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Isolation and characterization of *Streptomyces* sp. NMF76 with potential antimicrobial activity from mangrove sediment, Red Sea, Egypt.

Nayer M. Fahmy
National Institute of Oceanography and Fisheries, Egypt.
Author email: nmfahmy6@yahoo.com

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ABSTRACT

Streptomyce sp. NMF76 was isolated from mangrove sediment at the Egyptian Red Sea coast by serial dilution method and identified based on morphological and biochemical properties as well as 16s rDNA sequence analysis. The culturing parameters maximizing the antimicrobial activity and the MIC values for the tested pathogens were determined. The ethyl acetate extract was analyzed by GC-MS. Morphological and biochemical characteristics and 16s rDNA sequence analysis affiliated the strain to the *Streptomyces* genus with accession number, MT0199162. It exhibited the maximum antimicrobial activity when cultured in ISP5 medium containing 3% NaCl and incubated at 30 °C for 14 days with glycerol and L-asparagine as carbon and nitrogen sources, respectively. The strain exhibited antimicrobial activity against *Vibrio damsela*, *S. aureus*, *E. fecalis* and *C. albicans* and the MIC values were 285,400, 461 and 545 µg/mL, respectively. GC-MS analysis of the extract revealed the presence of Benzene, 1,2,4-trimethyl (21.47 %), 2H-Pyran-3-ol,tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-, [2S-[2à, 5á (R*)]]- (18.35 %) , Benzene, 1-ethyl-3-methyl- (15.76 %), and undecane (9.96 %) as major components. Further studies are required to purify and elucidate the structure of the antimicrobial agent.

INTRODUCTION

The continuous demand to develop novel antibiotics is increased to replace the currently ineffective antibiotics and combat infectious diseases- which represent one of the main causes of death worldwide- caused by antibiotic resistant pathogens **(de Lima Procópio *et al.* 2012).** Actinomycetes, the high GC Gram-positive bacteria, are the most valuable microbial group and have produced about half of the microbial bioactive secondary metabolites. The bioactive compounds of actinomycetes exhibit a wide range of biological activities including antibacterial, antifungal, anticancer, antioxidant, antiparasitic, enzyme inhibitors, and immunosuppressive (**Miao and Davies, 2010; Azman *et al.* 2015)**. Moreover, actinomycetes degrade the complex substances present in the natural environment and produce many enzymes of industrial interest: protease, lipase, cellulase, amylase, esterase, and L- asparaginase **(Usha *et al.* 2011; He *et al.* 2012; Yeager *et al.* 2017)**. Among actinomycetes, the genus *Streptomyces* is the primary producer of bioactive compounds, specially antibiotics **(Azman *et al.* 2015)**.

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During the past few decades, terrestrial microorganisms produced already known compounds, and the discovery of novel compounds has become extremely difficult; therefore, researchers have switched the search for novel pharmaceutical compounds to the promising extreme environments such as desert, marine, deep sea, and mangrove **(Xu *et al.* 2014)**, as the extreme conditions (pH, temperature, salinity, and pressure) in these environments promote a distinct microbial defense mechanisms and lead to the biosynthesis of novel molecules **(Wilson and Brimble, 2009)**. Mangrove ecosystem is a highly productive ecosystems with soil conditions- high salinity, high moisture, high organic matter content and low oxygen- extremely different from normal soil conditions; therefore, mangrove actinomycetes produce unique bioactive compounds **(Manivasagan *et al.* 2014; Azman *et al.* 2015)**. About 86 new species of actinobacteria (including 8 novel genera) have been isolated from mangrove environment. Mangrove actinobacteria have produced 84 new compounds with different biological activities (antimicrobial, antitumor, antiviral, antifibrotic, and antioxidants) including salinosporamides, xiamycins, and novel indolocarbazoles **(Xu *et al.* 2014; Li *et al.* 2019)**.

The biosynthetic gene clusters in microbial genome regulate the production of secondary metabolites, but the physico-chemical factors- nutrient supply, pH, temperature, and oxygenation- affect the expression of these genes under laboratory conditions **(Bhatnagar and Kim, 2010)**. Accordingly, even metabolically-active Streptomycete cultures can synthesize antibiotics only under suitable nutritional and cultivation conditions: Many studies reported the influence of substrate composition and growth conditions on the antibiotic production by different *Streptomyces* species **(Singh *et al.* 2009; Thakur *et al.* 2009; Singh *et al.* 2014)**.

Mangrove ecosystem in Egypt extends along the Egyptian Red Sea coast and covers about 225 ha; *Avicennia marina* dominates this ecosystem **(Madkour and Mohamed, 2008)**. Little work has been done on the bioactivity of actinomycetes from this habitat. The present study aimed to isolate actinomycete strains from mangrove sediment collected from the Egyptian Red Sea coast, with potential activity in antimicrobial agents productivity. In addition to the characterization and optimization of the growth conditions.

MATERIALS AND METHODS

Samples collection

Marine sediment samples were collected in April, 2017 from the mangrove area located about 17 km to the south of Safaga between latitudes 26°36'53"N–26°37'07"N and longitudes 34°00'46"E–34°00'27'E **(Figure 1)**. Five Samples were collected from the top 10 cm from mangrove rhizosphere sediments in sterile plastic bags and stored at 4°C until return to the laboratory.

Isolation and purification of marine actinomycetes.

For isolation of actinomycetes, about one-gram of each sediment sample was homogenized in 100 mL of sterile sea water by shaking for 12 hours in rotary shaker at 180 rpm. Serial dilutions of the supernatant were carried out up to 10^{-5} and plated on starch nitrate agar supplemented with nalidixic acid (25 mg/l) and cycloheximide (75 mg/l). Plates were incubated at 30 °C for 14- 21 days. Actinomycete colonies were

selected based on the morphological appearance and purified by streaking on the same medium **(Baskaran *et al.* 2011; Jagan *et al.* 2013)**.

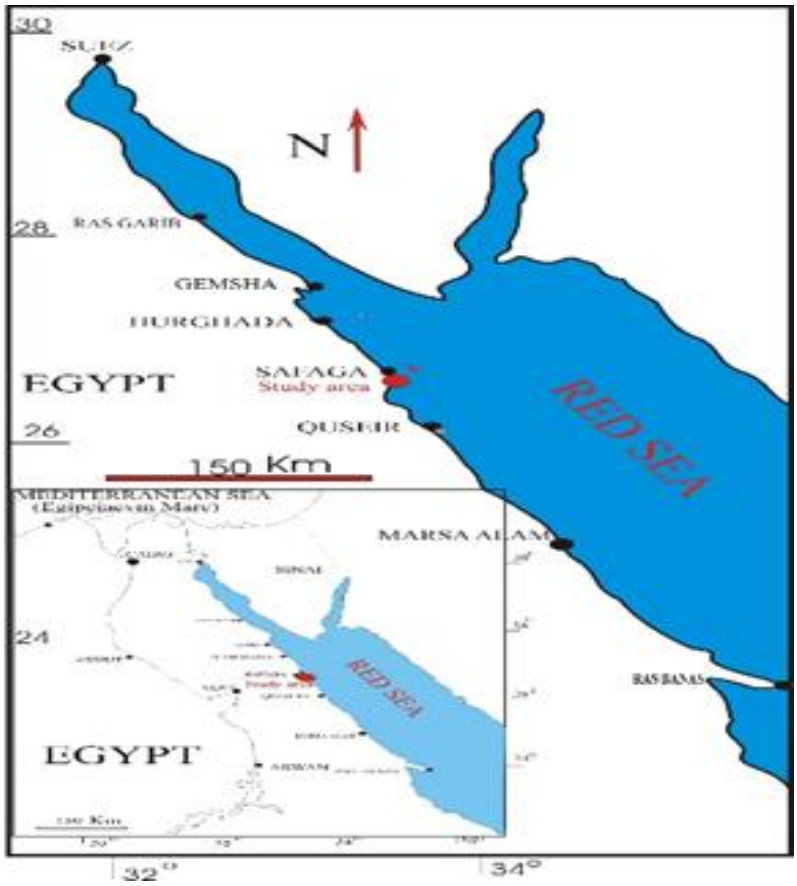

Figure 1. The staudy area ((Madkour, and Mohamed, 2008).

Assessment of the antimicrobial activity of NMF76 strain.

Microbial indicators

The microbial indicators included: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATTC 25923, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 4027, *Vibrio damsela*, *Candida albicans* ATCC 10231, *Fusarium* sp., *Rhizoctonia solani,* and *Aspergilus niger*. These microbial strains were kindly provided by the staff members of microbiology lab., National Institute of Oceanography and Fisheries, Alexandria, Egypt.

Preparation of inoculums

The inoculums of bacterial and fungal strains were prepared by growing cells in nutrient broth at 32 °C for twenty-four hours. The cells were collected by centrifugation at 7000 rpm, washed with sterile saline, and suspended in saline at 0.1 OD at 600 nm using JENWAY ٦٨٠٠ spectrophotometer **(Wayne, 2002; Cwala *et al.* 2011)**.

Antimicrobial activity

The antimicrobial activity of NMF76 strain was evaluated by cultivation on ten solid media such as ISP1, ISP4, ISP5, ISP7, starch casein agar, Bennett's modified agar,

Waksman's Glucose agar, Thronton's agar, and GLM media prepared in 50% sea water and adjusted to pH 7. After incubation for 14 days at 30 °C, Cork borer plugs of the whole culture of each medium were transferred to nutrient agar or potato dextrose agar media previously seeded with bacterial or fungal test microorganisms, respecively **(Ball *et al* . 1957)**. The plates were incubated at 37 °C for 24 h for bacteria and 48 h for fungi and the inhibition zones were measured **(Thomas *et al.* 2014)**. The medium that supported the highest antimicrobial activity was selected for cultivation in submerged culture. The antimicrobial potential of the ethyl acetate extract of strain NMF76 was determined by disc diffusion method using sterile 6mm filter paper discs impregnated with the 0.5 mg/disc of the crude extract **(Thanigaivel *et al.* 2014)**.

Determination of minimum inhibitor concentrations (MICs).

To determine the minimum inhibitory concentrations (MICs), sterile nutrient broth (for bacteria) and potato dextrose broth (for fungi) supplemented with different concentrations of ethyl acetate (EA) extract of NMF76, dissolved in DMSO, were inoculated separately with the microbial indicators and incubated for 24 hours. The lowest concentration inhibiting the growth of the test microorganism was recorded as the MIC **(Andrews, 2001; CLSI, 2019)**.

Characterization of NMF76 strain.

Cultural characteristics

The cultural characteristics of NMF76 strain were studied by growing the strain on different culture media. Micromorphology was examined by cover slip technique using starch nitrate agar medium **(Williams *et al.* 1989)**. Biochemical tests including nitrate reduction; gelatin liquefaction; production of cellulase, amylase, and lipase were evaluated according to **Gordon *et al.* (1974)**. Growth at different pH, different NaCl concentrations, and different temperatures were determined according to **Shirling and Gottlieb (1966)**. Behavior towards ten standard antibiotics was tested by disc diffusion method **(Kumar *et al.* 2014)**.

Molecular identification

DNA extraction

Genomic DNA was extracted using PrepMan™ Ultra Sample Preparation Reagent supplied by Applied Biosystems, USA (PN 4322547) according to the manufacturer's instructions.

PCR amplification

PCR reaction was carried out to amplify a 500-bp 16S ribosomal DNA (rDNA) from the 5' end of the gene in a reaction volume of 30 µl (15µl of McroSeq. PCR master mix and 15µl of working stock of genomic DNA). The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min.; followed by 30 cycles at 95 °C for 30s, 60 °C for 30s, and final extension at 72 °C for 10 min. Sequencing analysis was carried out in sanger sequencer 3500 Applied Biosystems. Molecular characterization of the strain was carried out at Colors medical lab, Egypt.

Phylogenetic analysis.

The BLAST program (www.ncbi.nlm.nih.gov/blst) was utilized to identify the similarity with the sequences available in the database and the top 10 similar sequences were selected for multiple sequence alignment by CLUSTAL W program **(Thompson *et al.* 1997)**. Phylogenetic tree was constructed by the neighbor-joining method **(Saitou and Nei, 1987)** using Mega-X software version 10.1.7. Bootstrap values were performed using

1000 replicates **(Felsenstein, 1985)** and the evolutionary distances were computed using the Jukes-Cantor method **(Jukes and Cantor, 1969)**. All positions containing gaps and missing data were eliminated **(Hall, 2013)**.

Optimization of the culture medium.

Stock culture slants of NMF76 strain were used to prepare general and washed inoculums for different inoculations during all optimization experiments according to **Shirling and Gottlieb (1966)**. At the end of the incubation period of each experiment, the whole culture was centrifuged. Cell-free broth was used for determination of antimicrobial activity against S. *aureus* by agar well diffusion method **(Thanigaivel *et al.* 2014)** and the pellets were used for dry weight determination **(Thakur *et al.* 2009)**.

Effect of pH

To evaluate the effect of initial pH on growth and antimicrobial activity, the basal medium was adjusted to different pH (4-10), inoculated and incubated for 14 days at 30 ^{o}C. Growth and antimicrobial activity were determined as stated above **(Thakur *et al.* (2009).**

Effect of carbon source

To study the effect of carbon source on growth and antimicrobial agent(s) production, different carbon sources (glucose, glycerol, maltose, and starch) were sterilized and supplemented separately to twenty-five milliliters of ISP5 medium (pH,5) in 250 mL Erlenmeyer flasks at 1% concentration. Each flask was inoculated with 0.5 mL of washed inoculum and incubated at 30 ^{o}C for 14 days under static conditions. Similarly, the effect of nitrogen source was studied by replacing the L- asparagine with different nitrogen sources in ISP5 medium- L- Arginine, potassium nitrate, sodium nitrate, tyrosine and ferric ammonium citrate **(Thakur *et al.* 2009).**

Effect of NaCl concentration.

The effect of NaCl concentration on growth and antimicrobial agent (s) production by NMF76 strain was studied by cultivation in various NaCl concentrations (0- 10%) in to ISP5 medium (pH,5) supplemented with 1% glycerol as a sole carbon source. Biomass accumulation and antimicrobial activity were assessed as mentioned above after incubation for 14 days at 30 ^{o}C **(Singh *et al* 2009).**

Effect of temperature and incubation period.

The effect of temperature was studied by incubating the inoculated basal medium (pH,5) at different temperatures (10-45 ^{o}C for 14 days). Similarly, the effect of incubation period was studied by incubating the inoculated basal medium (pH,5) for up to 20 days. The growth and antimicrobial activity were assessed as stated above **(Singh *et al.* 2009).**

Production and extraction of secondary metabolites.

Strain NMF76 was inoculated to 1000 ml Erlenmeyer flasks containing 200 ml of the optimized ISP5 medium. The flasks were incubated at 30 $^{\circ}$C for 14 days under static conditions. The cultures were filtered through Whatman filter No.1, extracted with equal volumes of ethyl acetate, and the organic phase was collected and evaporated using HAHNVAPOR HS-2005 rotary evaporator (Hahnshin Scientific**,** Korea**)**. The crude extract was weighed dissolved in methanol at known concentration **(Sharma *et al.* 2016)**.

G- GC-MS analysis of NMF-76 EA extract

The EA extract of NMF76 was analyzed by gas chromatography- mass spectrometry (GC-MS). The analysis was carried out using Thermo Scientific Trace GC1310 gas chromatograph attached with ISQ LT single quadrupole mass spectrometer

and fitted with Agilent J&W DB-5 column (length-30 m, thickness–0.25 μm, internal diameter–.0.25 mm). The temperature program was started at 40 °C, held for 5 min, and raised to 275 °C at 5 °C/min and held for 5 min. Sample was injected at 300 °C using helium as carrier gas (1 mL/min). The MS was operating at 70 eV. The peaks were identified by matching the mass spectra with WILEY 09 and NIST 11 mass spectral databases. The analysis was carried out at the Regional Center for Mycology and Biotechnology, Al Azhar University, Cairo.

RESULTS

1- Isolation of actinomycete

Isolation of actinomycetes from soil samples collected from mangrove ecosystem at the Egyptian Red Sea coast by serial dilution method resulted in the isolation of fifteen different actinomycete isolates. Among them, one isolate exhibited a strong antimicrobial activity against Gram-positive, Gram-negative and unicellular fungi was selected for further characterization and designated as strain NMF76.

2- Morphological characteristics of the potent isolate.

The strain NMF76 grew on both synthetic and complex media. The growth was abundant on ISP1, Bennet's modified agar, Waksman's glucose agar and Thronson's agar media and moderate on, ISP4, ISP5, ISP7, starch casein agar, GSYP-ME agar and GLM agar. The strain produced white aerial mycelium and yellow to gray substrate mycelium; the color of the spore mass was grey and spore chains were rectiflexous with warty surface **(Figure 2 and Table1).**

Table 1: Culture characteristics of NMF76 on different media.

Medium	Growth	*S. mycelium	**A. mycelium	Diffuslible pigment
Tryptone yeast extract agar (ISP1)	+++	Pale yellow	Grayish white	-
Inorganic salt starch agar (ISP4)	++	Pale yellow	Grayish white	-
Glycerol asparagine agar (ISP5)	++	Grayish white	yellowish white	-
Tyrosine agar (ISP7)	++	Grayish white	yellowish white	-
Starch casein agar	++	Pale yellow	Grayish white	-
Bennet's modified agar	+++	Yellow	yellowish white	-
Waksman's glucose agar	+++	Yellow	yellowish white	-
Thronson's agar	+++	Yellow	Grayish white	-
CSYP-ME agar	++	Pale yellow	Grayish white	-
GLM agar	++	Pale yellow	Grayish white	-

*S. mycelium: substrate mycelium; **A. mycelium : Aerial mycelium

3- Physiological and biochemical charateristics

Strain NMF76 was Gram-positive, non-motile and could hydrolyse starch, carboxymethyl cellulose, protein and tween80. Catalase was positive, but nitrate reduction, urea decomposition, H_2S production, were negative. The strain utilized glucose, maltose, starch and glycerol but was unable to utilize fructose, lactose, mannitol, sucrose or arabinose. The strain failed to transform tryptophan to indole or produce acid from glucose and was resistant to Flucloxacillin 5mcg, Amoxicillin/clavulanic acid 20/10mcg, Ciprofoxacillin 5 mcg, and Trimethoprime/Sulphamethoxazole 1.5/23.75 mcg

and was sensitive to Amikacin 30mcg, Clindamycin 2mcg, Streptomycin 10mcg and Tetracyclin 30 mcg. It grew at different pH (4-10), different temperatures (15- 45 °C) and at1-10 % of NaCl concentrations **(Table 2)**.

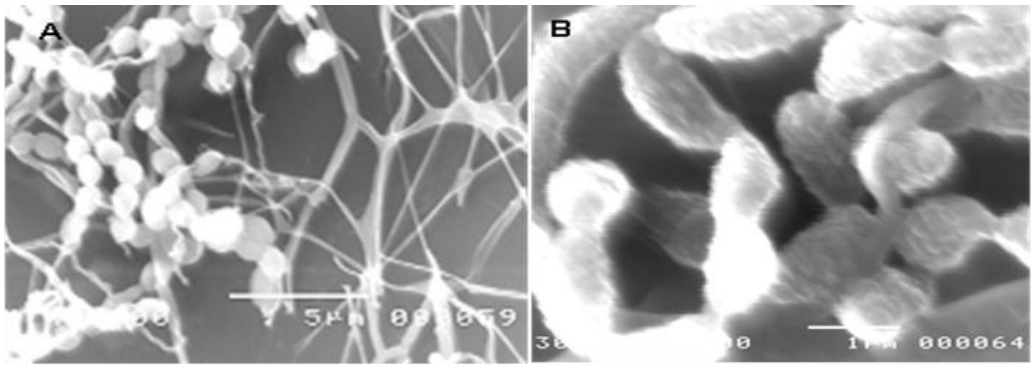

Figure 2. Scaning electron micrograph of NMF76 strain showing rectiffexous spore chain with warty to rough spore surface. Bars, 5µm (A) and 1 µm (B).

Table 2. Morphological, physiological and biochemical characteristics of NMF76.

Characteristics	Result	Characteristics	Result
Morphological characteristics		Arabinose	-
Gram	positive	Starch	++
Spore chain	rectiflexous	Sucrose	-
Spore mass	White	Glycerol	+++
Spore surface	Smooth	Manitol	-
Color of substrate mycelium	Gray	**Resistance to:**	
Diffusible pigment	-	Amikacin 30mcg	-
Motility	-	Flucloxacillin 5mcg	+
Physiological and biochemical properties		Clindamycin 2mcg	-
Production of:		Erythrocin 15 mcg	-
Amylase	+	Amoxicillin/clavulanic acid 20/10mcg	+
Cellulase	+	Streptomycin 10mcg	-
Protease	+	Ciprofoxacillin 5 mcg	+
Lipase	+	Tobramycin 10 mcg	+
Catalase	+	Trimethoprime/Sulphamethoxazole 1.5/23.75 mcg	+
Urease	-	Tetracyclin 30 mcg	-
H_2S production	-	**Growth at different pH values**	
Nitrate reduction	-	4-10	+
Indole	-	**Growth at different NaCl (w/v, ‰)**	
Methyl red	-	0	-
Triple sugar	-	1-10	+
Utilization of carbon source		**Growth at different temperatures**	
Glucose	+++	10	-
Fructose	-	15-45	+
Maltose	++	50	-
Lactose	-		

4- Molecular phlogeny.

Partial 16s rDNA sequence was determined and submitted to NCBI GenBank database under the accession number, MT0199162. Phylogenetic analysis of the 16s

rDNA sequence of strain NMF76 revealed the affiliation of the strain to the genus *Streptomyces*; it exhibited the highest similarity with *Streptomyces fenghuangensis* strain GIMN4.003 (GenBank accession no. (NR_117502; 100%), **(Figure 3).**

5- Antimicrobial activity of strain NMF76.

Comparing the antimicrobial activity of NMF76 cultivated on different culture media, the strain exhibited antimicrobial activity against *Vibrio damsela*, *S. aureus*, *E. fecalis* and *C. albicans* when grown in all culture media except for ISP1 and Thronson's media, however, it exhibited no antimicrobial activity against the tested filamentous fungi (*A. niger*, *R. solani* and *F. moniliform*). Among the culture media, ISP5 media supported the highest antimicrobial activity and the inhibition zones were 35, 29, 25 and 20 mm for *V. damsela*, *S. aureus*, *E. faecalis* and *C. albicans*, respectively **(Figure 4)**.

Effect of culture conditions on growth and antimicrobial activity.

Effect of pH

Although *Streptomyces* sp. NMF76 grew at pH ranging from 4-10, the biosynthesis of the antimicrobial agent occurred only under acidic condition and was maximum at pH 5; however, the growth was maximum at pH 7 **(Figure 5).**

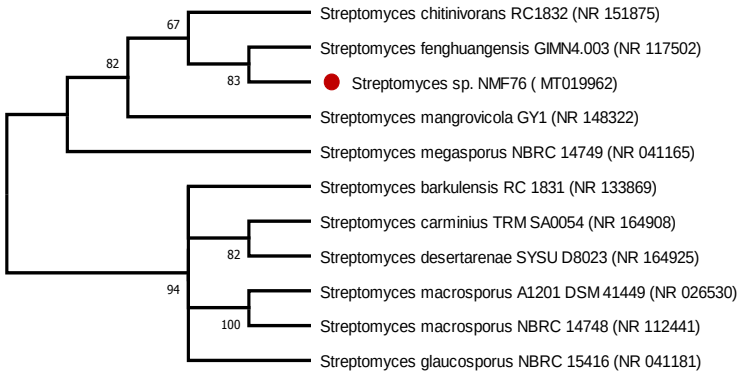

Figure 3. unrooted, neighbor-joining phylogenetic tree of *Streptomyces* sp. NMF76 showing phylogenetic relationship with related *Streptomyces* species. Numbers at nodes are bootstrap percentages based on 1000 resamplings, only values above 50 are given.

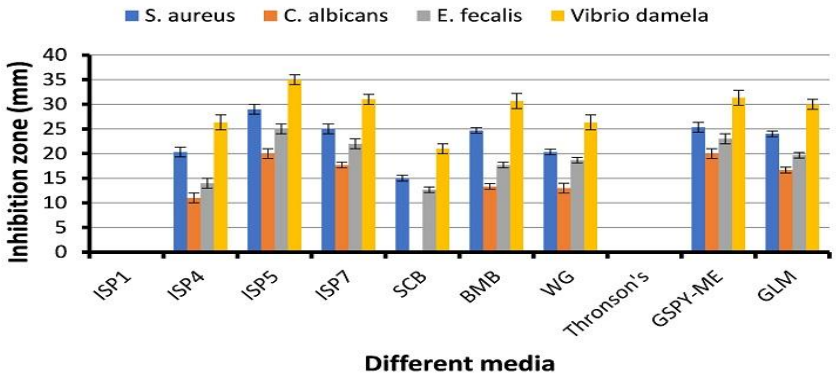

Figure 4. Effect of different media on antimicrobial activity of *Streptomyces* sp. NMF76.

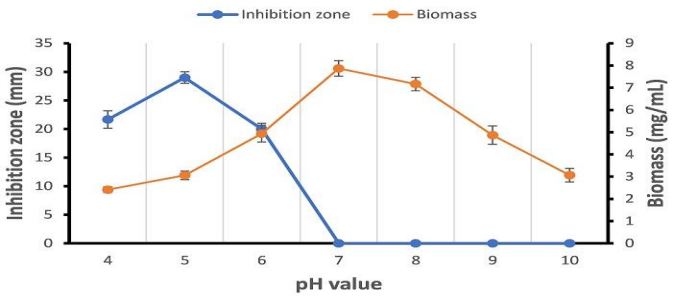

Figure 5. Effect of different pH on growth and antimicrobial activity of strain NMF76 *against S. aureus*.

Effect of carbon and nitrogen sources

Among the tested carbon sources, only four -glucose, glycerol, maltose and starch- supported the growth of NMF76 strain when supplemented as a sole carbon source in both ISP5 or carbon source utilization media (described in materials and ethod). Glycerol was the best carbon source for growth (3.9 mg/mL) and antimicrobial activity (inhibition zone, 22mm). Only glycerol and glucose stimulated the antimicrobial agent production when supplemented to ISP5 medium **(Table 2 and Figure 6)**. Of all examined nitrogen sources, L- Asparagine was the best nitrogen source for growth (10.5 mg/ml) and antimicrobial activity, (inhibition zone, 30 mm). The other tested nitrogen sources supported lower growth and antimicrobial activity compared to L- Asparaginase with ferric ammonium citrate being the less efficient nitrogen source **(Figure 7)**.

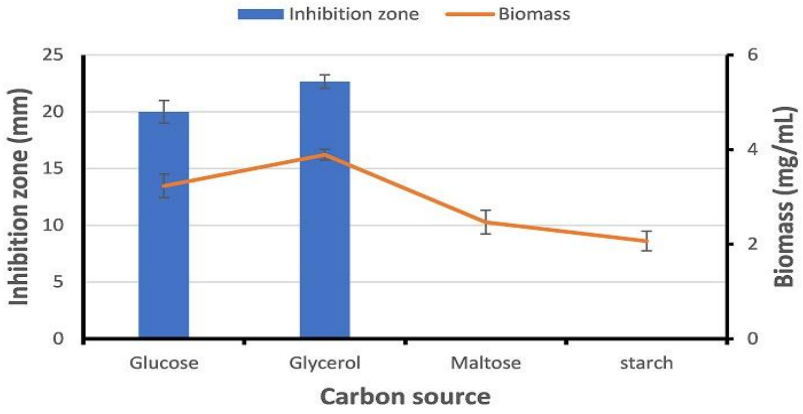

Figure 6. Effect of different carbon sources on growth and antimicrobial activity of strain NMF76 against *S. aureus.*

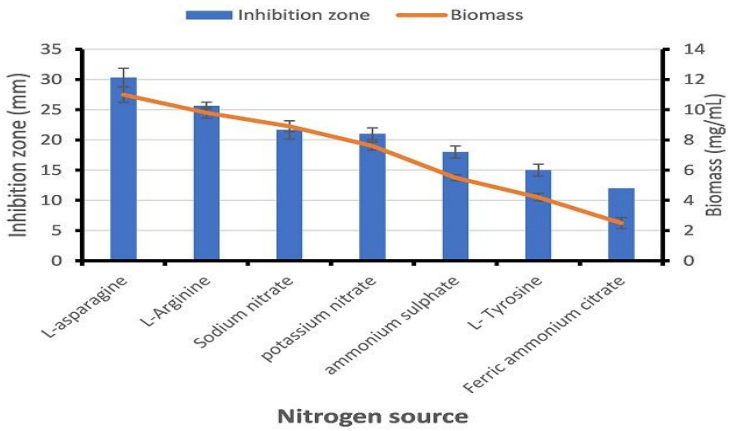

Figure 7. Effect of different nitrogen sources on growth and antimicrobial activity of strain NMF76 against *S. aureus.*

Effect of NaCl concentration.

Figure 8 shows the effect of salinity on growth and antimicrobial agent production by *Streptomyces* sp. NMF76 cultivated in ISP5 medium, 3 % NaCl was the optimum concentration for antimicrobial activity (inhibition zone, 24.6 mm), and 7% NaCl was the optimum concentration for maximum growth (10.4 mg/mL).

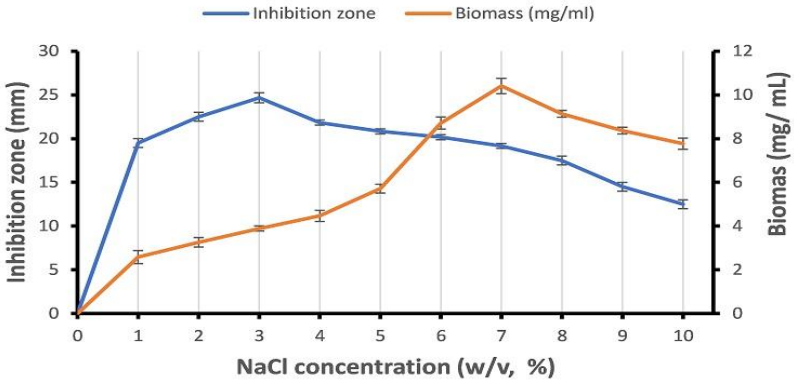

Figure 8. Effect of NaCl concentration on growth and antimicrobial activity of strain NMF76 against *S. aureus*.

Effect of incubation period.

Strain NMF76 cultivated in ISP5 under static conditions exhibited antimicrobial activity after incubation for 6 days which increased gradually and peaked (inhibition zone, 28.7) after 14 days of incubation **(Figure 9)**. The growth was also maximum after 14 days of incubation (10.3 mg/mL).

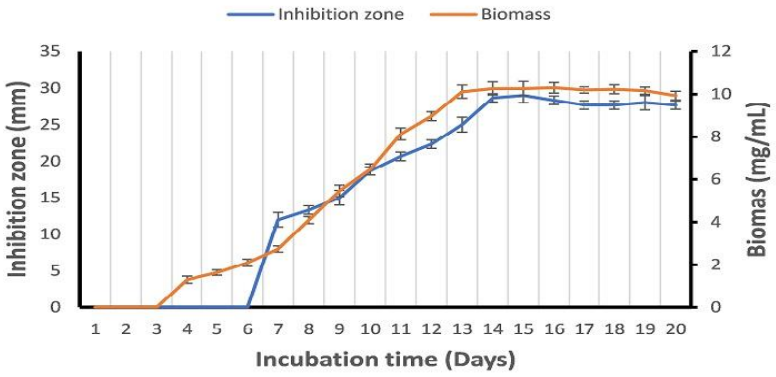

Figure 9. Effect of incubation period on growth and antimicrobial activity of strain NMF76.

Effect of temperature.

Strain NMF76 showed a narrow range of incubation temperature for good growth and antimicrobial activity. The optimum temperature for growth and antimicrobial activity was 30 °C, however, growth of the strain remained near the optimum up to 35 °C **(Figure 10)**.

Antimicrobial activity of EA extract.

Cell- free broth of NMf76 strain cultivated in ISP5 medium for 14 days was extracted with ethyl acetate and yielded 200 mg of crude extract. The effectiveness of the extract against the microbial indicators was evaluated as diameter of inhibition zone and MIC value and it exhibited the maximum inhibition zone and the lowest MIC value against *S. aureus* followed by *E. faecalis* and *C. albicans* **(Table 3)**.

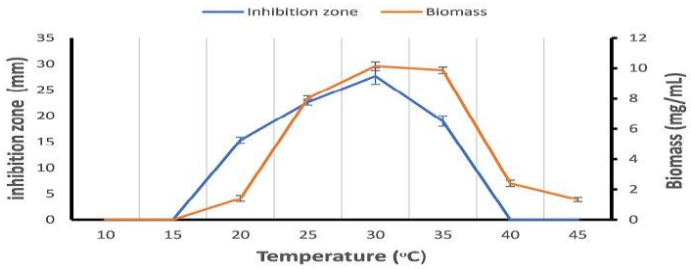

Figure 10. Effect of temperature on growth and antimicrobial activity of strain NMF76.

Table 3. Antimicrobial activity and MIC (µg/mL) of EA extract of NMF76 strain.

Test microorganism	*Inhibition zone (mm)	MIC (µg/ml)
Vibrio damsela	21± 1.2	285
S. aureus	15.66 ± 1.15	400
E. fecalis	13.33 ± 1.15	461
C. albicans	11.66 ± 0.57	545

* Values are presented in mean ± SD (n =3).

GC/MS analysis of EA extract.

GC-MS analysis of the crude extract of NMF76 revealed the presence of twenty compounds belonging to different classes of compounds and exhibiting varying

percentages of peak areas. The dominant compounds were Benzene, 1,2,4-trimethyl (21.47 %), 2H-Pyran-3-ol,tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl)-,[2S-[2à,5á(R*)]]- (18.35 %) , Benzene, 1-ethyl-3-methyl- (15.76 %), and undecane (9.96 %). The rest of the compounds had lower peak area percentages ranging from 0.74 to 6.17 % **(Table 4).**

Table 4: Compounds identified from NMF76 EA extract using GC-MS.

RT	Compound name	MW	Area (%)	MF
4.07	Benzene, 1,3-dimethyl	106	6.17	C_8H_{10}
4.78	Hexadecanoic acid, 3,7,11,15-tetramethyl-, methyl ester	326	0.93	$C_21H_{42}O_2$
5.21	Benzene, propyl	120	1.49	C_9H_{12}
5.81	Benzene, 1-ethyl-3-methyl-	120	15.76	C_9H_{12}
5.95	- ¹Butyl(dimethyl)silyloxypropane	174	2.46	$C_9H_{22}OSi$
6.21	1,3-Propanediol, TBDMS derivative	190	1.49	$C_9H_{22}O_2Si$
6.81	Benzene, 1,2,4-trimethyl	120	21.47	C_9H_{12}
7.55	10,13-Octadecadiynoic acid, methyl ester	290	1.43	$C_{19}H_{30}O_2$
7.73	Benzene,4-ethyl-1,2-dimethyl-	134	0.92	$C_{10}H_{14}$
8.42	2,3-Epoxicaran, trans	152	0.74	$C_{10}H_{16}O$
8.83	Undecane	156	9.96	$C_{11}H_{24}$
12.78	2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one	218	1.61	$C_{15}H_{22}O$
15.31	2,4,6-Trimethylmandelic acid	194	0.91	$C_{11}H_{14}O_3$
19.77	1,4-Benzenediol, 2-(1,1-Dimethylethyl)-5-(2-PrOpenyl)-	206	1.22	$C_{13}H_{18}O_2$
23.07	2-Furanmethanol, tetrahydro-à,à,5-trimethyl-5-(4-met hyl-3-cyclohexen-1-yl)-,[2S-[2à,5á(R*)]]-	238	2.65	$C_{15}H_{26}O_2$
23.7	cis-7,10,13,16-Docosatetraenoic acid, methyl ester	346	1.3	$C_{23}H_{38}O_2$
25.08	2H-Pyran-3-ol,tetrahydro-2,2,6-trimethyl-6-(4-met hyl-3-cyclohexen-1-yl)-,[3S-[3à,6à(R*)]]-	238	18.35	$C_{15}H_{26}O_2$
28.88	Hexadecanoicacid,methyl ester	270	4.53	$C_{17}H_{34}O_2$

DISCUSSION

We isolated a halotolerant antibiotic producing actinomycete isolate from mangrove sediment designated NMF76 and identified as *Streptomyces* sp. NMF76 and found that the culturing conditions- pH, carbon and nitrogen sources, NaCl concentration, incubation period and temperature- affect the growth and antimicrobial activity.

Streptomycetes are competitive microorganisms and dominate many environments including marine and freshwater ecosystems: they produce the hydrolytic enzymes necessary to utilize complex substrates, produce antibiotics required to antagonize other microbes and form the desiccation-resistant hydrophobic spores to withstand the adverse conditions **(van der Heul *et al.* 2018)**. Strain NMF76 produced hydrolytic enzymes such as amylase, lipase, and cellulase and exhibited antimicrobial activity. Strain NMF76 tolerated up to 10% NaCl, grew at different pH (4-10) when incubated at 15-45 °C. **Shrivastava *et al.* (2015)** and **Abdul Hamid *et al.* (2015)** isolated halotolerant actinomycetes from mangrove environment growing at pH (6-9) with optimum temperature 25-37 °C. *Streptomyces* synthesize the antibiotics through microbial fermentation in response to environmental signals including the nature and level of carbon and nitrogen sources, temperature, oxygen concentration, pH, and light. Therefore, both the type of nutrients formulating the culture medium and the culturing conditions influence the synthesis of these compounds by the producing microorganism

(Ruiz *et al.* 2010). Our results showed that the strain produce the antimicrobial agent(s) under acidic conditions with maximum antimicrobial activity at pH 5, but the maximum growth occurred at pH 7. Streptomycetes are neutrophilic showing maximum growth at pH range 6- 8, but microorganisms synthesis secondary metabolites in response to reduced growth, and they lose this property at increased growth **(Kim *et al.* 1992; Kim *et al.* 2000).**

Kim *et al.* (2000) investigated kasugamycin production and cell growth by *Streptomyces kasugaensis* and found that the strain grew well under neutral conditions and the growth was maximum with no kasugamycin production, but switching to the acidic conditions, pH 3.5-4, enhanced kasugamycin production and decreased the growth; they concluded that acidification of the medium was necessary to enhance kasugamycin synthesis. The initial pH of the culture medium affects the activity of many enzymes involved in microbial metabolic reactions, cell morphology and membrane permeability, and consequently the growth and antibiotic production **(kim *et al.* 2000; Elmahdi *et al.* 2003)**. It has been reported that acidification of the culture medium enhanced the production of nikkomycins by *Streptomyces tendae* **(Mohrle *et al.* 1995)** and methylenomycin by *S. coelicolor* A3(2) **(Hayes *et al.* 1997)**. Several regulatory mechanisms affect the secondary metabolite production by microorganisms. Among these mechanisms, carbon source regulation controls the production of antibiotics and the presence of certain carbon source in the culture medium may stimulate or suppress the production of antibiotic via activation or suppression of the biosynthetic enzymes involved **(Ruiz *et al.* 2010)**. *Streptomyces clavuligerus* produce two closely related beta-lactam antibiotics - cephamycin C and clavulanic acid- derived from two different precursors. Glycerol inhibited cephamycin c and induced the formation of clavulanic acid by this strain **(Saudagar and Singhal, 2007)**. **Leulmi *et al.* (2019)** reported that, while D- glucose enhanced nigericin production by *Streptomyces youssoufiensis* SF10 strain, ribose or mannitol decreased nigericin production, but sucrose or starch inhibited nigericin production. In the present study, both glycerol and glucose stimulated the production of the antimicrobial agent(s), but maltose and starch suppressed the synthesis of the antimicrobial agent(s) by NMF76 strain when supplemented separately to the culture medium.

In our study, strain NMF76 grew at 1-10% NaCl supplemented in ISP5 medium but failed to grow in the absence of NaCl, yet it grew in complex media prepared by deionized water. The optimum NaCl concentration for growth and antimicrobial activity were 7% and 3%, respecively. **Singh *et al*. (2009)** found the optimum NaCl concentration for antimicrobial activity of the halotolerant *Streptomyces tanashiensis* strain A2D to be 2% and **Saha *et al.* (2005)** isolated an actinomycete isolate that grew at 20% NaCl and exhibited the maximum antimicrobial activity at 7% NaCl. **Jensen and Fenical (1996)** reported that some marine bacteria could grow and produce secondary metabolites when grown in complex fermentation medium prepared by deionized water. Strain NMF76 produced the antimicrobial agent after incubation for 4 days and peaked after 14 days. **Ng *et al*. (2014)** detected Rifamycin B production by a species *Salinispora* after 6 days of incubation and found the maximum production after 16 days of incubation.

The ethyl acetate extract of NMF76 strain exhibited antimicrobial activity againt Gram positive and Gram- negative bacteria and C. *albicans* with MIC values ranging from 285 to 545 µg/mL. These results are in agreement with **Al-Dhabi *et al.* (2020)** who

reported that the ethyl acetate extract of *Streptomyces* sp. strain Al-Dhabi-97 derived from marine habitat showed MIC values ranging from 62.5 to 500 µg/mL against the tested Gram negative and Gram positive bacteria, but **Akhter *et al.* (2018)** found a lower MIC values - 16 to 100 µg/mL against *P. aeruginosa, S. aureus, E. coli* and *K. pneumoniae*- from *Streptomyces pratensis* NA-Zhou-S1- isolated from marine region.

Benzene, 1,2,4-trimethyl and Benzene, 1-ethyl-3-methyl and 2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cyclohexen-1-yl were major components of the ethyl acetate extract of NMF76 strain. These compounds have been detected from natural sources: **Wang *et al.* (2013)** reported Benzene trimethyl from *Streptomyces alboflavus*TD-1 exhibiting antimicrobial activity, **Oyekunle (2017)** detected Benzene, 1-ethyl-3-methyl in essential oil of *Thevetiaperuviana* seeds, and **(Nisha and Rao (2018)** identified 2h-pyran-2-on, tetrahydro-4-(2-methyl-3-met from *Trigonella foenum-graecum* L. Further studies are required to purify the antimicrobial agent and elucidate the chemical structure by detailed chemical analysis.

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