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Production, Purification and characterization of bioactive compounds from locally *Streptomyces*rochei M78

Nadhim H. Hayder*, Marwa S. Mahmood
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

The aim of the current study was to optimize different cultural and environmental conditions for production the antibacterial bioactive metabolites by *Streptomyces rochei* M78 isolated from agriculture soil, in Baghdad, Iraq. The effect of various parameters such as, culture media, incubation time, pH, carbon and nitrogen sources, C: N ratios and inducers on antibacterial metabolite production was studied by varying single parameter at a time. It was found from the results that higher metabolite production by isolate observed using starch casein broth (SCB) as the best production medium, at initial pH 7.0.Starch andcasein +yeast extract + peptone appeared to be the best carbon and nitrogen sources respectively and C: N ratio of 4: 1 after 72 h of incubation for optimal production of antibacterial metabolites.Optimization studies indicated that antibacterial metabolites production was associated with bacterial growth, and that the presence of inducers, such asedible oils and diesel oil as well as amino acids in the medium also enhanced antibacterial metabolites production.The most bioactive compounds were produced with soybean oil as the sole carbon source, and leucine as amino acid, yielding an inhibition zone more than 35 mm against all tested pathogenic bacteria.Among different solvents used for the extraction of antibacterial metabolite, ethyl acetate was found to be the best for solvent extraction of the metabolites yielding 2.18 g /l of red to brown extract with oily nature. The antibacterial activity of different extraction fractions of the metabolites showed that the ethyl acetate extract was the most active agent against tested pathogenic bacteria.Physiochemical characteristic of antimicrobial metabolites revealed that the antimicrobial metabolite was red to brown in color, having gummy and oily nature. The purified metabolite was soluble in different solvents, with a melting point of 150 °C. The metabolites of isolate M78 were stable at pH that varies from 4 – 11, maximum antibacterial activity was found at pH 7 and at temperatures ranging from 25 to 100 °C, maximum at 25 °C. Higher bactericidal concentration (BC) of the compound against Gram positive and Gram negative bacteria was determined as 250 µg/ml. The results showed that MBC values of the active metabolite had an impact at lower concentrations than those of standard antibiotic against tested pathogenic bacteria, suggesting that the metabolite was more effective.Theminimum inhibitory concentration value of compounds was 500 µg/ml against all tested bacterial isolates. Thin layer chromatography analysis of active metabolites showed two spots having an Rf value = 0.72 and 0.80.The FTIR spectrum of antibacterial compounds exhibited the presence of OH, C=O functional ester group, and C-H and CH_3 groups in the structure. GC-MS analysis of active metabolites detected a total of 23 peaks; two major hydroxylated fatty acids were then identified as octadecanoic acid, 2-(2-hydroxy ethoxy) ethyl ester and tridecanoic acid, 3 methyl-, methyl ester with relative abundance of 100 and 33.63 % respectively.

Keywords: Bioactive compounds, *Streptomyces sp.*, production conditions, MIC, Hydroxylated fatty acids.

*Email: Nadhemwandawy@yahoo.com

انتاج تنقية وتوصيف المركبات الفعالة حيويا من عزله محليه لبكتريا *Streptomycesrochei*M78

ناظم حسن حيدر*، مروة شاكر محمود

قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

هدفت الدراسة الحالية الى تحديد الظروف الزراعية والبيئية المثلى لإنتاج المركبات الفعالة ضد البكتيرية وتوصيفها من العزلة M78 *Streptomyces rochei* المعزولة من الترب الزراعية، في بغداد، العراق. درست تأثير العوامل المختلفة، مثل تأثير الوسط الزرعي، فترة الحضانة، الرقم الهيدروجيني، المصادر الكربونية والنيتروجينية، نسبة الكاربون : النتروجين والمحفزات على انتاج المركبات ضد البكتيرية . وجد من النتائج بان افضل انتاج للمركبات الفعالة كانت باستخدام وسط الانتاجي النشأ والكازئين، عند الرقم الهيدروجيني 7، والنشأ وكازئين+ مستخلص الخميرة+ البيبتون كأفضل وسط كاربوني ونتروجيني على الترتيب ونسبة الكاربون : النتروجين 4:1 بعد72 ساعة من الحضانة. اكدت دراسة الظروف المثلى بأن المركبات ضد البكتيرية المنتجة غير مرتبطة بالنمو، و وجود المحفزات مثل زيوت الطعام وزيت المحركات والاحماض الامينية في الوسط حفزت عملية الانتاج. معظم المركبات الفعالة حيويا انتجت بوجود زيت فول الصويا كمصدر وحيد للكاربون، والحامض الاميني الليوسين مع الحصول على قطر تثبيط اكثر من 35 ملم ضد البكتريا الممرضة. استخدمت عدة مذيبات لاستخلاص المنتوج الايضي الفعال، وجد بأن خلات الاثيل افضل مذيب للاستخلاص مع الحصول على منتوج بلغ 2.18 غم/لتر. كما اثبتت نتائج الفعالية ضد البكترية بأن مستخلص خلات الاثيل اعطى افضل فعالية مايكروبية. اظهرت الخصائص الفيزيائية- الكيميائية بأن المنتوج الفعال ذا لون احمر مائل الى البني، لزج وبطبيعة دهنية. كما وجدت من خلال النتائج بأن المنتوج الايضي المنقى جزئيا ذائب في مذيبات مختلفة، مع درجةأنصهار بلغت 150°م وذو ثباتية عالية عند الرقم الهيدروجيني من 4-11، والعظمى عند الرقم الهيدروجيني 7، وبدرجة حرارية من 25 الى 100 °م والعظمى عند 25 °م. حددت التركيز القاتل الاعلى للمنتوج ضد البكتريا الموجبة والسالبة لصبغة غرام لتكون 250 مايكروغرام/مل والتركيز المثبط الادنى للمركبات الفعالة بلغ 500 مايكروغرام/مل ضد جميع البكتريا الممرضة تحت الاختبار. بينت نتائج تحليل كروموتوغرافيا الطبقة الرقيقة للمنتج الايضي حزمتين مع قيمة ال Rfمساوية 0.72 و0.80. أظهرت نتائج طيف الFTIRبأحتواء المركبات الفعالة بكتيريا على مجموعة ال OH، C = O مجموعة الاستر، C-H ومجموعة CH_3. كما ان نتائج تحاليل الGC-MS كشفت أحتواء المركبات الفعالة حيويا على 23 قمة، مع قمتين اساسيتين من الاحماض الدهنية الحاوية على الهيدروكسيل وشخصت بأنها حامض الديكانويك (2-هيدروكسي ايثوكسي) مثيل الاستر وحامض الترايديكانويك، 3 مثيل، مثيل الاستر مع وفرة نسبية بلغت 100 و33.67% على الترتيب.

Introduction

Actinomycets are Gram – positive bacteria which occupy a large proportion of the soil microbial biomass and play an important role in the production of bioactive metabolites. *Streptomyces* spp. represents at least 90% Actinomycetes isolates, which differ significantly in their morphological and physio- chemical properties [1]. This group of microorganisms is responsible for the production of over 6000 types of bioactive secondary metabolites obtained from different species of *Streptomyces*.Many of these compounds are extracellular enzymes, antibiotics, antimicrobial, anticancer, insecticides, herbicides, etc. [2, 3]. Moreover, for the biosynthesis of these bioactive compounds, different types and concentration of medium components are used, such as carbon, nitrogen and other critical constituents as well as inducers. The nature and concentration of fermentation medium have a marked effect on the biosynthesis and the chemical nature of bioactive compounds [4, 5]. Most of bioactive compound biosynthesis takes place in the stationary phase, when the growth of microorganisms is rapidly reduced,while, on the other hand, bioactive metabolites may be growth- associated and maximum production can be obtained in log phase [6].

Fatty acids (FAs) have been known to display an antimicrobial activity since the 1920s.[7, 8] As more microorganisms become resistant to current antibiotics, there is a great need for new antimicrobial agents. FAs inhibit the growth of a wide variety of microorganisms, for example, Gram negative bacteria, Gram-positive bacteria, and fungi [9,10and11]. FAs tend to have a better antimicrobial activity against Gram-positive bacteria than against Gram negative bacteria.[12; 13]. Even with these generalizations, there are always exceptions. For example, long-chain FAs are active against *Neisseria gonorrhoeae*, a Gram-negative bacterium. [10] On the other hand, antibacterial activity reported by both saturated and unsaturated FAs against *S. aureus* and Methicillin Resistant *Staphylococcus aureus*,MRSA [14].Previous studies reported that bioactive metabolites, hydroxyl fatty acids [15] and poly hydroxylated saturated fatty acids [16] produced from *Streptomyces* strains exhibited antibacterial and antifungal activity.

The current study aims at, screening of *Streptomyces* isolates for bioactive compounds production. Identification of active isolates which exhibited higher active metabolite production using cultural, the nucleotide sequence of the corresponding *16S rRNA*encoding gene analysis, followed by optimization of fermentation conditions. Semi purification of bioactive metabolites and determination of their structure. The antibacterial activity of bioactive metabolites was studied as well.

Methods

*Streptomyces*isolates

Seventy six isolates of *Streptomyces* which previouslyisolated from agriculture, garden soil and sediments were obtained from the biotechnology department/Baghdad university, and were identified to the level of genus according to Bergey' s Manual of determinative Bacteriology[17], and Bergey' s Manual of Systematic Bacteriology [18].These isolates were prepared for screening experiments by adding one gram of soil containing *Streptomyces* isolates to 5ml of sterilized D.W in test tubes, mixed vigorously and left to stand for a few minutes, then 100µl of each soil suspension was streaking in duplicates on soybean agar plates. The plates were incubated for 14 days at 30 ºC.

Screening of isolates for production of antibacterial metabolites

Seventy six pure cultures of *Streptomyces* spp. Isolates were screened for their ability to produce bioactive compounds depending on antibacterial activity against several clinical bacteria, namely gram positive (*Staphylococcus area*sand *Bacillussubtilis*) and gram negative (*Escherichia coli*and*Klebsiella pneumonia*) bytheagar diffusion methods. The isolates were grown in 250 ml flasks containing 50 ml of fermentation broth (glucose 2.5 %,soybean 0.5%,NaCl 0.5%, K_2HPO_4 2 %, $MgSO_4$0.05% and $CaCO_3$0.01%) [19]. Two pieces (6 mm diameter) of agar from each sevenday culture of *Streptomyces* isolates grown on a soybean agar medium was used for inoculation of flasks. The cultures were incubated at 30°C in a rotary shaker (JSSI-200C) at 160 rpm for 3 days. The culture broth was centrifuged at 8000 rpm at 4 °C for 20 min. The supernatant was filtered using 0.45 µm filter paper (Millipore). The filtrate was used for antibacterial activity [20]. To determine the antibacterial activity, pathogenic bacteria were grown overnight in nutrient broth at 37°C for 24 h (O.D = 0.5) McFarland 1* 10^8 CFU/ ml); the cultures were streaked on Moller Hinton agar medium. Wells (6 mm in diameter) were prepared in each seeded agar plate and each well was filled with 75µl of the active metabolites. The plates were kept at 4°C for 24 h for the diffusion of the metabolites. The plates were incubated at 37 °C for 24 h. The diameter of inhibition zones was measured [21]. Each test was repeated two times and the activities were shown as the mean of the diameter of the inhibition zone. Six isolates which exhibited higher production of bioactive compounds (zone of inhibition more than 20 mm) were screened for antimicrobial activity against four pathogenic bacteria *S.aureus*e and *B.subtilis*) and gram negative (*E. coli* and *K. pneumonia*) using the agar well diffusion method as well as determination of remaining sugar in the culture filtrate. The active isolate was chosen for identification and antimicrobial metabolite production in optimization and characterization studies.

Identification of *Streptomyces* M78 isolate

Streptomyces isolate was cultured in 30 ml of glucose soybean medium GSM (glucose 25, soybean 5, NaCl 5, K_2HPO_4 2 , $MgSO_4$ 0.5 and $CaCO_3$1)[19] supplemented with 0.5% (w/v) glycine for 46 h with shaking at 28° C. Cells were harvested by centrifugation (5 min, 4000× *g*), washed [2× 10 mL of 10% (w/v) sucrose] asdescribed by Jasmina*et al*., [22] Genomic DNA extraction and purification were carried out by HiPura (HiMedia), Lysozyme (10 mg) (Sigma, USA) was added to the bacterial suspension and incubated at 37°C for 20 min. The pellet was suspended with the lysisbuffer and incubated at 37 ° C for 30 min thencenterifugated in a cooling centrifuge (10000 rpm), then the

supernatant was loaded to extraction cartridge, DNA was eluted by 50 µl elution buffer (Provided by manufacturing company). The DNA samples were measured for their concentration and purity usingamicro volume UV Spectrophotometer (ACT Gene, USA).

DNA sequencing and *inseleco*

The primers *16rRNA*encoding gene F: **TCACGGAGAGTTTGATCCTG** and *16rRNA*encoding gene R: **GCGGCTGCTGGCACGTAGTT** were used for identification of*Streptomycessp*. M 78 as reported by Masakazu *et al*., [23]. PCR was performed in a 50 µl mixture containing 1× PCR buffer (10 mMTris–HCl, 1.5 mm MgCl2, 50 mMKCl [pH 9]) (Merck, India), 100 µM (each) deoxynucleoside triphosphates, 1 U of Taq DNA polymerase (Merck, India), 10 pM each of forward and reverse primers, and 100 mg of template DNA. The program for PCR included an initial denaturation 94°C for 5min, 30-40 cycles of denaturation at 97°C for 30s, annealing at 50°C for 1min, extension at 72°C for 1min and a final extension at 72 °C for 7min. The PCR products were loaded on a 1.5% agarose gel, stained with ethidium bromide (5 ng/ml) and bands were observed using a gel documentation system (ATTA, Japan). PCR products were sent for sequencing at Bioneer, Korea. The obtained sequence was compared for similarity with sequences present in the genomic database banks, using the "NCBI Blast" program available at the ncbi.nlm.nih.gov web site; highest matching sequences were downloaded.

Determination of remaining sugar

The residual amount of glucose in the fermentation broth filtrate was determined using a phenol sulfuric acid method [24].

Optimization of culture conditions for the production of antimicrobial metabolites

The effect of various cultural and environmental conditions on productions of antibacterial metabolites by the isolate M78 was studied. The influence of fermentation media on the production of antibacterial metabolites was studied by cultivating the isolate M78 in different media containing (g/l) A:(glucose 25 , soy bean 5, NaCl 5 , K_2HPO_4 2 , $MgSO_4$ 0.5 and $CaCO_3$ 1)[19] B:(glucose 10, soy bean 10 and NaCl 10)[25] C:(glucose 15 , glycerol 2.5 , soy bean meal 25 , NaCl 2.5 , $NaNO_3$ 4, K_2HPO_4 5, $ZnSO_4$ 0.04 and $CaCO_3$ 0.4)[26] D: (starch 10, malt extract 10 , casein 3, peptone 1 , yeast extract 1 and K_2HPO_4 0.5)[27]E:(malt extract 10, Dextrose 4 , yeast extract 4)[27]F:(Beef extract 3 , peptone 5 and NaCl 5)[28]. All these media were adjusted to pH 7 and sterilized by autoclave at 121°C for 15 min. Inoculated cultures were grown in a rotary shaker at 160 rpm at 30°Cfor 3 days. In order to determine the effect of various carbon and nitrogen sources on the antimicrobial metabolite production , basal medium was supplemented with various types of carbon sources (starch , maltose , sucrose , glucose , inositol and fructose at a concentration 2%) and nitrogen (KNO_3 , NH_4NO_3, NH_4SO_4, casein +yeast extract +peptone , urea and peptone at 0.5% concentration). The influence of C: N ratio of antimicrobial metabolite production was investigated by inoculating the isolate M78 in to starch malt extract broth adjusted with different C: N ratios *via.* ,1:1, 2:1, 3:1, 4:1, 5:1 and 6:1. The effect of pH was studied by inoculating of isolate M78 in to flasks containing starch, malt extract broth adjusted with different pH values *via.* , 5, 6, 7, 8, 9 and 10 (using 0.1 N HCl and 0.1 N NaOH). To determine the best incubation time for antimicrobial metabolite production, the starch, malt extract broth was inoculated by isolate M78 and incubated at 30°C, pH 7 up to 6 days. Cultures 0, 1, 2, 3, 4, 5 and 6 days were tested for viable counts of isolate M78 and the filtrate for antimicrobial activity and remaining sugar determination. To study the effect of inducers on antimicrobial metaboliteproduction, different sources of oil wastes (seas am oil, soybean oil, corn oil, sunflower oil, olive oil) and diesel oil at a concentration 1% as a sole source of carbon were add to basal medium; for amino acids, 0.05% of various amino acids sources (L-leucine, L-proline, L-arginine, L-glycine and L-phenyl alanine)were added in starch malt extract broth. In all of the previous experiments, the content of the flasks were centrifuged at 8000 rpm at 4°C for 20 min, and the culture filtrates were tested for antimicrobial activity and the remaining sugar.

Viable count assay

The total viable *Streptomyces* counts (CFU/ml) were made on a soybean agar, 1ml of the culture was drawn periodically at time intervals 0, 1, 2, 3, 4, 5 and 6 days from each of the flasks in duplicate and diluted using 10-fold dilution. Different sterilized pipettes were used for each of the dilution. 0.1 ml of the diluents was taken from dilution factors: 1:10 to $1:10^6$ were transferred into 2 sets of Soybean agar plate. The whole set of plates were incubated at 30ºC for 2-7 days for growth of

Streptomyces colonies. Cultures showing "between" 30-300 colonies were calculated using the colony counter [29].

Extraction, purification and characterization of antimicrobial metabolites

The isolate M78 from the 7- day culturewas cultured in 250 ml Erlenmeyer flasks containing 50 ml of an optimized production medium (starch malt extract broth, casein + peptone + yeast extract broth in basal medium, pH 7) and incubated at 30°C for 3 days in a shaker incubator at 160 rpm. After the incubation, one liter total volume of the culture broth was filtered through Whatman no.1 filter paper followed by centrifugation at 8000 rpm at 4 °C for 15 min. The spent culture broth (supernatant) was aseptically transferred into 250 ml flasks and equal volume 1:1 (v/v) of six different solvents (n-butanol, petroleum ether, n-hexane, ethyl acetate, methanol: chloroform (1:2) and diethyl ether) were separately added to the filtrate. The mixture (filtrate and solvent) was shaken vigorously for 20 min and kept stationary for another 15 min to phase separation. The aqueous and organic layer of crude extract was concentrated to solvent free content by evaporation in oven at 40°C to obtain a gummy crude extract. The remaining residues were dried in vacuum desiccator, weighed and re-dissolved in a small volume of methanol (1mg/ml) and tested for their antimicrobial activityusing the well diffusion method [30]. Methanol was used in each test as control against tested pathogenic bacteria.

Solubility of the antimicrobial metabolite

The solubility of antimicrobial metabolites was followed using different solvents (petroleum ether, diethyl ether, methanol, chloroform, methanol: chloroform (1:2 v/v), ethyl acetate, n-butanol, n-hexane, dimethyl sulfoxide (DMSO) and distilled water) by dissolving 100 µg (1mg) of crude extract in 5 ml of each solvent, and the results were recorded [15].

Melting point

The melting point of antimicrobial metabolite was determined by a Melting point apparatus (Stuart /England).

Stability of antimicrobial metabolite

The stability of antimicrobial metabolite for different pH and temperature values was studied according to [27]. For pH stability, 25 ml of culture supernatant of *Streptomyces* isolate M78 was taken in 50 ml Erlenmeyer flask (in duplicate) and the pH was adjusted to (4, 5, 6, 7, 8, 9, 10 and 11) and keeping it in a refrigerator for 30 min. Then the supernatant in each flask was tested for antimicrobial activity against (*S. aureus* and *K. pneumonia*) using the agar well diffusion method. For temperature stability, 25 ml of culture supernatant was heated in a water bath at different temperature degrees (25, 30, 40, 50, 60, 70, 80, 90 and 100 °C) for 15 min, cooled. Then the supernatant was tested for antimicrobial activity against (*S. aureus* and *K. pneumonia*) using thewell diffusion method.

Minimum Bactericidal Concentration of partial crude extract (MBC)

The antimicrobial activity of ethyl acetate extract was tested in terms of bactericidal activity against (*S. aureus,B. subtilis, and E. coli* and *K. pneumonia*) by using agar well diffusion method [31]. The compound was dissolved in methanol at different concentrations (0, 40, 50, 75, 100,125, 150and 250 µg/ml) and employed for antimicrobial activity test. Methanol was used in each test as control against tested pathogenic bacteria.

Minimum Inhibitory Concentration of partial crude extract (MIC)

Minimum inhibitory concentration of the active compounds was determined by the serial dilution against pathogenic bacterial isolates (*S. aureus, B. subtilis, and E. coli* and *K. pneumonia*). Different concentrations of bioactive compounds were preparedindimethylsulphoxide (DMSO) which ranged from 50 µg/ml to 1000 µg/ml in test tubes containing 5 ml of nutrient broth. Overnight grown bacterial isolate suspensions in nutrient broth were standardized to 10^8 CFU/ml using McFarland no. 0.5 standard solutions. A 100 µl of bacterial suspension was used to inoculate for each tube. DMSO (100 µl) and the medium which contains (100 µl) test bacteria serve as controls. After 24 hof incubation at 37 °C, 100 µl from each concentration was streaked over Muller Hinton agar plates. MIC values were recorded as the minimum concentration that inhibits the growth of test bacteria [31].

Thin layer chromatography (TLC) of bioactive metabolite

The crude ethyl acetate extract of the isolate *Streptomyces*M78 was determined using TLC on a (20 * 20 cm) silica gel plate (60F254,0.2 mm, Merck). A small drop of a sample was spotted onto TLC plate, with a capillary tube and dried; the spotting process was repeated by concentrating the spot to obtain approximately (2 to 5 µg) of the sample on the plate. The TLC plate was developed with

chloroform: methanol (9: 1 v/v) as solvent system and was sprayed with, ninhydren, ferric chloride, iodine and sulfuric acid and visualized using UV light [27].

Fourier transform infrared (FTIR) spectrum of bioactive metabolite

FTIR spectra of the ethyl acetate extract were analyzed after homogenization of the sample with KBr. Potassium bromide (AR grade) was dried under vacuum at 100 °C for 48 h and 100 mg of KBr were mixed with 1 mg of crude extract to prepare KBr pellet. UV spectra at 200 – 400 nm were recorded on a Shimadzu-IR affinity-1 spectrophotometer. The spectra were plotted as intensity versus the wave number[32].

Gas chromatography – mass spectroscopy (GC – MS) analysis of crude extract

GC-mass chromatography analysis was performed to identify the active antibacterial compounds in the extract. Identification of bioactive compounds was done by injection 1µl of sample into a RT * 5 column (30 *0.32 nm) of GC-MS model (Perkin Elmer, Claus 500, USA); helium (3ml/ min) was used as a carrier gas . The following temperature gradient program was used (75 °C for 2 min followed by an increase from 75 to 175 °C at a rate of 50 °C per min and finally 7 min at 175 °C). The m/z peaks representing mass to charge ratio characteristics of the antibacterial fractions were compared with those in the mass spectrum library of the corresponding organic compounds[33].

Results

Screening of isolates for antimicrobial activity

Seventy six *Streptomyces* isolates already isolated from different sources were screened for bioactive compound production. In the primary screening process, 40 isolates (56.6%) were active against test microorganisms gram positive and gram negative bacteria. Six isolates (*Streptomyces*M24,M35, M39, M78, M 119 and M188) showed higher activities against the test microorganisms (zones of inhibition more than 20 mm). These six isolates underwent secondary antimicrobial activity against four clinical bacterial isolates. Antibacterial activity of isolate *Streptomyces* sp. M78 was higher than all of the isolates and standard antibiotic (Gentamycin CN 30) used as a positive control Table-1, Figure-1. The results of antibacterial screening assays indicated that *Streptomyces* M78 was the most antagonistic isolate with an inhibition zone ranging from 23.4 –30 mm for human pathogenic bacteria. The most active isolate *Streptomyces* M78 was selected for further study on the basis of higher antibacterial agent producer. Previous studies showed that many species of *Streptomyces* had antibacterial activity against pathogenic microorganisms[34, 35].

Table 1-Secondary screening of *Streptomyces* isolates for antimicrobial metabolite production.

No. of Isolates	Antimicrobial Activity (mm) Gram negative Bacteria		Antimicrobial Activity (mm) Gram positive Bacteria	
	E. coli	*K. . pneumonia*	*S. aureus*	*B. subtilis*
M 24	29	10.6	28	0
M 35	23	0	24	0
M 39	25	13.4	21	0
M 78	**30**	**23.4**	**29**	**7.22**
M 119	24	16.64	22	22
M 188	24	12.5	30	0
Gentamycin CN 30	9.5	9.2	8.1	7

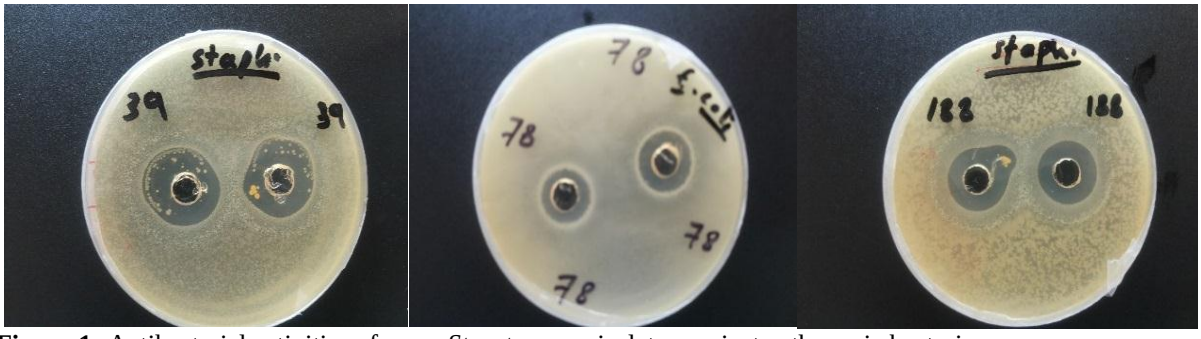

Figure 1- Antibacterial activities of some *Streptomyces* isolates against pathogenic bacteria.

Identification of *Streptomyces*M78

The taxonomic identity was confirmedas*Streptomyces sp.* by PCR and sequencing of 16S rDNA as reported by Kandhasamyand Sun [36]. Hence the isolate was designated as *Streptomyces sp.* after recovering 500-bp from the isolate by partial amplification of *16S rDNA* gene, variable region according to the*S. ambofaciens* as described by Masakazu*et al*., [23]. In addition to sequenceidentity as shown in Figure-2. In conclusion the specie belongs to the *Streptomyces rochei*99% by using PCR and DNA sequence partially encompassing the 16S DNA gene in species detection, as that used byXuechang*et al.* [37].

Streptomyces rochei strain M78 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|GU998798.1|Length: 847Number of Matches: 1
Range 1: 13 to 458GenBankGraphics

Score	Expect	Identities	Gaps	Strand
819 bits(443)	0.0	**445/446(99%)**	0/446(0%)	Plus/Plus

Query 11GGCGTGCTTACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACG 70
Subject 13GGCGTGCTTACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACG 72
Query 71GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCT 130
Subject 73GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCT 132
Query 131 AATACCGGATACTGATCCTCGCAGGCATCTGCGAGGTTCGAAAGCTCCGGCGGTGCAGGA 190
Subject 133 AATACCGGATACTGATCCTCGCAGGCATCTGCGAGGTTCGAAAGCTCCGGCGGTGCAGGA 192
Query 191 TGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTA 250
Subject 193 TGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTA 252
Query 251 GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG 310
Subject 253 GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG 312
Query 311 AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG 370
Subject 313 AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG 372
Query 371 GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTAC 430
Subject 373 GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTAC 432
Query 431 CTGCAGAAGAAGCGCCGGGTAACTAC 456
Subject 433 CTGCAGAAGAAGCGCCGGCTAACTAC 458

Figure 2-Sequences producing significant alignments.

Optimization of cultural and environmental conditions for bioactive compounds production

Effect of production media

A large number of factors affect the growth and the production of bioactive compounds by *Streptomyces* sp. such as culture media, incubation time, initial pH, carbon and nitrogen sources have a direct effect on production of bioactive compounds. The effect of these factors with a combination of media components also influences the growth and metabolite production [38]. The influence of fermentation media on the production of bioactive metabolites was tested by cultivating the isolate *Streptomycesrochei*M78 in six different media, namely A:soya bean glucose broth (SGB), B: soya bean glucose – glycerol broth (SGGB), C: starch casein broth (SCB), D: yeast extract malt extract broth (YEMEB) and beef extract broth (BEB). Among the five media used, the media SCB was found to be the best medium for bioactive compound production Figure-3. In the current study, SCB medium was shown to be the best medium for the production of bioactive compound by isolate *Streptomycesrochei*M78 as indicated by higher zones of inhibition produced by filtrate of SCB medium.

Similar results were recorded in earlier studies. Ismail and Asia, (2008) [39]found that*Streptomyces* DS-104 produce inhibitory compounds optimally in SCB. Besides, SCB was the best medium for the production of bioactive metabolites by *Streptomyces* sp. MP 525[40].

Effect of incubation period of growth and bioactive compounds production

In the current study, it was observed that growth and bioactive compound production started after the first day of incubation. During fermentation, the number of cells of isolating gradually increased reaching a maximum onthesecond day, 139 CFU/ml Figure-4. Bioactive metabolites started on the second day of incubation. The maximum of antimicrobial activity was shown on the third day, that is in the stationary phase, indicating growth independent production of bioactive compounds, and rendering them secondary metabolites of the isolate *Streptomycesrochei* M78. On the other hand, the remaining sugar concentration in the medium had reduced to a minimum. There was no antimicrobial activity production on first day as indicated by no inhibition zones of the test bacteria. The maximum bioactive compounds production was observed at the end of incubation period of 3 days. This activity remained stable between 3 to 4 days, then decreased slowly until 5 days. Similar results had been recorded by various workers [41and 42]. Viana*et al*., 2010 [43] reported that the highest antibiotic

(Clavulanic acid) production 494 mg/l was obtained by *Streptomyces* spp. ERI-26 after 48 h of incubation time. So, too, Osman *et al*., 2011 [6] reported that the production of antibiotics by *S. plicatus*reachs its maximum after 3 days of incubation.

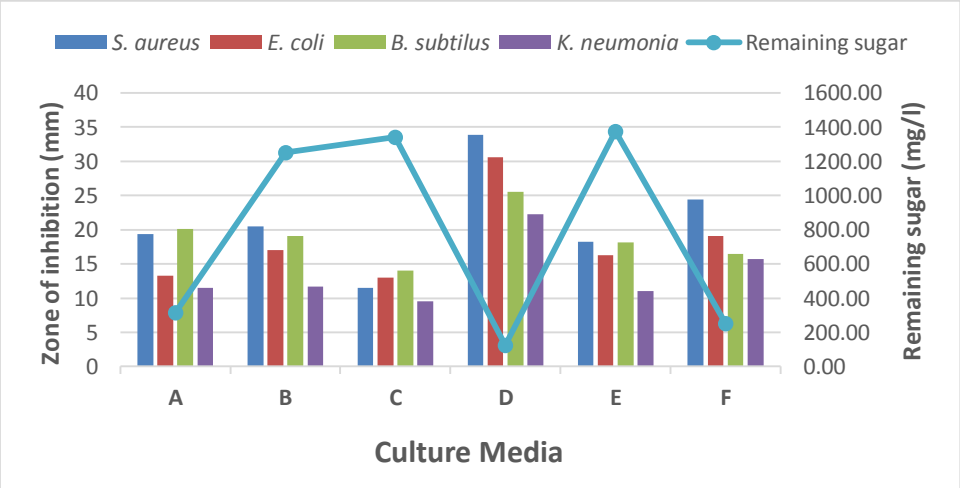

Figure 3-Production of bioactive compounds in different culture media by *Streptomycesrochei* M78. A: SGB, B: SGGB, C: SCB, D: YEMED and F: BEB.

Effect of pH

Many cellular processes, such as regulation and the biosynthesis of secondary metabolites, affected the changes in the initial pH [44]. The effect of pH on bioactive compound production for *Streptomycesrochei* M78 is presented in Figure-5.Amaximum inhibitory activity and minimum remaining sugar were observed in the case of a medium adjusted to pH 7, since the diameter of inhibition zones resulted from antibacterial activity which reached up to 30, 26.5, 30 and 26 in case of *B. subtilis; S. aureus; E. coli and K. pneumoni* respectively. Above pH 7, a decline in the antibacterial activity was observed. Thakur *et al*., [45] and Mustafa, [46] reported that pH 7 was optimum in their respective studies on antibiotic production by *Streptomyces* species. Generally, in most published researches, optimum pH for bioactive compounds production in *Streptomyces* cultures has been reported to be near neutral.

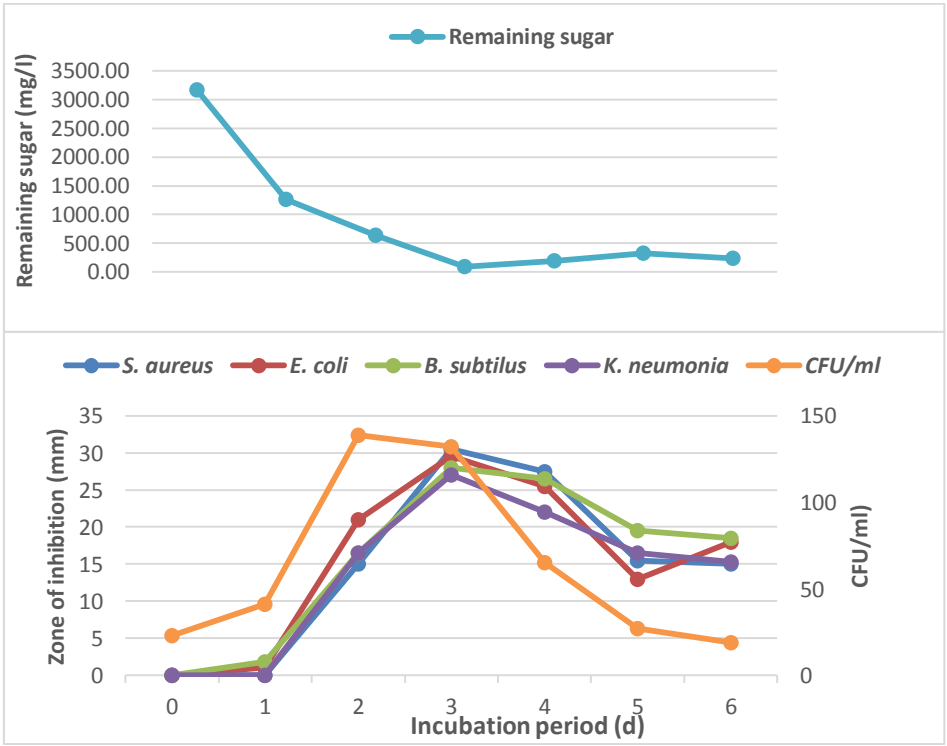

Figure 4- Effect of incubation period of growth and bioactive compounds production by *Streptomycesrochei* M78 (pH: 7, temperature: 30, rpm: 160).

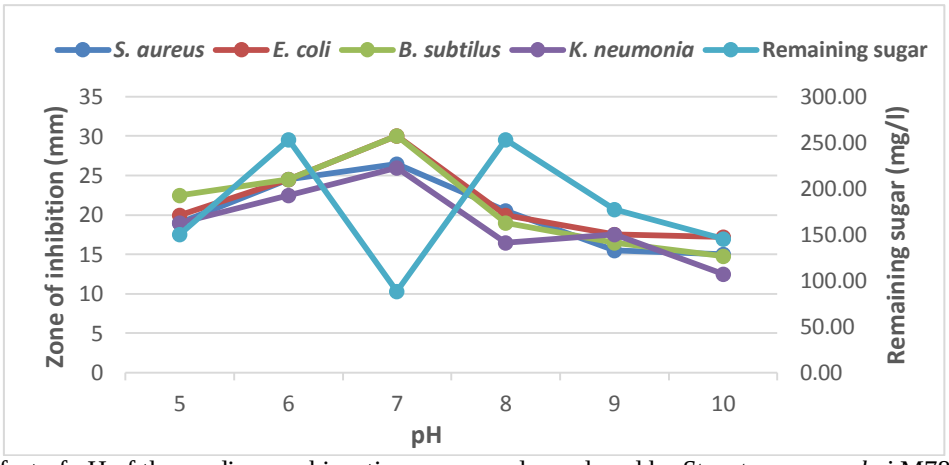

Figure 5-Effect of pH of the medium on bioactive compounds produced by *Streptomycesrochei* M78.

Effect of carbon sources on bioactive metaboliteproduction

The effect of different carbon sources of production of active compounds is shown in Figure-6. All the carbon sources tested supported the production of antibacterial metabolites, whereas, starch was the best carbon source for biosynthesis of antimicrobial substances with a concentration 2 g/100 ml medium. The effect of the tested carbon sources in the production of antibacterial agentscan be arrange in the following descending manner; for *Streptomycesrochei* M78, starch> maltose >fructose > inositol >sucrose >glucose. The production of antimicrobial compounds was maximal in medium containing starch as a sole carbon source, in which minimum, reducing sugar was observed,whereas glucose was the least carbon source for antimicrobial activity. Therefore, starch was used as a sole source of carbon in the remainingstudies.However, the production of secondary metabolites is often stimulated by slowly assimilated complex carbon sources like polysaccharides. This phenomenon may be explained by the fact that glucose causes catabolic repression, in which the enzymes responsible for secondary metabolite biosynthesis are inhibited [46]. Similarly,Gao*et al*., [47] found that starch is the best carbon source for antibiotic production by *Streptomyces avermitilis* 14-12 A.

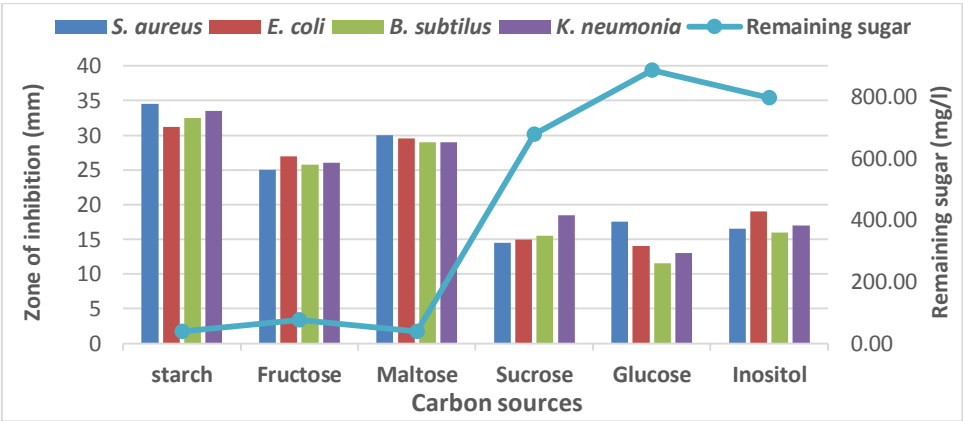

Figure 6-Influence of some carbon sources on bioactive compounds production by *Streptomycesrochei* M78.

Effect of nitrogen sources

The effect of different nitrogen sources(0.5 % w/v) at 2% starch in antibacterial metabolite production by the isolate *Streptomycesrochei* M78 was studied. The results in Figure-7 showd that(casein +yeast extract + peptone), KNO_3 and peptone enhanced the antimicrobial activity against pathogenic bacteria,while,urea, NH_4NO_3 and NH_4SO_4 were observed to suppress the antimicrobial activity. Casein +yeast extract + peptone which was already used as nitrogen sources, was found to be the best nitrogen source for bioactive metabolite production. It was clear from the results observed that the isolate *Streptomycesrochei* M78 was influenced by the nature and type of the nitrogen source amended in the medium. In comparison with inorganic nitrogen sources, the results found that organic nitrogen sources stimulated relatively higher antimicrobial metaboliteproduction. The results were in accordance with Vahidi*et al*., [48] and Yu *et al*., [49]who reported that organic nitrogen sources exhibited an increase in the level of antimicrobial metabolite production.

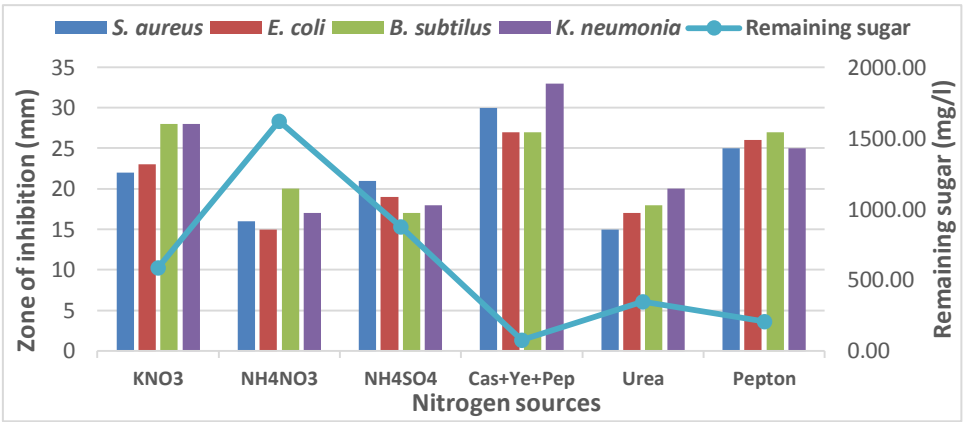

Figure 7- Effect of different nitrogen sources on bioactive compounds production by *Streptomycesrochei* M78.

Effect of C: N ratios on production of bioactive compounds

The initial concentration of starch was found to exhibit a marked influence on the production of bioactive substances by the isolate *Streptomycesrochei* M78. It was found that increasing C: N (starch: casein + yeast extract + peptone) ratios resulted in an increase of antibacterial metabolites as indicated by the high antimicrobial activity of crude extracts against test pathogenic bacteria Figure-8. In the present study, the isolate *Streptomycesrochei* M78 was found to produce bioactive compounds to a higher extent, increasing the starch concentration. Maximum bioactive metabolites were obtained at C: N ratio (4:1) starch concentration,i.e., 2%.

The concentration of carbon and nitrogen plays an important role in controlling the process of bioactive metabolites biosynthesis in *Streptomyces* [50]. The elevation of nitrogen level affected the synthesis of enzymes involved in the production of primary and secondary metabolites as reported by Sanchez and Demain [51]. Voelker and Altaba, [52] illustrated that complex nitrogen sources could enhance the production of secondary metabolites. They also mentioned that these sources could maintain high metabolite titer due to slow release of nitrogen compounds during the fermentation process. According to results obtained above, it could be concluded that the use of the starch casein medium along with the optimized carbon and nitrogen ratio could maximally support the bioactive metabolite production by the isolate *Streptomyces* sp. M78.

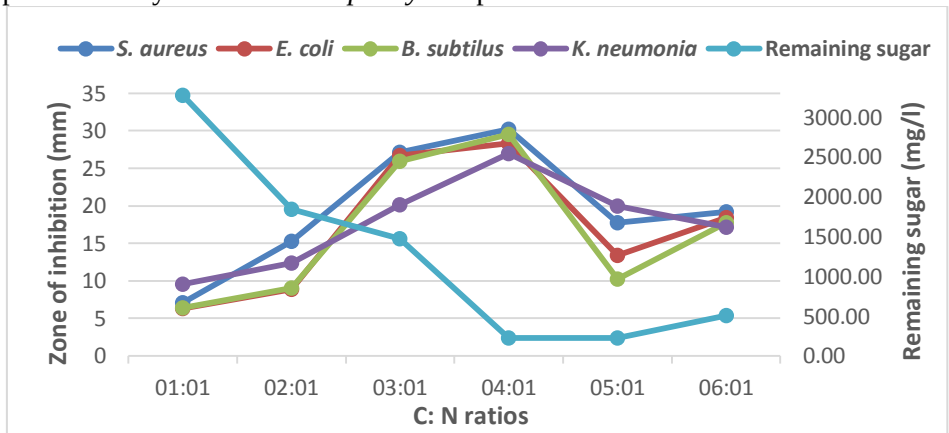

Figure 8- Effect of different carbon: nitrogen ratios on bioactive compounds produced by *Streptomycesrochei* M78.

Effect of Oil sources

The type, quality, and quantity of bioactive metabolites are influenced by the nature of the carbon substrate. Media containing edible oils were used for the production of bioactive metabolites from the isolate. *Streptomycesrochei* M78. In general, the edible oils and frying diesel oil were found to be more suitable for bioactive compounds production. Out of the different edible oils and diesel oil used, the most bioactive compounds were produced with soybean oil as the carbon source, yielding an inhibition zone more than 35 mm against all tested pathogenic bacteria Figure-9. The same results were obtained by Park *et al.*, 1994 which tested 7 different oils as substrate for Cephamycin C production by *Streptomyces* sp. 6621.Soybean oil was found to be the optimal carbon source for

production of this antibiotic [53]. Also Ohta*et al* improved Neomycin production by *Streptomyces fradiae* by using soybean oil as a sole carbon source [54]. According to bioactive compounds produced, some reports have suggested, the necessity of supplementation with oils and hydrocarbons to induce production. Hydrophobic substrates like corn oil, lard (which are rich in unsaturated and saturated fat) and long chain alcohols induce microbial growth and metabolite production owing to their typical fatty acid composition by maximizing rhamnolipids production by *P. aeruginosa*UG2[55]

Effect of amino acid addition

The effect of different amino acids in the production of antimicrobial agent is shown in Figure-10. Marked inhibitory activity was observed in the case of culture grown in broth containing Leucine as an amino acid with a concentration (0.05 g / 100 ml),whereas Arginine was found to be the least amino acid source for antimicrobial metabolite production. It was clear from the results that the active metabolite production by isolate *Streptomycesrochei* M78 was greatly influenced by the type of amino acid supplement in the medium. In comparison with the type of amino acids, Leucineinduced a relatively higher antimicrobial activity reaching 36, 35.73, 36.9 and 35.5 nm against *B. subtilis; S. aureus; E. coli and K. pneumonia* respectively**.** The use of amino acids as inducer materials was also recorded by Rius*et al*., (1996), who used the amino acid lysine as an inducer for the production of lysine Aminotransferase in the Cephamycin pathway of *Streptomyces clavuligerus*[56]; Nguyen *et al*., (1995) used Valine for Tylosin production in *Streptomyces fradiae* [57].

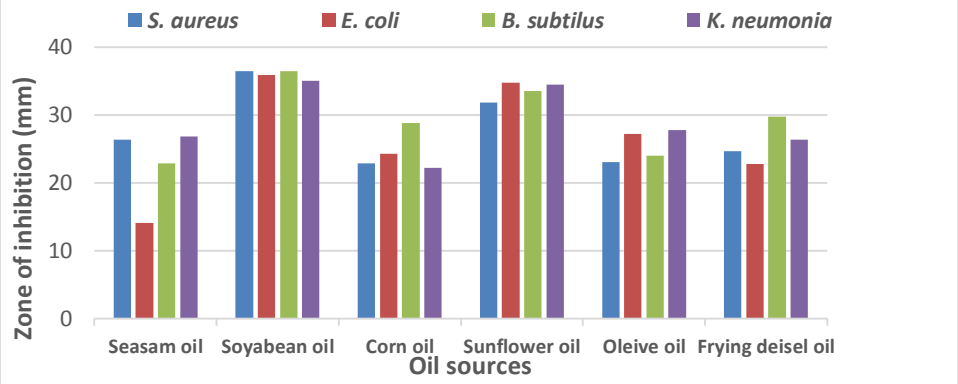

Figure 9-Theeffect of oil sources as inducers on bioactive compounds produced by *Streptomycesrochei* M78 (pH: 7, temperature: 30, carbon source: oils, rpm: 160).

Extraction and purification of metabolites and antimicrobial activity

The fermentation process was carried out for three days under optimum conditions (pH 7, temperature 30°C and rpm 160) using starch malt extract broth as production medium. One litter total volume filtrate was prepared, followed by centrifugation at 10000 rpm at 15°C for 20 minutes. The filtrate was extracted using different solvents to recover metabolites from culture. The obtained organic extracts were concentrated in a vacuum to dryness. The results in Table-2 showed that, among different solvents, ethyl acetate was found to be the best for solvent extraction of the metabolites, yielding 2.18 g /l of red to brown oily nature extract. The antibacterial activity of different extraction fractions of the metabolites were test by agar diffusion method, against four pathogenic bacteria. The results in Table-2 showed the ethyl acetate extract was the most active agent against pathogenic bacteria. Similar results were arrived at by Elleuch, et *al*., [15], who found ethyl acetate to be the best solvent for the of the recovery the metabolites from the culture of *Streptomyces* sp. TN 272; they concentrated crude extract in vacuum to dryness, affording 1.26 g / l. Also Maataoui*et al*., (2014) [27] observed a higher antibacterial activity against *S. aureus* with an inhibition zone of 30 mm with ethyl acetate extract.

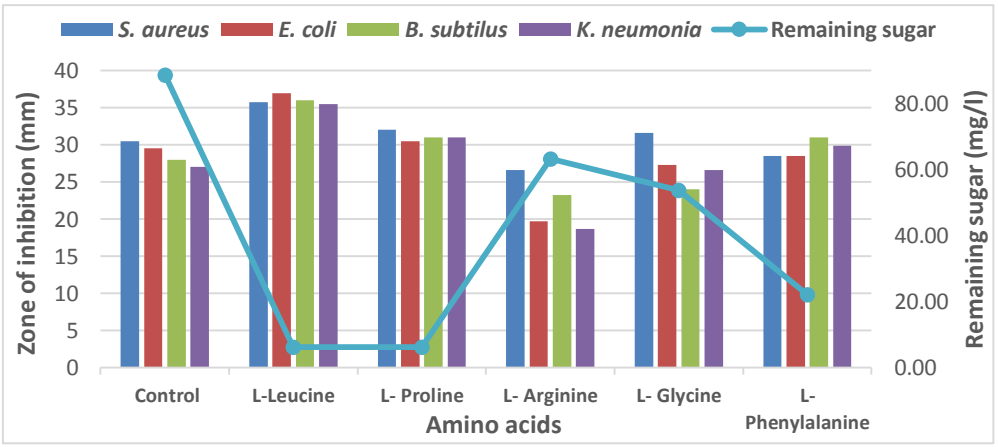

Figure 10-Effect of amino acid sources as inducers on bioactive compounds produced by *Streptomycesrochei* M78 (pH: 7, temperature: 30, carbon source: starch, rpm: 150).

Physiochemical characteristic of antimicrobial metabolites

The results showed that the antimicrobial metabolite was red to brown in color, with gummy and oily nature. The purified metabolite is soluble in methanol, chloroform, diethyl ether, ethyl acetate, DMSO and water with heating, but insoluble in hexane, butanol and petroleum ether with a melting point of 150 °C. Similar results were recorded in previous studies [58,59].

Stability of antimicrobial metabolites

In the current study, the stability of the active metabolite at different pH and different temperature was examined. The results in Figure-11 showthat the metabolite was stable at pH ranging from 4 – 11. Maximum antibacterial activity was found at pH 7 and the activity decreased with the increase or decrease of pH value. The metabolites of isolate M78 were stable at temperature ranging from 25 to 100 °C. Maximum activity was found at 25 °C. The antibacterial activity was not eliminated upon treatment of temperature of 60 to 100 °C. It could be concluded that the active metabolites might be of a non-protein nature; the activity of the compound gradually decreased with the increase in temperature;thisdid not, however, lead to a complete loss of antibacterial activity. Uddin*et al*., [27] found that bioactive metabolites produced by *Streptomyces albolongus* were stable at pH 4 – 11 maximum of 7 and temperature ranged from 60 to 100 °C maximal at 35 °C. Similar results were also found by Maataoui*et al*., [27].

Table 2- The yield and antibacterial activity of different extraction fractions of the metabolitesproduced by *Streptomycesrochei* M78.

Solvents	Crude extract (g/l)	Zone of Inhibition (mm)			
		B. subtilis	*S. aureus*	*E. coli*	*K. pneumonia*
N- butanol	1.80	22.1	26.345	23.265	28.535
Petroleum either	1.90	24	25.49	22.05	19.715
N- hexane	1.42	0	0	0	0
Ethyl acetate	2.18	38.5	33.815	33.59	37.24
Methanol: chloroform	2.08	26.5	24.435	28.065	25.75
Diethyl either	1.21	21.5	20.245	19.5	18.5

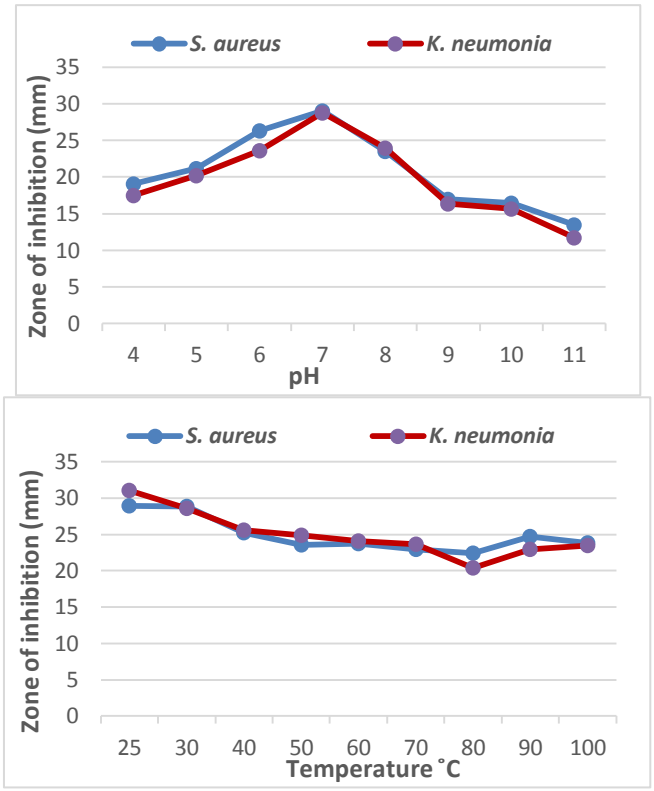

Figure 11-Stability of the crude extract from the isolate *Streptomycesrochei* M78 with different pH and temperature values.

Minimum Bactericidal Concentration of ethyl acetate extract

The biological activity (MBC) of the crude extract showed that the extract was active against Gram positive and Gram negative bacteria. The MBCs values exhibited by the extract in this study ranged between 40 to 250 µg/ml Figure-12. Higher bactericidal concentration of the compound against Gram positive and Gram negative bacteria was 250 µg/ml. The results also showed that MBC values of the active metabolite had an impact at lower concentrations than those of standard antibiotic (gentamycin) against tested pathogenic bacteria,suggesting that the metabolite was more effective. These results may suggest that this active metabolite, after its further purification, could be used for the development of the valuable pharmaceutical application as shown by the results of antibacterial activity.

Minimum inhibitory concentration

The bioactive compounds of the isolate *Streptomycesrochei* M 78 exhibited a broader and higher antimicrobial activity Figure-12. MIC value of compounds was 500 µg/ml against all tested bacterial isolates. Both MIC and MBC values of bioactive compound had an impact at lower concentrations than those of standard antibiotics against tested pathogenic isolates, suggesting that the bioactive compounds were more effective. Similar results were observed by Vieglmann*et al*., 2014 [16] who found that MIC of polyhydroxylated saturated fatty acids ($C_{20}H_{40}O_6$) produced by *Streptomyces* strain SM8 exhibited higher antimicrobial activity at a concentration of 400 and 210 µg /ml against *B. subtilis* and *Candida albicans* respectively. Also a mixture of four hydroxy fatty acids; 7-hydroxy-tetradecanoic acid, 7-hydroxy-pentadecanoic acid, 9-hydroxy-hexadecanoic acid and 9-hydroxy-heptadecanoic acid produced by *Streptomyces* sp. TN272 showed antifungal activities against the two tested fungi, *Fusarium* sp. and *Candida tropicalis*

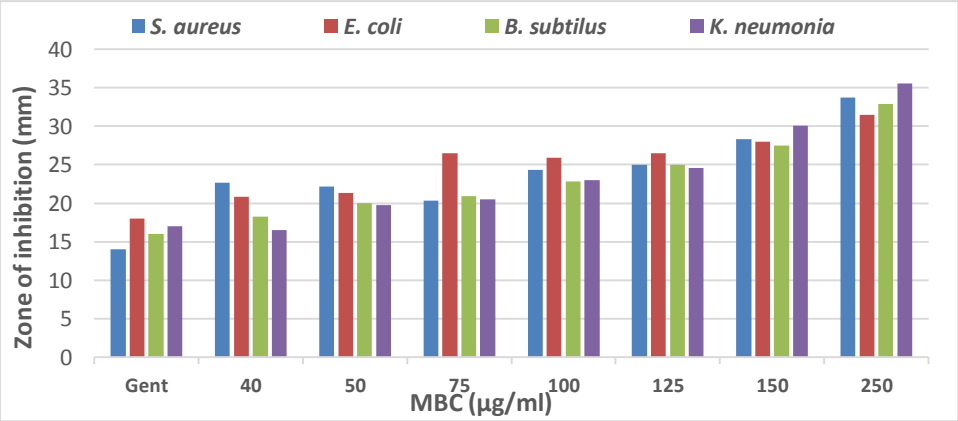

Figure 12-Minimum bactericidal concentration of crude extract produced by *Streptomycesrochei* M78 against pathogenic bacteria.

Thin layer chromatography (TLC)

TLC is often used for the preliminary characterization of antimicrobial compounds from producing microorganism organic extract, especially the determination of the position of the spot on the TLC plate [60; 27]. In the current study, chloroform: methanol (9: 1 v/v) was used as the solvent system to study ethyl acetate extract of the isolate M78. TLC analysis showed two spots having an R_f value = 0.72 and 0.80 Figure-13. The antimicrobial compound exhibited positive results with the iodine, ferric chloride, and negative result with ninhydrin reagent. Similar results were observed by Ilic*et al*., [61] and Maataoui*et al*., [27], they observedthatR_f value ranged from 0.70 to 0.78 of ethyl acetate extract active compounds developed in chloroform: methanol (9: 1 v/v).

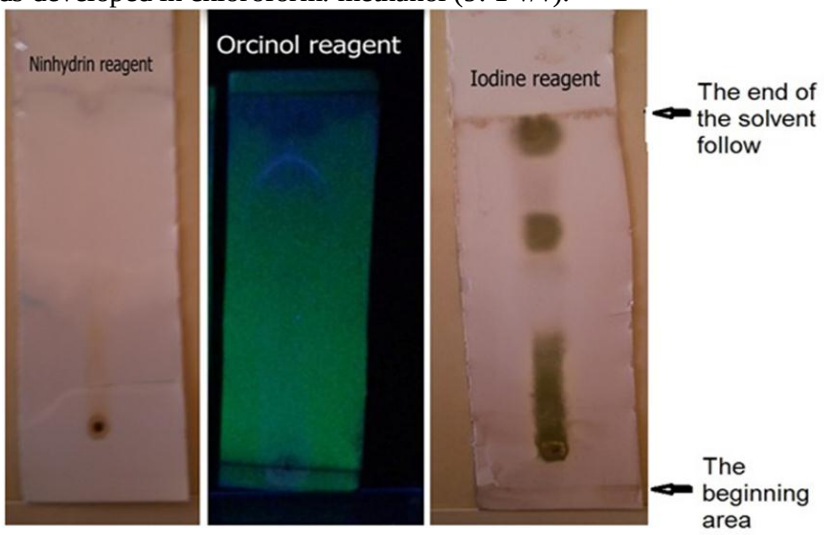

Figure 13-TLC analysis of partial purified bioactive metabolites produced by *Streptomycesrochei* M78

FTIR spectrum of antibacterial metabolite

The FTIR spectrum of antibacterial compounds in figure 14showed an absorption band at Vmax 3442.9 and 1039.6, which indicates (OH) groups in the structure, 1074 and 1645.5 (C=O) functional ester group, 2856.5 and 2927.9 (C-H) groups, 1404.1 and 1458.1 indicates the presence of CH_3 group [31,62].

GC- Mass analysis of antibacterial metabolites

The chemical composition of semi-purified bioactive extracts was analyzed by GC-MS.In comparison with the constituents of the NIST library, a total of 23 peaks were observed, out of which 13 active peaks were predicted Table-3. Of the 13 compounds identified, the data revealed the occurrence of two major separable components with the molecular formulas of $C_{22}H_{44}O_4$ and $C_{15}H_{30}O_2$ with relative abundance of 100 and 33.63 % respectively Table-3. The two major hydroxylated fatty acids were then identified as Octadecanoic acid, 2-(2-hydroxy ethoxy) ethyl ester and Tridecanoic acid, 3 methyl-, methyl ester with molecular weight 372 and 242 (g/mole) respectively. Both peaks with GC analysis indicated the aliphatic and hydroxylated lipid nature of the structures. Similar results

were mentioned by Viegelmann*et al*., [16] with slight differences regarding the chemical structure.They observed polyhydroxylated saturated fatty acid from *Streptomyces* sp. (SM8), and elucidated as 3,6,8,11-tetrahydroxy-16, 17 dimethyl octadecanoic acid with the chemical structure $C_{20}H_{40}O_6$ and molecular weight of 376.2 (g/mole). They also mentioned that the product polyhydroxylated fatty acid belong to glykenin antibiotics. 3-hydroxy saturated fatty acids had previously been reported to have antifungal activity against some fungi strains [63];Kitahara*et al*., [64] observed an antibacterial activity of saturated fatty acids against methicillin-resistant *Staphylococcus aureus*. The proposed fragmentation pathway for metabolite octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester ($C_{22}H_{44}O_4$) as suggested by Viegelmann*et al*., (2014) include Eicosanoic acid (C_{20} H_{40} O_2) (312 g/mol), Hexadecanoic acid (C_{16} H_{32} O_2) (256 g/mol) and Tridecanoic acid (C_{13} H_{26} O_2) (241 g/mol).

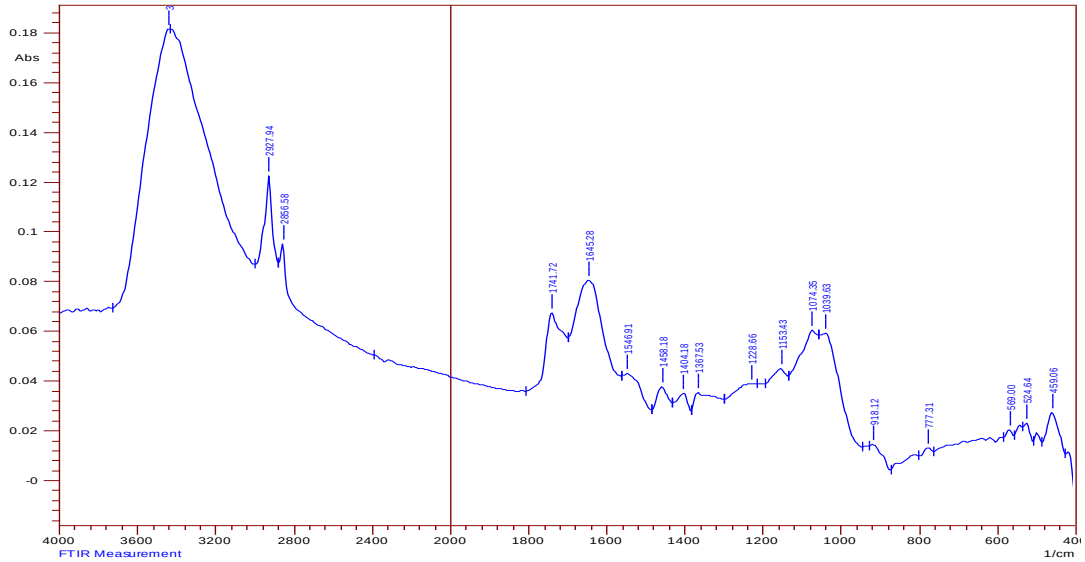

Figure 14-FT-IR spectra of antibacterial metabolites produced from *Streptomycesrochei* M78.

The compounds were identified from the GC analysis of the extract Table-3, whichmight be responsible for the antibacterial activity. Similar results were observed by Kumar *et al*.,[65] and Joseph –Selvin*et al*., [58].Compounds such as 1,4 Dioxane, Hexanoic acid and Oleic acid produced by *Streptomyces lavendulas* strain SCA5 and Actinomycetes*Nocadiopsisdassonvillei* MAD08 respectively were reported to possess an antimicrobial activity.

Table 3-GC mass profile of the *Streptomycesrochei* M78ethyl acetate extract.

No.	Retention time (min)	Compounds	Molecular formula	Molecular weight (g/mole)	Area %	Relative Abundance %
1	2.38	Cyclobutene, 2-propenylidene	C_7H_8	92	4.99	12.88
2	9.22	4-Hepten-3-one, 4-methyl	$C_8H_{14}O$	126	2.56	6.68
3	12.97	Pentanoic acid	$C_5H_{10}O_2$	102	2.42	6.25
4	15.82	Propionic acid, 2-methyl	$C_4H_8O_2$	88	2.35	6.06
5	16.96	1,3Dioxan -4-one, 2-(1-methyl-ethyl) -5-methyl	$C_8H_{14}O_3$	158	2.35	6.06
6	17.06	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.65	14.58
7	17.63	Tridecanoic acid,12methyl-, methyl ester	$C_{15}H_{30}O_2$	242	4.50	11.61
8	18.066	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	$C_{22}H_{44}O_4$	372	38.75	**100.00**
9	18.433	Tridecanoic acid, 3-methyl -, methyl ester	$C_{15}H_{30}O_2$	242	13.03	**33.63**
10	19.15	Octanoic acid, 4-methyl	$C_9H_{18}O_2$	158	3.07	7.92
11	20.19	3-Undecene	$C_{11}H_{22}$	154	3.5	9.03
12	20.44	Tetradecanoic acid, butyl ester	$C_{18}H_{36}O_2$	284	2.05	5.29
13	23.79	1,2-Benzen dicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	5.43	14.01

The GC analysis of the ethyl acetate extract of the new *Streptomyces* sp. Isolate TN272 delivered eighteen bioactive compounds. Among them were the compounds: 1-hexadecene, 5-octadecene,hexadecanoicacid, 1-nonadecene, 7-hydroxy-tetradecanoic acid, 7-hydroxy-pentadecanoic acid,9-hydroxy-hexadecanoic acid and 9-hydroxy-heptadecanoic acid which possessesantifungalactivities [15].

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