investigate their biological potential. Therefore, the primary aim of this work is to uncover and develop new antimicrobial agents sourced from natural marine environments, thereby expanding the repertoire of available treatments against microbial infections.

Materials and methods

Sampling area and collection

In Alexandria, Egypt, eight seawater samples were taken from various locations throughout the Mediterranean Sea. El-shatpy (31.210819°N 29.913783°E), El-max (31.163753°N 29.863214°E), El-montazh (31.2886°N 30.0159°Eand Cleopatra (31°20′N 27°13′E) were the collection locations. In order to further analyze the samples, they were taken from around 10 meters below the surface of the water, far about 2 km. from each shore and stored at 4°C.

Pretreatment of samples

A 1 mL sample of seawater was mixed with 9 mL of sterile water and vortexed for a duration of two minutes. The samples were heated for 10 min in a water bath at 60 ^{o}C to remove non-sporulating bacteria. After the suspension was serially diluted (10−1, 10−2, and 10−3) with sterile water. The actinomycetes isolation agar medium was made using natural seawater, and a 100-µL aliquot was put on top. The plates were then cultured for two to three weeks at 28°C **(Al-Dhabi *et al*., 2016 and Abdelfattah *et al*., 2016).**

Isolation, purification, and maintenance of actinomycete

Actinomycetes were isolated using a starch nitrate agar medium. Antifungal antibiotics (amphotericin B) were added to prevent fungal contamination **(Narendra *et al.,* 2010)**. Every sample included three plates incubated for seven to fourteen days at 37°C. The actinomycete colonies that were separated from the crowded plate were labeled and chosen for further investigation, while agar slants with starch nitrate agar were used to isolate, subculture, and store the pure colonies at 4 °C **(Zeatara *et al*., 2022)**.

Screening of Actinomycetes isolates for antimicrobial activity:

Several test microorganisms were used, including Gram-positive bacteria (*Streptococcus pyogenes MTCC 655, Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 6633). Gram-negative bacteria (*Salmonella typhi* ATTC14028, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATTCC 13315, *Enterobacter cloacae* LMG 2683, *Klebsiella pneumoniae* ATCC53637, and *Escherichia coli* ATCC 25922,) and test of yeast and fungal strains, (*Alternaria alternata* Te19, *Aspergillus terrus* SQU14026, *Fusarium chlamydosporum* F25, and *Candida albicans* MTCC183, *Candida albicans* ATTC90028). Microorganisms were obtained from Greater Cairo Water Company's Central Water Quality Laboratory and Ain Shams University's Faculty of Agriculture. Both are located in Cairo, Egypt. The preliminary screening of the isolated actinomycetes for antimicrobial activity was carried out using the Cork-borer method (**Cooper 1963, and Muharram *et al*.,2013)**. Plugs taken from actinomycete cultures were placed on an agar medium that was seeded with the test organisms. Agar well diffusion and starch nitrate broth were used to secondary screen isolates showing antimicrobial activity obtained from the preliminary screening **(Gebreselema *et al.,* 2013)** . Further experiments were conducted on the most effective isolate.

Identifying the most active isolate of actinomycete:

The most active actinomycetes isolates were identified by comprehensively examining their chemical, morphological, and physical properties. The identification process followed the conditions and guidelines outlined in Bergey's Manual of Determinative and Bacteriology Bergey's Manual of Systematic Bacteriology **(Williams *et al.,* 1989; Hensly, 1994 and Whitman *et al.,* 2012)**. Using starch nitrate agar medium and ISP methods, morphological characteristics were studied (**Shirling and Gottlieb, 1966)**, and the coverslip culture technique **(Kawato and Shinobu, 1959)**. A

cell wall analysis was performed using established methods **Becker *et al*. (1964); Lechevalier and Lechevalier, (1968)**, and microscopic examinations were performed using a light microscope and a scanning electron microscope (SEM)(JSM-5400; JEOL, Jeol 2100 High resolution, Musashino, Akishima, Tokyo, Japan), in Cairo, Egypt, the National Center for Radiation Research and Technology (NCRRT) carried out the research. Sigma Company for Genetics and Scientific Service Company, Cairo, Egypt, performed a molecular identification using 16S rRNA gene sequences by aligning sequencing products with similar sequences from the Gene Bank database using Cluster X **(Thompson, *et al.,* 1997)** and NCBI/BLAST **(Altschul *et al.,*1997)** to determine the highest similarity.

The effect of optimal cultural conditions on the growth and antimicrobial activity of the selected actinomycete isolates

The optimization process involved conducting a series of experiments where one variable was changed at a time while keeping other factors constant. In this study, the goal was to identify the conditions that maximize antimicrobial activity. As a measure of antimicrobial activity, the inhibition zones were measured 24 hours after each experiment using the ethyl acetate extract as the most potent bioactive compound.

Optimization of physical parameters

This step involved optimizing the incubation time, temperature, and pH. The fermentation starch nitrate broth medium (SNB) was used to test and select the best bioactive compounds in terms of production **(Saadoun *et al*., 2008 ;Suthindhiran *et al.,* 2009 and Ahmed *et al.,* 2016).**

Optimization of biochemical parameters

Various biochemical parameters were considered for optimization, including different sources of sodium chloride concentrations, nitrogen and carbon compounds, different concentrations of the best nitrogenous and carbon sources, and different concentrations of $MgSO_4.7H_2O$ and K_2HPO_4, different amino acids. Additionally, bioactive compounds were tested for their effects on different heavy metals these heavy metals are zinc sulphate, cobalt chloride, cadmium sulphate, and copper sulphate (**Singh *et al.,* 2009; Usha *et al*., 2010 ; Li *et al.,* 2010 and Ahmed *et al.,* 2016)**.

Fermentation and extraction of active antimicrobial compounds produced from the most potent actinomycete isolate.

The active antimicrobial compounds from the most potent actinomycete isolate were produced by submerged fermentation **(Syed and Rekha 2019)**. The fermentation broth of isolates was prepared and centrifuged at 5000 rpm for 20 min. The supernatant was filtered; the filtrate was subjected to different solvent extraction to recover antimicrobial metabolites in pure form. Organic solvents (chloroform, ethyl acetate and sum of chloroform and ethyl acetate) were used in the extraction of the antimicrobial metabolites. Chloroform and ethyl acetate solvents were added to the filtrate in a ratio of 1:1 (v/v) and were shaken vigorously for 1 h for complete extraction. Also, the

filtrate was collected, extracted by a mixture of chloroform: ethyl acetate (1:1 v/v). The mixture was added to the broth filtrate by 1:4 respectively**.** (**Zeinab, 1987;Noura *et al.*,2017 and Mohamed *et al*., 2017)**. A microbial test was conducted using the filter paper disc diffusion method to test the antimicrobial activity of the crude extract. The best solvent for producing antimicrobials was found to be ethyl acetate.

Detection of antimicrobial active compounds in actinomycete isolates crude extracts by TLC.

To visualize the number of compounds and investigate the purity degree of the crude ethyl acetate extract present in most active actinomycetes isolates. Thin layer chromatography (TLC) using silica gel plate was performed. Aluminum plates pre-coated with silica gel (20×20 cm, 0.25 mm Alugram® SIL G/UV 254, Macherey and Nagel, Duren) and more than nine mobile phases tested to select the perfect solvent system to further separated by column chromatograph. Different solvent systems were used, consisting of ethyl acetate solution (Dichloromethane: Ethyl acetate = 100:1, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1, v/v), ethyl acetate-methanol solution (Ethyl acetate: Methanol = 30:1, 20:1, 10:1, 5:1, 2:1, 1:1, v/v), and Dichloromethane : Ethyl acetate : Methanol (70:20:10 v/v) (**Attimarad *et al.,* 2012).** TLC analysis was performed to visualize the number of compounds present and assess the purity of the crude ethyl acetate extract. Column chromatography was used to further separate the samples using multiple mobile phases **(Atta *et al.,* 2009).** The antimicrobial activity-guided fractionation of bioactive fractions was tested using the agar well diffusion method (0.1 mm.) against tested microorganisms. And all the individual fractions show antimicrobial agent were analyzed by TLC for homogeneity, The compounds separated by TLC were visualized by spraying with vanillin/sulphuric reagent, 0.5 g vanillin in 100 ml sulphuric acid/ ethanol (40: 10). The TLC plates were examined under a UV lamp at a wavelength of 254–365 nm. Finally, the purified compound was collected and evaporated using a rotary evaporator, (SENECO Technology Co., Ltd., Taiwan). for characterization purposes. at **Nawah-Scientific compony AlMokattam, Cairo, Egypt.**

Identification and characterization of pure active compounds

An analysis of the physiochemical properties of the purified substances obtained from the most active isolate of actinomycete was conducted. Using UV analysis, 1H NMR spectroscopy, IR spectroscopy, and GC-MS analysis, we evaluated their elemental content, solubility, spectroscopic characteristics and empirical formula. A study conducted at Cairo, Egypt's Nawah-Scientific Company provided the data for this study.

Testing the cytotoxicity of purified active substances extracted from the most active actinomycete isolate on Vero cell line

The purified active substances of the most active isolate of actinomycete were tested for their cytotoxic effects on Vero cells (ATCC CCL-81). Actinomycete extracts were tested at different concentrations (control, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 150, 250, and 500 g/mL). **(Andrighetti-Fröhner *et al.,* 2003 and Todorov *et al.*,2015**) determined the maximum tolerated concentration without causing toxicity or

morphological changes. Utilizing GraphPad Prism software (San Diego, CA, USA), the 50% inhibitory concentration (IC50) was calculated using absorbance values and dose-response curves.

2.10 Antiviral assay

The herpes simplex virus (HSV-1), Vero cell line (ATCC: CCL-81), and corona 229E virus were used in this study **(Khaled *et al.*,2021; Mostafa, *et al.*,2020 and Hanan *et al.*, 2022)** . The antiviral activity of the purified active compounds was assessed using these cell line and viruses. All viruses and vero cell were obtained from **Nawah-Scientific compony AlMokattam, Cairo, Egypt.**

Results

Actinomycetes that produce antimicrobials: isolation and screening:

Based on colony morphology and microscopic appearance, twenty-six actinomycetes were isolated. A total of eleven actinomycetes were isolated from Cleopatra, six from Elmontazh, five from Elshatpy, and four from Elmax. Even after six weeks of cultivation, several collected water samples did not produce actinomycetes. Out of 26 actinomycetes, only 9 isolates exhibited potent potency to inhibit at least one of the test microorganisms under study after 24 hours of incubation. A secondary screening was performed on these 9 isolates; one isolate from Cleopatra (A7) showed strong antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and fungi. (Table 1)

Table (1): Secondary screening for antimicrobial activities of the nine isolates against the tested bacterial and fungal species

		Mean diameter of inhibition zone (mm)								
		Isolates No.								
	Test Strains	1	2	3	4	5	6	7	8	9
Gram positive	*B. subtilis* ATCC 6633	15	28	30	25	-ve	15	38	26	23
	S. aureus ATCC 6538	20	37	28	15	23	12	39	31	22
	S. pyogenes MTCC 655,	9	30	11	17	14	14	24	12	22
Gram negative bacteria	*P. aeruginosa ATCC 27853*	11	9	-ve	16	17	12	12	-ve	13
	E. coli ATCC 25922	19	16	20	15	-ve	10	22	20	18
	S. typhi ATTC14028	15	28	12	17	11	9	30	12	15
	K. pneumonia ATCC53637	18	19	10	-ve	11	9	29	11	15
	P. vulgaris ATTCC 13315	13	18	28	12	21	23	31	10	18
	E. cloacae LMG 2683	9	28	11	15	25	12	27	-ve	-ve
Fungi	*F. chlamydosporum F25*	35	37	33	27	11	-ve	38	35	-ve
	A. alternata Te19	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	terrus SQU14026	29	18	20	9	10	11	25	-ve	-ve
Yeast	*C. albicans ATTC90028*	18	11	12	-ve	16	-ve	16	14	15
	C. albicans MTCC183	14	15	12	13	29	20	30	17	12

Identification of the most promising actinomycetes isolates A7

Characterizations of the actinomycete isolate A7

Taxonomic characterizations were conducted to identify the most active isolate, A7. Morphological characteristics, such as spore production color of substrate and aerial mycelia, were observed in different cultures and ISP media. The identification process followed international keys and specific characteristics of the actinomycetes (Tables 2a. and 2b, Fig. 1).

Table (2a.): cultural characteristics of the actinomycetes isolate A7

Types of media	Growth	Substrate mycelium	Aerial Mycelium	Diffusible pigment
Starch nitrate agar	Excellent	Light gray (ISCC-NBS-264)	White (ISCC-NBS-263)	Brilliant orange yellow (ISCC-NBS-67)
Yeast- malt extract agar (ISP2)	Moderate	White (ISCC-NBS-263)	Light grayish yellowish brown (ISCC-NBS-79)	Moderate brown (ISCC-NBS-58)
Oatmeal agar (ISP3)	Good	White (ISCC-NBS-263)	Light gray (ISCC-NBS-264)	Brownish gray (ISCC-NBS-64)
Inorganic salts-starch agar (ISP4)	No growth	-	-	-
Glycerol- asparagine agar (ISP5)	No growth	-	-	-
Melanine production medium 1-Tryptone-yeast extract broth (ISP 1)	Good	Deep orange yellow (ISCC-NBS-69)	Deep orange yellow (ISCC-NBS-69)	Deep orange yellow (ISCC-NBS-69)
2- Peptone iron agar (ISP 6)	Good	Grayish yellowish brown (ISCC-NBS-80)	Dark grayish yellowish brown (ISCC-NBS-81)	Grayish yellowish brown (ISCC-NBS-80)
3- Tyrosine agar medium (ISP7)	Good	Light grayish yellow (ISCC-NBS-79)	Grayish yellowish brown (ISCC-NBS-80)	Light grayish yellow (ISCC-NBS-79)

***ISP:** I n t e r n a t i o n a l journal o f s y s t e m a t i c bacteriology

***ISCC-NBS**: Intersociety Color Council- National Breau of Standards.

Table (2b.): Morphological, physiological, and biochemical characteristics of actinomycetes isolate A7.

Character	Results
1- morphological characteristics:	
- Spore chain	Retinaculiaperti type
- Spore mass	White
- Spore surface	Irregular rugose
2- Chemotaxonomic analysis:	
- Cell wall hydrolysis for: Diaminopimelic acid	LL- DAP
3-Physiological characteristic:	
a- Melanin pigment:	
- Tryptone-yeast extract broth (ISP 1)	+
- Peptone iron agar (ISP 6)	+
- Tyrosine agar medium (ISP7)	-
b-Carbon utilization:	
- starch,	+++
- D-glucose,	+++
- dextrose,	+++
- sucrose,	++
- D-fructose,	+++
- Maltose,	+++
- Galactose,	++
- L-arabinose,	-
- D-xylose,	-
- cellulose	-
- D-mannitol	-
c-Nitrogen utilization	
- Tryptophan	++
- Arginine	++
- Alanine	++
- Proline	++
- Tyrosine	++
- Glycine	+
• **Growth pH**	
2-3	WG
4-9	+
10-13	-
• **Growth temperatures ^{o}C:**	
20-45	+
• **Growth in presence of Na Cl conc. (%)**	
1-12	+

All results compared to control with standard starch nitrate agar medium

(+) =positive, **(-)** =negative, (**WG**) =weak growth, **(+++)** =very good growth, **(++)** =good growth

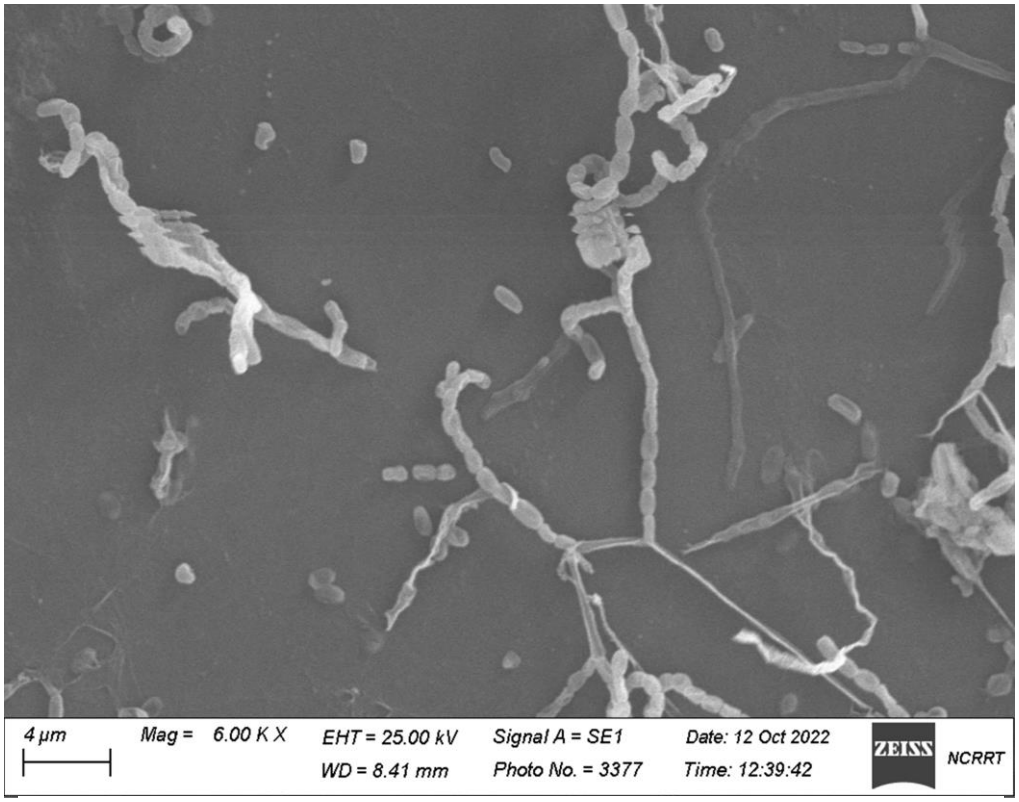


Fig. (1): Scanning electron micrograph of isolate A7 showing the irregular rugose spore surface.

Identification of selected isolate A7 based on its molecular composition

Molecular identification of isolate A7 was performed by amplifying the DNA sample using PCR and targeting the 16S rRNA gene. The obtained partial sequence was compared to the NCBI database using BLAST, revealing a 99.87 % similarity to *Streptomyces mutabilis* strain NBRC 12800, It was given the name *Streptomyces mutabilis* strain AZHD22 and deposited in the NCBI GenBank with accession number OR632696.1. A phylogenetic tree was constructed to visually represent the relationship between isolate A7 and other *Streptomyces* species (Fig.2).

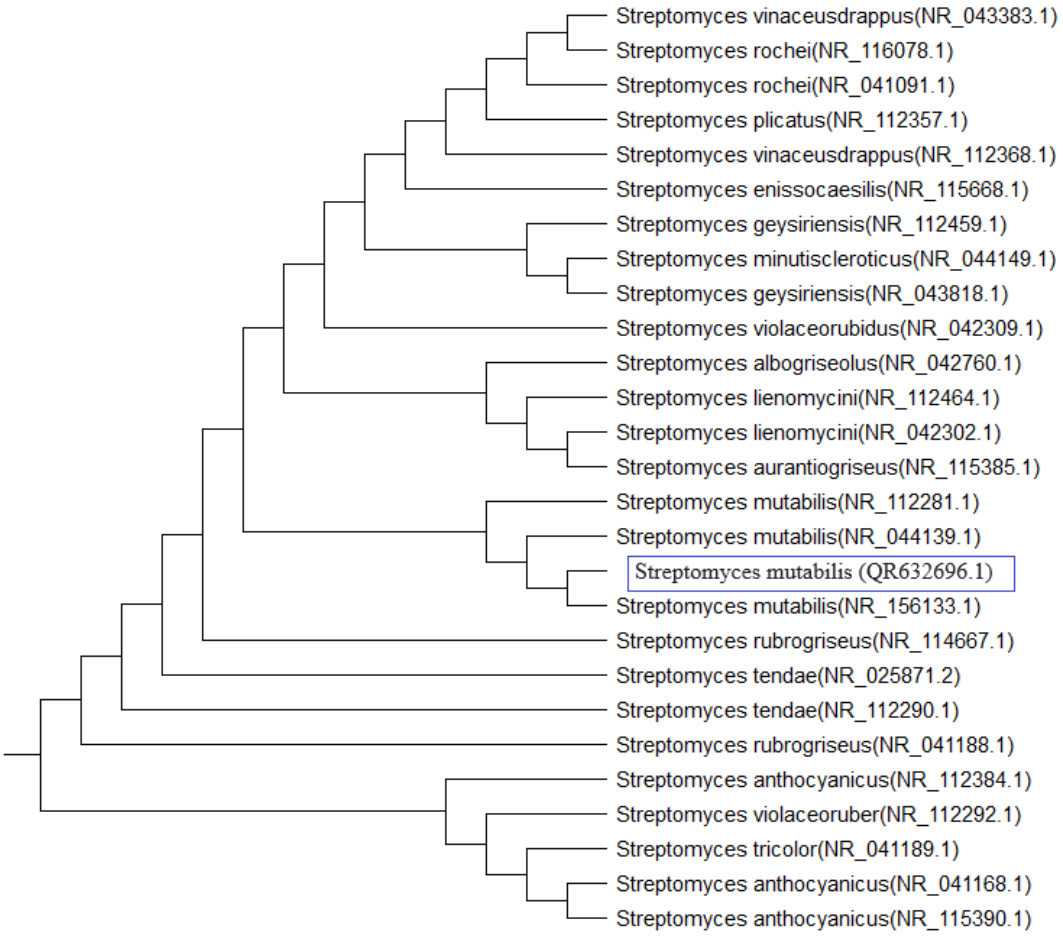


Fig. (2): Phylogenetic tree (dendrogram) showing the sequence relationships between isolate A7 strain and other strains related *Streptomyces* sp. based on the 16S rRNA gene sequences.

Genetic identification of *Streptomyces mutabilis* isolate A7 by optimization of biochemical and physical parameters

The optimization of biochemical and physical parameters for antimicrobial production of *Streptomyces mutabilis* AZHD22 was investigated. The initial test compared static and shaking conditions for extracting bioactive substances from the isolate, and submerged cultivation resulted in significantly higher antimicrobial activity compared to static conditions (Table 3)

Table (3): Effect of different cultivation methods of *S. mutabilis* AZHD22 on tested microorganisms.

	Test Strains	Mean diameter of inhibition zone (mm) *S. mutabilis* AZHD22	
		St.	Sh.
Gram positive	*B. subtilis* ATCC 6633	35	44
	S. aureus ATCC 6538	25	39
	S. pyogenes MTCC 655,	12	24
Gram negative bacteria	*P. aeruginosa ATCC 27853*	12	16
	E. coli ATCC 25922	19	22
	S. typhi ATTC14028	28	32
	K. pneumonia ATCC53637	29	30
	P. vulgaris ATTCC 13315	29	32
	E. cloacae LMG 2683	13	19
Fungi	*F. chlamydosporum F25*	34	38
	A. alternata Te19	30	35
	A. terrus SQU14026	21	25
Yeast	*C. albicans ATTC90028*	13	19
	C. albicans MTCC183	27	32

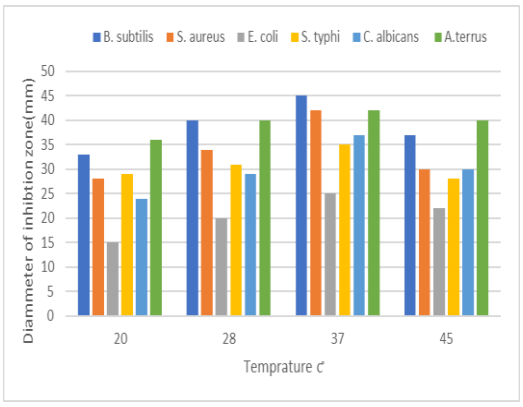
St.=static/ sh.=shaking

The effect of different initial pH values on the production of active metabolites by *S. mutabilis* AZHD22 was studied. The optimal pH value for the maximum inhibition zone was found to be 6, resulting in antimicrobial activity ranging from 30 to 47 mm. (Fig.3a.). Temperature was identified as a critical parameter. The maximum antimicrobial metabolite production occurred at an incubation temperature of 37°C, with inhibition zones ranging from 25 to 45 mm. (Fig.3b.). The suitable incubation period for maximum active metabolite yield was determined to be 7 days on SNB medium, resulting in inhibition zones ranging from 25 to 45 mm. (Fig.3c.). The optimal NaCl concentration for antimicrobial compound production was found to be 4 g/L. Increasing the salt concentration beyond this point led to a reduction in antimicrobial agent biosynthesis, which showed antimicrobial activity ranging between 30 and 45 mm (Fig.3d.).

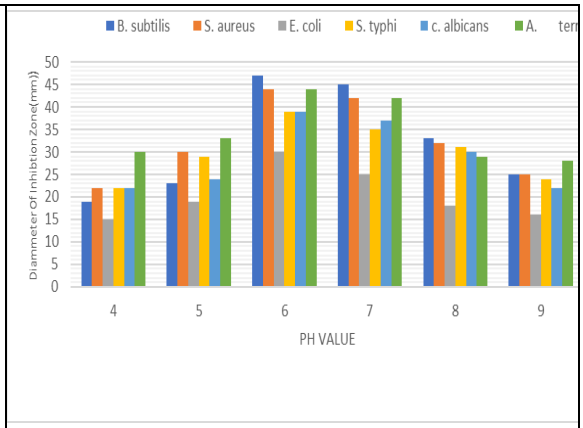
Starch was performed to detect the best carbon source, with the highest antimicrobial metabolite productivity observed at a concentration of 1.5 % (Figs. 3e. and 3f.). Of all examined nitrogen sources, sodium nitrate was the most effective for antimicrobial metabolite production, with the concentration of 0.4% determined to be the optimal nitrogen source for antimicrobial activity, resulting in inhibition zones ranging from 25 to 45 mm (Fig.3g. and 3h.). K_2HPO_4 at a concentration of 2.5 g/L was found to promote the highest yield of active metabolites, with inhibition zones ranging from 25 to 45 mm (Fig.3i.).

The results presented in Figure (3j.) indicate that the highest biosynthesis of bioactive compounds was achieved for *S. mutabilis* AZHD22 in the presence of 0.5 g/L of $MgSO_4 \cdot 7H_2O$, with inhibition zones ranging from 30- 47 mm. Also, between different amino acids, tryptophan had an inhibitory effect, while other amino acids had either slight effects or slight retarding effects on antimicrobial productivity, with inhibition zones ranging from 25- 45 mm (Fig.3k.).

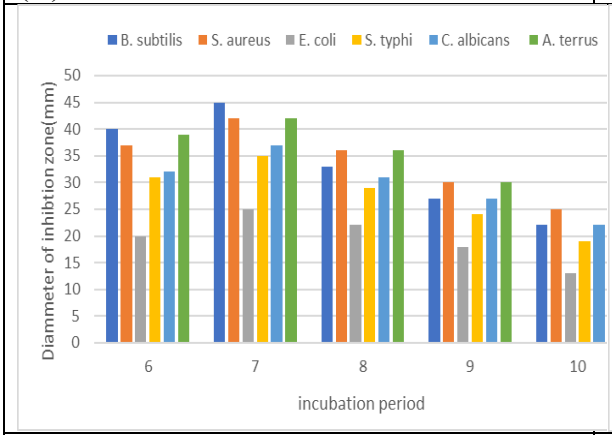
As it is evident from the results given in Fig. (3l.), zinc sulfate-induced maximum bioactive compounds biosynthesis by *S. mutabilis* AZHD22, with antimicrobial activity ranging between 25 to 45 mm.



(A)



(B)



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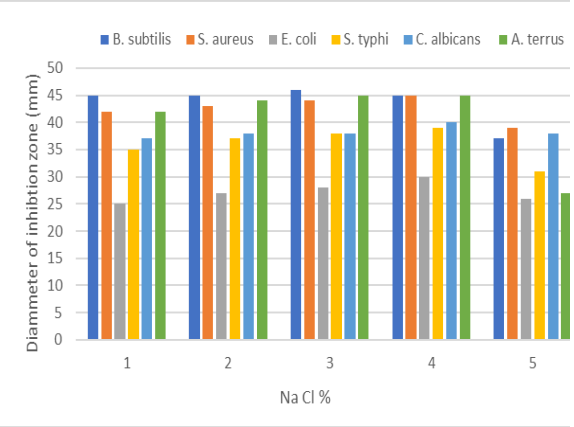
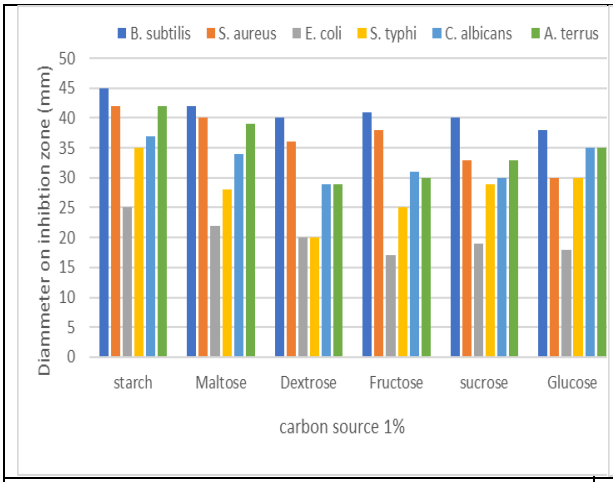
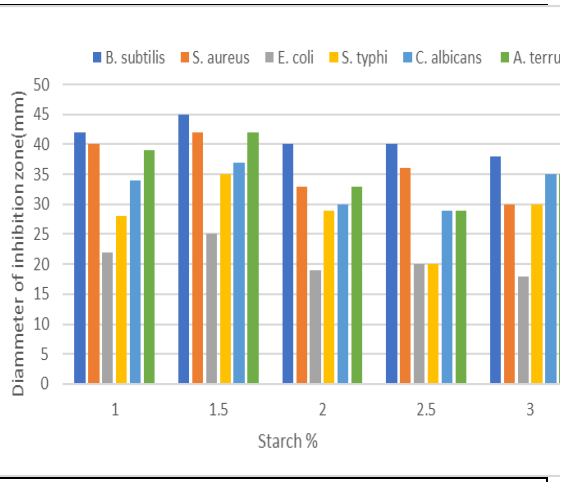


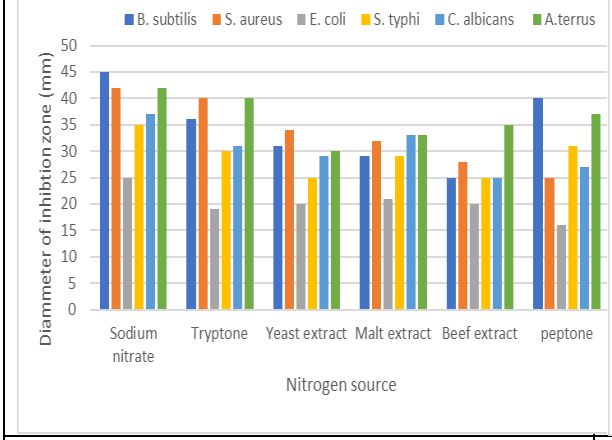
(D)


B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter on inhibtion zone (mm)
starch
Maltose
Dextrose
Fructose
sucrose
Glucose
carbon source 1%

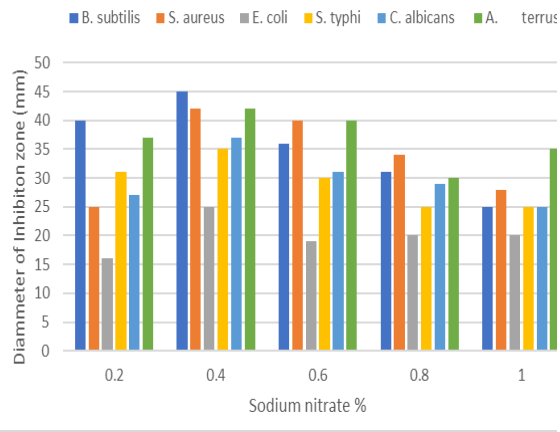
(E)


B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terru
Diammeter of inhibition zone(mm)
Starch %

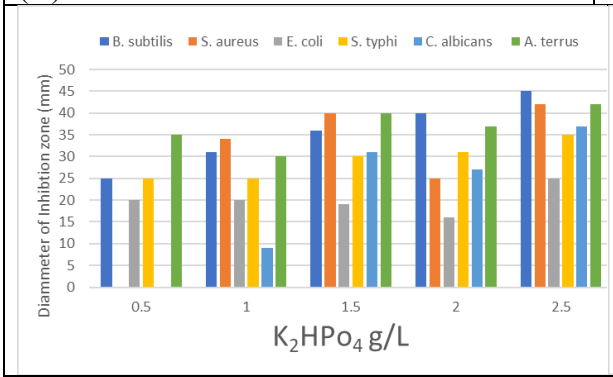
(F)


B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A.terrus
Diammeter of inhibtion zone (mm)
Sodium nitrate
Tryptone
Yeast extract
Malt extract
Beef extract
peptone
Nitrogen source

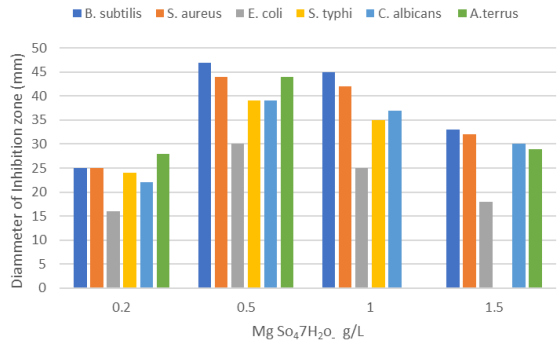
(G)


B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A.
terrus
Diammeter of Inhibiton zone (mm)
Sodium nitrate %

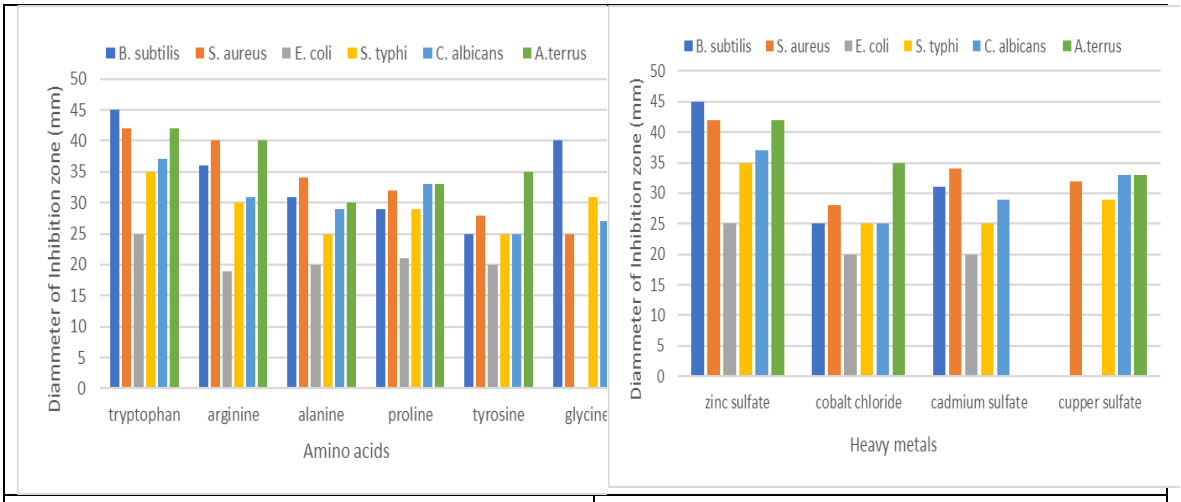
(H)


B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter of Inhibtion zone (mm)
K2HPo4 g/L

(I)


B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A.terrus
Diammeter of Inhibition zone (mm)
Mg So4 7H2O. g/L

(J)



(K) (L)

Fig.(3): Effect of different physical and biochemical parameters for antimicrobial production of *Streptomyces. mutabilis* AZHD22., **(a)** pH, **(b)** Temperature, **(c)** Incubation period, **(d)** Na Cl %, **(e)** Carbon source 1%, **(f)** Glucose %, **(g)** Nitrogen source, **(h)** Sodium nitrate %, **(i)** K_2HPO_4 a conc. **(j)** $MgSO_4 \cdot 7H_2O$ conc. **(k)** Amino acid, **(l)** Heavy metals.

Production, extraction, and purification of active antimicrobial compounds

To produce the active antimicrobial substances, *S. mutabilis* AZHD22 was cultivated in large-scale (15 L) submerged fermentation cultures under the optimized conditions mentioned previously. Shaken flasks containing 50 mL of starch-nitrate broth (SNB) were used, and sterile conditions were maintained throughout the process. The most potent antimicrobial-producing actinomycetes were selected for this study. Different solvents were evaluated for the extraction of the active antimicrobial compounds, and ethyl acetate was found to be the most effective solvent. The antimicrobial compound produced by *S. mutabilis* AZHD22 was extracted using an equal volume of ethyl acetate, resulting in the formation of a white-yellowish powder precipitate. Thin-layer chromatography (TLC) analysis was performed on the extract using a solvent system composed of dichloromethane (DCM), ethyl acetate, and methanol (70:20:10 v/v). This solvent system provided the best elution and separation of bands. Purification of the antimicrobial compounds was carried out using silica gel column chromatography, with the D.C.M.:ethyl acetate: methanol (70:20:10 v/v) solvent system used as the eluting solvent. Using agar well diffusion assays, 11 fractions (5 mL each) were collected and tested for antimicrobial activity. Fraction 6 (F6) from *S. mutabilis* AZHD22 exhibited the highest antimicrobial activity, as evidenced by the largest inhibition zone. This bioactive fraction was further examined for homogeneity using TLC, which confirmed the presence of a single spot. The selected fraction was then evaporated under a reduced vacuum to obtain a dried sample for further studies.

Identification and characterization of F6 obtained from *S. mutabilis* AZHD22.

Physicochemical Characteristics of F6 .

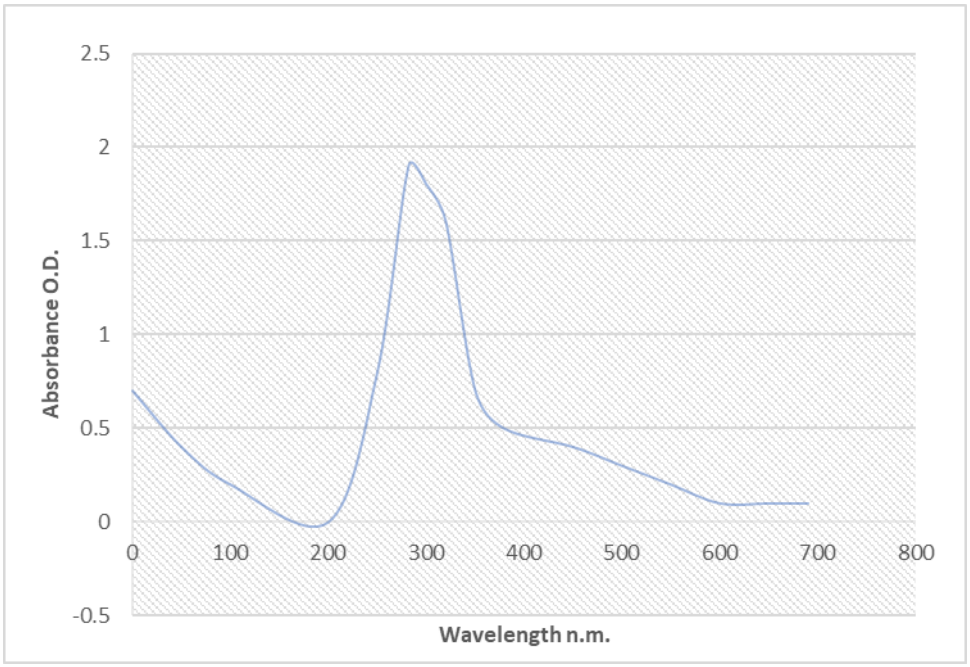
F6 obtained from *S. mutabilis* AZHD22 exhibited favorable solubility in various solvents, including ethyl acetate, chloroform, acetone, toluene, butanol, benzene, ethanol, methanol, and diethyl ether. The purified active substances (100 µg or 1 mg) dissolved in 5 mL of each solvent and were precipitated by petroleum ether.

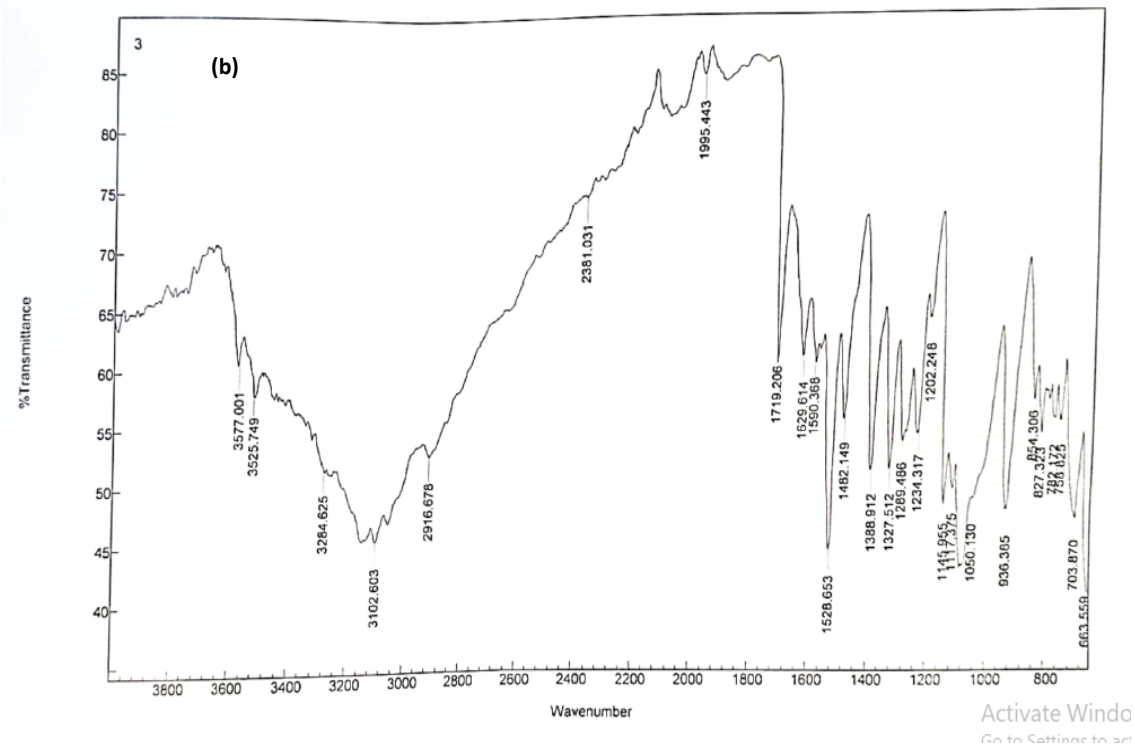
The spectroscopic characteristics of F6 from *S. mutabilis* AZHD22.

The ultraviolet (UV) spectrum of Fraction 6 obtained from *S. mutabilis* AZHD22 was examined, as shown in Figure (4a.). The spectrum displayed a peak absorbance at 282 nm, indicating a high intensity at this wavelength within the range of 200 to 800 nm. Furthermore, the Fourier-transform infrared (FTIR) absorption of Fraction 6 was analyzed and depicted in Figure (4b.). The IR spectrum exhibited characteristic bands, including a broad O-H (hydroxyl) stretching band in the range of 3300-3600 cm-1, representing phenol groups. A strong, sharp C=C stretching band at approximately 1600-1620 cm-1 indicated the presence of an aromatic ring. Additionally, a weak C-H bending band at 700-900 cm-1 suggested the existence of aromatic carbon-hydrogen bonds. Other notable bands included a C-O stretching band at 1000-1300 cm-1, indicating the presence of a methoxy (O-CH3) group, and a C-H stretching band at 3000-3100 cm-1, indicating aliphatic carbon-hydrogen bonds. The C=C-H bending band at 1000-1300 cm-1 suggested the presence of a propenyl group (CH2=CH-CH3). Moreover, the proton nuclear magnetic resonance (H NMR) spectrum of Fraction 6 extracted from *S. mutabilis* AZHD22 was analyzed (see Figure 4c.). The spectrum revealed the presence of aromatic protons (H in the phenyl ring) in the range of δ 6.5-7.5 ppm, methoxy group (CH3O-) in the range of δ 3.5-4.5 ppm, and allyl group (CH2=CH-CH2) in the range of δ 5.5-6.5 ppm.

The purified active substance, known as fraction 6, was subjected to elemental analysis, revealing the following composition (%w/w): 73.14% carbon (C), 7.37% hydrogen (H), and 19.49% oxygen (O). Based on previous analyses and empirical calculations, the active substance was determined to have the empirical formula $C_{10}H_{12}O_2$. The mass spectrum of fraction 6, obtained through gas chromatography separation, is presented in Figure (4d.). The compound displayed a retention time of 13.49 and matched the chemical formula $C_{10}H_{12}O_2$ with a molecular weight of 164. Through analysis using a GC-mass spectrophotometer device, it was confirmed that the compound is Phenol, 2-Methoxy-4-(2-Propenyl), as depicted in the mass spectrum figures (4d. and 4e.). The major peaks accounted for 89.5% of the total peaks, with Phenol, 2-Methoxy-4-(2-Propenyl) exhibiting the highest peak area at an RT (retention time) of 13.49 and an MF (molecular formula) of 860.

(a)


Absorbance O.D.
Wavelength n.m.
2.5
2
1.5
1
0.5
0
-0.5
0
100
200
300
400
500
600
700
800


(b)
%Transmittance
Wavenumber
3577.001
3525.749
3284.625
3102.603
2916.678
2381.031
1995.443
1719.206
1629.614
1590.368
1528.653
1482.149
1388.912
1327.512
1289.486
1234.317
1202.248
1144.955
1117.375
1050.130
936.365
854.306
827.323
788.172
750.625
703.870
663.559

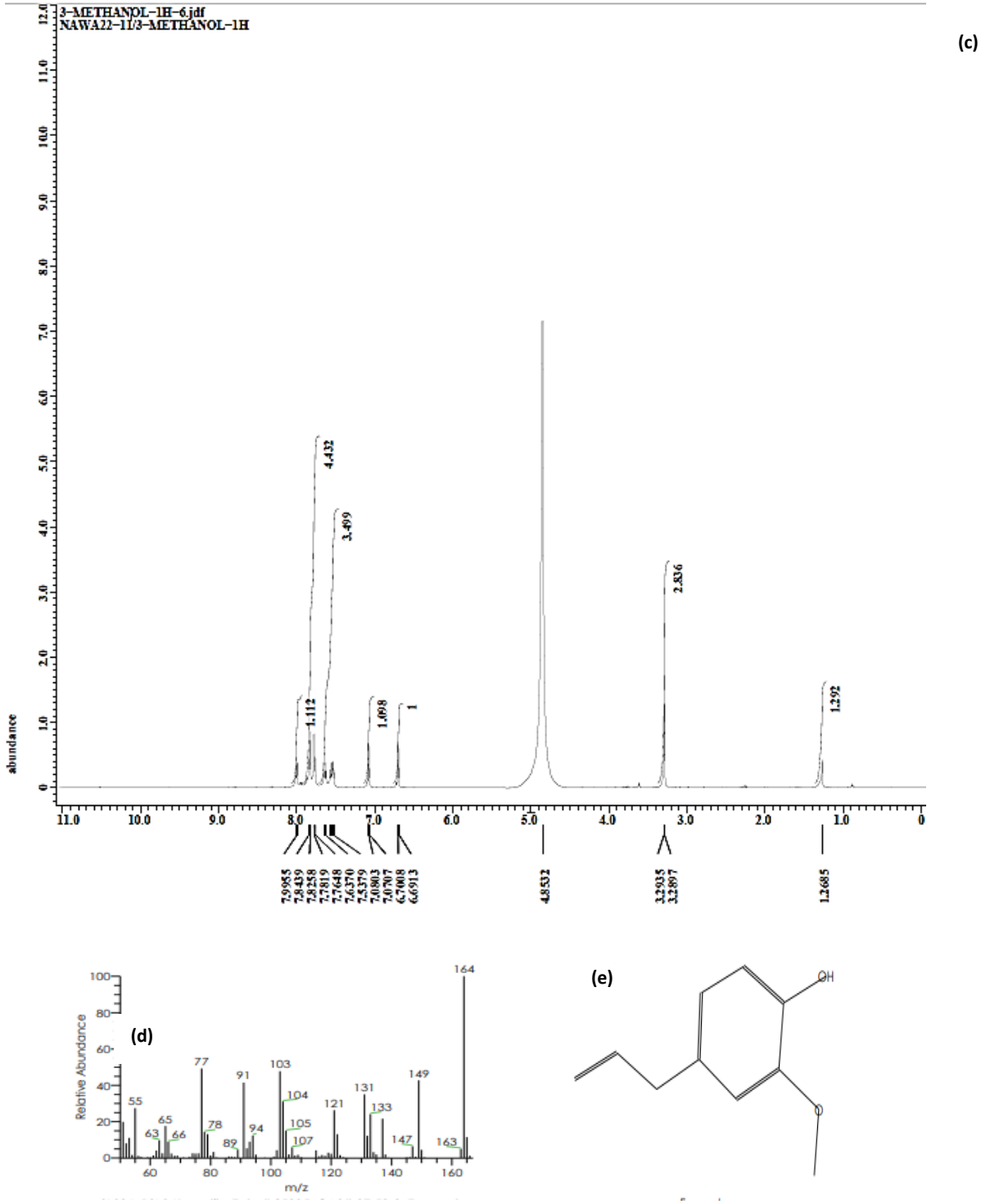


Fig. (4): The spectroscopic characteristics of F6**, (a)** UV Spectrum, **(b)** The infrared spectrum (IR), **(c)** ^{1} H NMR, **(d)** GC-MS analysis, **(e)** Chemical structure of Phenol, 2-Methoxy-4-(2-Propenyl), formula $C_{10}H_{12}O_2$ with a molecular weight of 164.

Cytotoxic assay of Phenol, 2-Methoxy-4-(2-Propenyl).

To evaluate the cytotoxicity of Phenol, 2-Methoxy-4-(2-Propenyl), a cytotoxic assay was conducted using Vero cells, a non-tumor cell line derived from African green monkey kidney. Different dilutions of the purified compound were incubated with the cells for 24 hours at 37°C. In addition to demonstrating antibacterial and antifungal activities, the pure bioactive compound produced by *S. mutabilis* AZHD22 exhibited antiviral activity within a non-toxic dilution range of 0 to 500 µg/mL. Figure (5) presents the results, indicating an IC50 value of 150 µg/mL for the purified compound, which did not cause cell death in Vero cells. However, at a dosage of 500 µg/mL, the compound was found to be toxic to the Vero-E6 cell line.

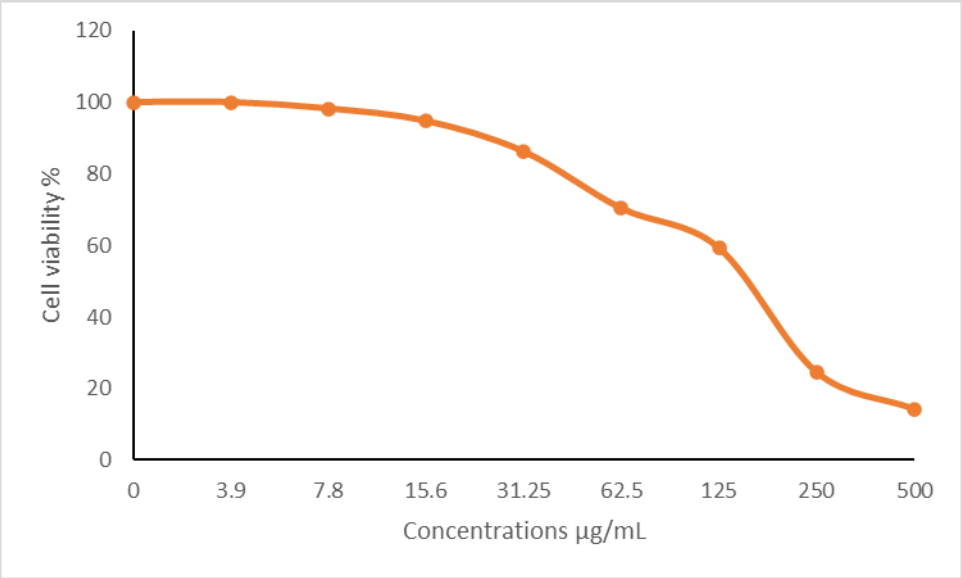


Fig. (5): Cytotoxicity assay of the purified active compound (Phenol, 2-Methoxy-4-(2-Propenyl) on the cell line.**Antiviral activity of Phenol, 2-Methoxy-4-(2-Propenyl).**

Regarding the antiviral activity, Table (4) shows that at a dosage of 150 µg/mL, the pure active metabolite Phenol, 2-Methoxy-4-(2-Propenyl) produced by *S. mutabilis* AZHD22 inhibited human coronavirus 229E by 18.5%. But only 38.5% of it shown antiviral effectiveness against the type 2 Herpes simplex virus.This indicates that the purified substance exhibits significantly stronger antiviral activity against Herpes simplex virus type 2 compared to human coronavirus 229E.

Table (4): Antiviral activity of Phenol, 2-Methoxy-4-(2-Propenyl) by rapid antiviral activity against Human Coronavirus (229E) and Herpes simplex virus type-2 tested on Vero cell line

Type of virus	**Fraction 6 concentration (µg/ml)**	Virus Control (PFU/ml)	Virus titer & treatment (PFU/ml)	Viral Inhibition (%)
Herpes simplex virus type -2	150	$1.35* 10^3$	$8.33* 10^2$	38.58
Human Coronavirus (229E)	150	$1.3*10^4$	$1.06*10^4$	18.5

Discussion

The vast expanse of the Earth's seas and oceans, covering over 70% of the surface, represents a valuable reservoir of microorganisms and bioactive secondary metabolites **(Orlova *et al.*, 2015).** However, there have been limited studies on the isolation of actinomycetes from the Mediterranean Sea. This study collected eight seawater samples from various locations in the Mediterranean Sea near Alexandria. A total of twenty-six different actinomycete strains were isolated and characterized, with isolate A7 exhibiting potent bioactivity as evidenced by a wide range of inhibition zones in both primary and secondary screenings. International keys were employed to identify the selected actinomycete isolate (**Kawato and Shinobu, 1959;Shirling and Gottlieb, 1966; Williams *et al.*, 1989; Hensly, 1994 and Whitman *et al.*, 2012**). Based on biochemical, physiological, and morphological characteristics, the most potent isolate, A7, was identified as a species of *Streptomyces*. Further identification through 16S-rRNA analysis revealed a match to *Streptomyces mutabilis* strain NBRC 12800, and it was designated as *Streptomyces mutabilis* strain AZHD22 and deposited in the NCBI GenBank with accession number OR632696.1.

The study investigated the effects of different environmental and nutritional factors to optimize the cultural conditions for maximum antimicrobial compound production *by S. mutabilis* AZHD22. Submerged cultivation was found to significantly enhance antimicrobial activity compared to static culture, consistent with previous research (**Hassan *et al.*, 2001;Padma *et al.*, 2002 and Venkateswarlu *et al.*, 2004;)**, as *Streptomycetes* are obligate aerobic organisms **(Kämpfer *et al.*, 2012)**.

The effect of pH on bioactive compound production by *S. mutabilis* AZHD22 was observed to be optimal at an initial pH of 6. Most published studies have reported near-neutral pH as the optimum for bioactive compound production in Streptomyces cultures **(Nadhim and Marwa, 2016)**. **Hassan *et al.*, (2001) and Adinarayana *et al.*, (2003)** reported that *S. mutabilis* AZHD22 produced the most antimicrobial secondary metabolites at 37°C, which is in accordance with our findings**.** The antimicrobial activity reached its peak after 7 days of incubation, with no variation between 7 and 9

days, confirming the presence of two distinct phases (trophophase and idiophase) in antibiotic production **(Guimarães *et al.*, 2004 and Remya and Vijayakumar, 2008)**

Regarding salt concentration, the maximum bioactive compound production by *Streptomyces mutabilis* AZHD22 was achieved at a 4% NaCl concentration, in line with the findings of **Zhang *et al.*, (2016) and Sweetline and Usha (2021)**. Starch was identified as the best carbon source for *S. mutabilis* AZHD22, exhibiting maximum antimicrobial activity at a concentration of 1.5%, which is consistent with the studies conducted by **Nayera *et al.*, (2012) and Ahmed *et al.*, (2020).**

Nitrogen sources play a crucial role in regulating secondary metabolism. Sodium nitrate was determined to be the optimal nitrogen source, with a concentration of 0.4%, resulting in the highest biosynthesis of secondary metabolites by *S. mutabilis* AZHD22. These results align with the findings of **Gupte and Kulkarni, (2002) and Narayana and Vijayalakshmi, (2008), ,** who demonstrated that sodium nitrate and soya beans were ideal nitrogen sources for bioactive metabolite production by Streptomyces species.

Among the tested minerals, K_2HPo_4 was found to support high yields of bioactive compound production (2.5 g/L) from *S. mutabilis* AZHD22. This is in agreement with the observations of **Hassan *et al.*, (2001),** who reported that K_2HPo_4 was not favorable for antibiotic production by *S. violatus*, while K_2HPo_4 showed favorability at a concentration of 2.5 g/L with an inhibition zone of 22 mm. **Ahmed *et al.*, (2016)** also noted that an optimum concentration of 2.0 g/L of K_2HPo_4 was required for antimicrobial compound production, with a gradual decrease in production observed at higher concentrations.

more the one **(Kang *et al.*, 1998)** researchers have highlighted the significance of magnesium sulphate for antibiotic production by other *Streptomyces* species **.** The effects of magnesium availability are believed to be linked to the cation's requirement for protein synthesis, and its depletion may hinder enzyme synthesis and activity **(Natsume *et al.*, 1994).** Consistent with these findings, our study demonstrated that magnesium sulphate at a concentration of 0.5 g/L was suitable for producing antimicrobial compounds by *S. mutabilis* AZHD22.

This study found that tryptophan is the most effective amino acid for maximizing the yield of active metabolites produced by *S. mutabilis* AZHD22. Several researchers have highlighted the significance of amino acids in synthesizing certain antibiotics. Specifically, L-tyrosine and glycine play a crucial role in the production of Erythromycin, while tryptophan is involved in the biosynthesis of chlortetracycline. Additionally, tryptophan and glycine are necessary to produce Chloramphenicol **(Mccormick and Fl€Ardh, 2012).** Zinc sulfate was observed to induce the highest biosynthesis of bioactive compounds by the bacteria, including *Streptomyces*.

Ethyl acetate was identified as the optimal solvent for extracting antimicrobial metabolites from *S. mutabilis* AZHD22. Previous studies have also utilized ethyl acetate as a solvent for extracting antimicrobial metabolites from actinomycetes **(El-Naggar *et al.*, 2017;Retnowati *et al.*, 2018; Apsari *et al.*, 2019; Srivastava and Shanmugaiah**

2019). Fraction 6 extracted from *S. mutabilis* AZHD22 exhibited broad-spectrum activity against various bacteria, yeast, and fungi. The active purified metabolite from this fraction was identified as Phenol, 2-Methoxy-4-(2-Propenyl) based on its physicochemical and spectroscopic characteristics. Other studies (**Nesma *et al.*, 2022)** have also reported the production of this compound by *Streptomyces sp. LS1* and its antimicrobial activity against five species of marine bacterial pathogens of G +ve (*S. aureus* ATCC 6538 *and B. subtilis* ATCC 6633) and G −ve (*P. aeruginosa* ATCC 9027, *K. pneumoniae* ATCC 13883, *and E. coli* ATCC 10418*)* bacterial strains. Phenol, 2-Methoxy-4-(2-Propenyl) is commonly used in pharmaceuticals, food, and cosmetics and as a local antiseptic and analgesic due to its safety and multidirectional action **(Magdalena and Beata, 2021)**.

Phenol, 2-Methoxy-4-(2-Propenyl) has been found to possess potent antibacterial properties against various strains of Gram-positive and Gram-negative bacteria **Anees *et al.*, (2017).** This study demonstrated that Phenol, 2-Methoxy-4-(2-Propenyl) also exhibits antiviral activity against human herpesvirus. Previous studies have reported that this compound induces glutathione S-transferase (GST) in rat liver and inhibits the replication of herpes simplex virus-1 (HSV-1) in vitro. Furthermore, it has been shown that Phenol, 2-Methoxy-4-(2-Propenyl) does not have cytotoxic effects at tested concentrations (15–250 mg/mL) and does not alter the morphology of treated cultures. These findings are consistent with the research of **Benencia and Courreges (2000),** who demonstrated the potential of Phenol, 2-Methoxy-4-(2-Propenyl) in inhibiting viral infection and replication, particularly against HSV-1 and HSV-2exhibiting IC50 values between 25.6 mg mL-1 and 16.2 mg mL-1., the compound has also been validated as effective against clinical isolates of HSV-1.

Conclusion

The increasing incidence of multidrug-resistant organisms poses a significant challenge in treating infectious diseases. Consequently, there is an urgent need to develop new antimicrobial compounds that can effectively combat antibiotic-resistant pathogens. This study focuses on isolating Phenol, 2-Methoxy-4-(2-Propenyl) from *S. mutabilis* AZHD22 and demonstrates its antimicrobial activity against various microorganisms. Additionally, this study reports the antiviral activity of the compound for the first time. The results indicate that the antimicrobial agent produced by *S. mutabilis* AZHD22 is non-toxic. The findings of this investigation highlight the Mediterranean Sea as a valuable source of diverse actinomycetes species.

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عزل وتقييم النشاط الحيوي ل *Streptomyces mutabilis* AZHD22 من البحر الأبيض المتوسط. مصدر محتمل للمركبات المضادة للميكروبات

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تم الحصول على ما مجموعه ستة وعشرون أكتينوميسيتات متنوع من مناطق مختلفة من البحر الأبيض المتوسط في الإسكندرية، مصر. خضعت هذه العزلات للفحص الأولي ضد سلالات متعددة إيجابية الجرام وسلبية الجرام والخميرة والفطريات. في الفحص الثانوي، أظهرت تسع عزلات درجات متفاوتة من النشاط ضد الكائنات الحية الدقيقة التي تم اختبارها. من بينها، أظهر عزله رقم(A7) خصائص قوية مضادة للميكروبات وتم اختياره لمزيد من التحقيق. من خلال تقنيات تحديد الهوية المورفولوجية والكيميائية الحيوية والمجهرية والجزيئية، تم تحديد عزله A7 على أنه *Streptomyces mutabilis*. استكشفت الدراسة أيضًا تأثير العوامل الغذائية والجسدية على النشاط المضاد للميكروبات للعامل الناتج عن عزل A7. ووجد أن الظروف المثلى للنشاط المضاد للميكروبات هي قيمة الأس الهيدروجيني ٦، ودرجة حرارة الحضانة ٣٧ درجة مئوية، وتركيز الصوديوم كلوريد كان ٤ غرام/لتر، وتركيز النشا ١.٥٪، وتركيز نترات الصوديوم ٠.٤٪، و ٢.٥ غرام/لتر من $K_2HPO_4.3H_2O$. بالإضافة إلى ذلك، تم دمج ٠.٥ غرام/لتر من $MgSO_4$ في ميدا الغذاء. تم وضع الحضانة في حاله اهتزاز لمدة ٧ أيام في ١٥٠ دورة في الدقيقة. تم استخدام أسيتات الإيثيل مع كروماتوغرافيا الطبقة الرقيقة لاستخراج وتنقية العامل المضاد للميكروبات. أظهر الجزء رقم ٦ أعلى نشاط ضد الكائنات الحية الدقيقة التي تم اختبارها. تم التعرف على المركب المنقى على أنه الفينول،٢و٤ ميكوسي (٢-بروبينيل). أظهر نشاطًا كابحاً ضد الكائنات الحية الدقيقة المختلفة وأظهر نشاطًا كبيرًا مضادًا للفيروسات دون أي تأثيرات سامة للخلاياالمختبرة.

الكلمات الافتتاحيه:

Streptomyces mutabilis :، نشاط مضاد للميكروبات، جين S rRNA١٦، تحليلGC-MS