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# ISOLATION AND IDENTIFICATION OF *STREPTOMYCES BAARNENSIS* MH-133 PRODUCE BIOACTIVE METABOLITE INHIBITING MULTIDRUG RESISTANT BACTERIA (MDRB)

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## ABSTRACT

In the present study five agriculture soil samples were collected from Empoli, Tuscany, Italy. Thirty-seven actinomycete isolates were obtained and selected from these samples according to their morphological characteristics and purified on starch nitrate agar medium. These isolates were screened for antibacterial activity against Multidrug Resistant Bacteria (MDRB), (*E. faecalis* and MRSA) as Gram positive bacteria and (*E. coli, Klebsiella pneumonia, Acinetobacter baumanii, Pseudomonus aeruginosa*, and *Enterobacter cloacae*) as Gram negative bacteria in addition to standard strain *Bacillus subtilis* ATCC-6633, *Micrococcus luteus* ATCC-4698 and *Salmonella typhi* ATCC-6539. Among tested actinomycete isolates only MH-133 isolate could inhibit the growth of all tested bacteria except *Enterobacter cloacae.* Morphological, physiological and biochemical identification results of MH-133 isolate indicated that, it belongs to the genus *Streptomyces.* The comparative analysis of 16S rDNA sequence and phylogenetic relationship showed that MH-133 isolates lies in a separate clade with *Streptomyces baarnensis* strain NRRL B-1902. MH-133 isolate was submitted to the GenBank, NCBI under the accession number, KY698030. Factors affecting antibacterial metabolite(s) were optimized to enhance the antibacterial activity. The optimum conditions were pH 8, temperature 30ºC, inoculum size 6% (v/v) on modified marine medium under shaking condition (150 rpm). The fermentation kinetic study was performed and the result showed that the maximum antibacterial activity was significantly achieved at the eighth day while both of carbohydrate consumption and biomass production were not shown significant changes starting from the sixth day. This study clearly proves that our isolate exhibit antibacterial activity against MDRB.

**KEYWORDS:** *Streptomyces baarnensis*, antibacterial, Multidrug Resistant Bacteria, fermentation kinetic.

## INTRODUCTION

In past decades, the discovery of different antibiotics from various microorganisms and the near eradication of diseases such as tuberculosis led to the concept that infectious diseases may be something of the past.[1] However, the emergence of infectious diseases involving multidrug resistant (MDR) bacterial pathogens since the 1980s means that bacterial infections are still a major threat for human health.[2] According to the World Health Organization over-prescription and improper use of antibiotics has led to the resistance of many pathogens.[3] The explosive increase of infections by pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) and fluoroquinolone-resistant *Pseudomonas aeruginosa* is estimated to cause approximately 19,000 deaths per year in the USA.[4] Also, it was estimated about 2 million infections and 23,000 deaths caused by antibiotic resistant pathogens per year in United States.[5] Current antimicrobial therapies have reduced efficiency or became inactive against these isolates, which have led to increased mortality among patients[6] and more people now dies of these increasingly antibiotic resistant pathogens than of HIV/AIDS and tuberculosis combined.[7] This underlines the urgent need for new antimicrobials. To overcome this situation, there is an interest to improve or discover novel class of antibiotics that have activity against MDRB, actinomycetes provided many important bioactive compounds of secondary metabolites with varying biological activities such as antibacterial, antifungal, antiviral, anticancer, enzyme,

immunosuppressant and other industrially useful compounds.[8] About two-third of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes, especially from *Streptomycetes*. *Streptomyces* species are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics.[9] The present study aims to screen and isolate a promising strain of actinobacteria for the development of new bioactive compounds against MDRB. Innovative strategies were used for screening of novel types of secondary metabolites producing actinomycetes against antibiotic resistant bacteria. Fresh cultures of human pathogenic bacteria were isolated from hospitalized patients and their sensitivity assays were conducted to determine the resistance pattern as sensitive, intermediate and resistant and only the most antibiotic resistant bacteria were used as indicator strains. This strategy became fruitful for screening of most effective secondary metabolites producing actinomycetes from the selected isolates.

## MATERIAL AND METHODS

***Selection and identification of the most MDRB***

Twenty-six pathogenic bacterial isolates were obtained from microbiology laboratory at 57357 Hospital for treatment of cancer patient, Cairo, Egypt. Antibiotic susceptibility of all isolates was determined by means of the agar diffusion method, according to guidelines established by clinical and laboratory standard institute.[10] The isolates that exhibited resistance against most tested antibiotics were microscopically differentiated by Gram stain and identified biochemically using vitek2 instrument. These isolates were used in the screening for antibacterial activity from actinomycetes in addition to standard strains (*Bacillus subtilis* ATCC-6633, *Micrococcus luteus* ATCC-4698 and *Salmonella typhi* ATCC-6539).

***Collection of soil samples***

Agricultural soil samples (approximately 500 g) were collected from different localities (0.5 km interval) at various depth of surface, ranging from layers of 15 to 40 cm depth. The samples were collected in sterile plastic containers by using sterile spatula, stored in iceboxes and transported to the laboratory. Soil samples were air dried for 7-10 days at 40 ℃,[11] Crushed and sieved to remove the shells and debris at the bacteriology Laboratory, Department of Microbiology, Faculty of Science, Al-Azhar University, Cairo, Egypt.

***Isolation of actinomycetes***

Actinomycetes were isolated by serial dilution method from collected soil samples according to Janaki and Ganesan[12] with some modifications. Stock solution of prepared soil samples were performed using serial dilution method and inoculated on starch nitrate agar plates. The inoculated plates were incubated at 28ºC for 2 to 3 weeks. During incubation period, the appeared actinomycete colonies were picked according to color, dryness, rough, convex colony. The selected colonies were purified using repeated streak plate method.[13]

***Screening of actinomycete isolates producing antibacterial activity using solid medium***

All purified actinomycete isolates were preliminary screened for their antibacterial activity by agar plug method.[14] Fresh culture of purified actinomycetes were grown on starch nitrate agar at 28°C for 14 day. After incubation time, three discs (6 mm in diameter) were cut by sterile cork poorer and placed on surface of Mueller–Hinton agar plates (Hi-Media - Mumbai) seeded with MDRB at turbidity equal to 0.5 McFarland standards. The prepared plates were incubated at 37°C for 24 h. The inhibition zone of bacterial growth was observed if antibacterial metabolite(s) produced by the actinomycete.

***Second screening using liquid medium***

Based on the results of preliminary screening, the most promising isolates were selected for the assessment of their potentiality to produce antibacterial metabolite(s) using liquid starch nitrate medium.[15] A loopful of selected isolates were inoculated into a 250 ml Erlenmeyer flasks containing 50 ml of starch nitrate broth (seed broth) and incubated on a rotary shaker at 150 rpm at 28°C. After 48 h of incubation, the seed culture (4% v/v) was transferred to a 1000 ml Erlenmeyer containing 200 ml of the same fermentation medium and incubated on a rotary shaker (150 rpm) at 28°C for 14 days After the incubation time, the mycelium was collected by centrifugation. Only 100 µl of supernatant was used for assessment of antibacterial activity. Three replicates were made and the mean diameter of inhibition zones were calculated.

***Characterization of the most potent actinomycetes isolate***

The selected isolate was characterized using morphological, physiological and biochemical methods.[16] The microscopic characterization was done by light microscope (Optika, Italy) using cover slip technique[17] and scanning electron microscopy (JEOL Technics Ltd, Japan) at the regional center for mycology and biotechnology- Al-Azhar University, Cairo.[18] Growth characteristics were determined on various International Streptomyces Project (ISP) media such as tryptone yeast glucose agar medium (ISP-1) malt extract agar (ISP-2), oat meal agar (ISP-3), inorganic salts starch agar (ISP-4), glycerol asparagine agar (ISP-5), Peptone yeast extract agar (ISP-6) and Tyrosine agar (ISP-7) after incubation at 28◦C for 10–15 days. The ability of actinomycetes isolate in utilizing various carbon and nitrogen sources was studied at 28◦C for 10–15 days in ISP-9 media. Salt tolerance was determined on starch nitrate medium prepared with series of NaCl concentrations; 0, 1, 2, 3,4,5,6,7,8,9, and 10% (w/v). Results were scored after incubation at 28 ◦C for 10–15 days. Also, pH and temperature ranges for growth were studied on starch nitrate medium.[19] Tolerance to toxic

substances as Sodium azide 0.01 & 0.02% (w/v), Phenol 0.1%(w/v), Crystal violet 0.001%(w/v) and growth on czapek's medium was formed according to Atta *et al*.[20] Biochemical tests including oxidase, catalase, citrase, lipase, gelatinase, caseinase, urease, lecithinase production, decomposition of tyrosine, cellulose and pectin, $H_2S$ production were done.[21] The actinomycete isolate was identified according to Bergey's Manual of Determinative Bacteriology.[22] Further characterization was done using 16S rDNA analysis according to Atta *et al*.[20]

***Fermentation studies***

***Effect of different media on the production of antibacterial metabolite(s) produced by MH-133 isolate***

Twelve culture media were used in comparative studies to find the optimal culture medium for antibacterial metabolite(s) production. The designated media were starch casein medium,[16] Bennett's medium,[23] ISP-2 medium, ISP-4 medium, ISP-5 medium,[24] starch nitrate medium,[13] modified marine medium,[25] glucose nutrient medium,[26] soybean meal glucose medium,[27] glycerol casein medium,[28] potato dextrose medium[29] and Czapek's medium.[26] The prepared flasks from each media were inoculated with 4% (v/v) of seed culture. All flasks were incubated at 28°C on rotary shaker at 150 rpm for 15 days. After the incubation, the culture filtrate of each medium was assayed for antibacterial activity by agar diffusion assay to select an appropriate medium for further studies. The antibacterial activity was tested by agar well diffusion method against methicillin resistant *Staphylococcus aureus,* multidrug resistant *E. feacalis, K. pneumonia* and *E. coli* as models of Gram-positive and Gram-negative bacteria.

***Effect of cultivation method, different pH, temperature and inoculum size on the production of antibacterial metabolite produced by MH-133 isolate***

To study the effect of cultivation methods on the productivity of MH-133 isolate, 6 conical flasks containing the best production medium were inoculated with 4% (v/v) of seed culture and divided into 2groups (3 flasks into each group) the first group was incubated under static condition at 28℃ and the second group was incubated on rotary shaker (150 rpm) at the same temperature for 8 days.[30]

To determine the effect of initial pH value of culture medium on bioactive metabolite production; 4% (v/v) of seed culture of isolate MH-133 was inoculated into 250 ml conical flasks containing 50 ml of the sterile fermentation medium with different pH values (5 – 9) and incubated on rotary shaker at 30℃ for 8 days. The pH was adjusted using 0.1 N hydrochloric acid or 0.1 N sodium hydroxide. The optimum pH of culture medium was fixed for subsequent study.

The optimum temperature for bioactive metabolite production was assayed at optimum pH and shaking condition by incubating the production medium at different temperatures 15, 20, 25, 30, 35, 40 and 45℃.[31]

To estimate the effect of inoculum size on the antibacterial metabolite production by MH-133 isolate, the sterile fermentation medium was inoculated with 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% (v/v) of the previously prepared seed culture while maintaining all above parameters fixed at optimum condition.[32]

Antimicrobial activity of all parameters was evaluated as mentioned above. All experiments were performed in triplicate. The mean and standard error values were calculated.

***Fermentation kinetic study***

Time course study of activity, biomass and substrate consumption by shake flask fermentation was done. Three replicates of 1000 ml flask under optimum condition (250 ml modified marine broth, pH 8, seeded with 48 h inoculum size 6% (v/v) on a rotary shaker (150 rpm) at 30°C). The study was conducted for 8 days. Every day, 25 ml of culture was withdrawn for determining activity,[14] biomass weight (cell dry weight (CDW))[33] and substrate consumption (total carbohydrates).[34]

***Statistical analysis***

The data obtained were determined to be normally distributed. Homogeneity of variances was assessed using Levene's test. Statistical analysis was also performed using a one-way classification of ANOVA where differences were regarded as statistically significant with probability $P < 0.05$ sing Minitab 17 software extended with a statistical package and Microsoft™ Excel® 2013 were used to statistically analyze the data.

## RESULTS AND DISCUSSION

***Isolation, Identification and antibiotic susceptibility determination of Indicator pathogenic bacterial isolates***

Twenty-six bacterial isolates were purified from clinical samples. The antibiotic pattern of these isolates was determined using 19 antibiotics represent different classes of antibiotics that are commercially available in form of antibiotic discs. The most resistant bacterial isolates were selected according to their resistance to different antibiotics. Generally, the results of this screening were indicated to widespread emergence of multidrug resistance in the tested isolates where, twenty-three isolate (88.46%) among the tested isolates showed resistance against at least three different groups of antibiotics while only three isolates (11.56%) were sensitive to all used antibiotics. Among twenty-three isolates only seven bacterial isolates were selected as the most resistant bacterial isolates coded as MK-5, MK-9, MK-13, MK-16, MK-19, MK-20, MK-24. These antibiotic resistant bacterial.

**Table 1: Antibiotic profile of the selected multi-drug resistant bacteria.**

| No | Antibiotics | Abbreviations | *A. Baumanii* MK-5 | *E. Coli* MK-9 | *E. Cloacae* MK-13 | *K. Pneumonia* MK-16 | *P. Aeruginosa* MK-19 | *E. Feacalis* MK-20 | MRSA MK-24 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Trimethoprime-sulfomethoxazole | SXT 25 | R | R | R | I | R | R | I |
| 2 | Cefuroxime | CXM 30 | R | R | R | R | I | R | R |
| 3 | Penicillin G | P 10 | R | R | R | R | R | R | R |
| 4 | Kanamycin | K30 | R | R | R | R | S | R | R |
| 5 | Nalidixic Acid | NA 30 | R | R | I | R | I | R | R |
| 6 | Vancomycin | VA 30 | R | R | R | R | R | R | S |
| 7 | Gentamycin | CN 10 | R | R | R | R | S | R | R |
| 8 | Bacitracin | B 10 | I | R | R | R | R | R | I |
| 9 | Chloramphenicol | C 30 | R | S | R | R | R | R | R |
| 10 | Methicillin | MET 5 | R | R | R | R | R | R | R |
| 11 | ciprofloxacin | CIP 5 | R | R | I | R | S | R | R |
| 12 | Erythromycin | E 15 | R | R | R | R | R | R | R |
| 13 | Amikacin | AK 30 | R | I | R | I | S | R | R |
| 14 | Rifampin | RA 5 | R | R | R | R | R | R | R |
| 15 | Neomycin | N 30 | R | R | R | I | I | R | R |
| 16 | Clindamycin | DA 2 | R | R | R | R | R | R | R |
| 17 | Amoxicillin-clavulanic acid | AMC 30 | R | R | R | R | R | R | R |
| 18 | Tetracycline | TE 30 | R | R | R | R | R | R | R |
| 19 | Rifamycin | RF 30 | R | R | R | R | R | R | R |

"R" indicate resistant, "I": intermediate, "S": sensitive

Isolates were identified biochemically using automated vitek2 system as *Acinetobacter baumanii* (MK-5) with portability of identification 99% with excellent confidence, *Escherichia coli* (MK-9) with portability of identification 97% with excellent confidence, *Enterobacter cloacae* (MK-13) with portability of identification 99% with excellent confidence, *Klebsiella pneumonia* (MK-16) with portability of identification 99% with excellent confidence, *pseudomonas aeruginosa* (MK-19) with portability of identification 99% with excellent confidence, *Enterococcus feacalis* (MK-20) with portability of identification 99% with excellent confidence and *Staphylococcus aureus* (MK-24) with portability of identification 95% with very good confidence. The most resistant bacterial isolate was *Enterococcus feacalis* (MK-20) that resisted all the tested antibiotics while the isolate *pseudomonas aeruginosa* (MK-19) showed less resistant one that resist 12 of 19 tested antibiotics (Fig. 1) and (Table 1).

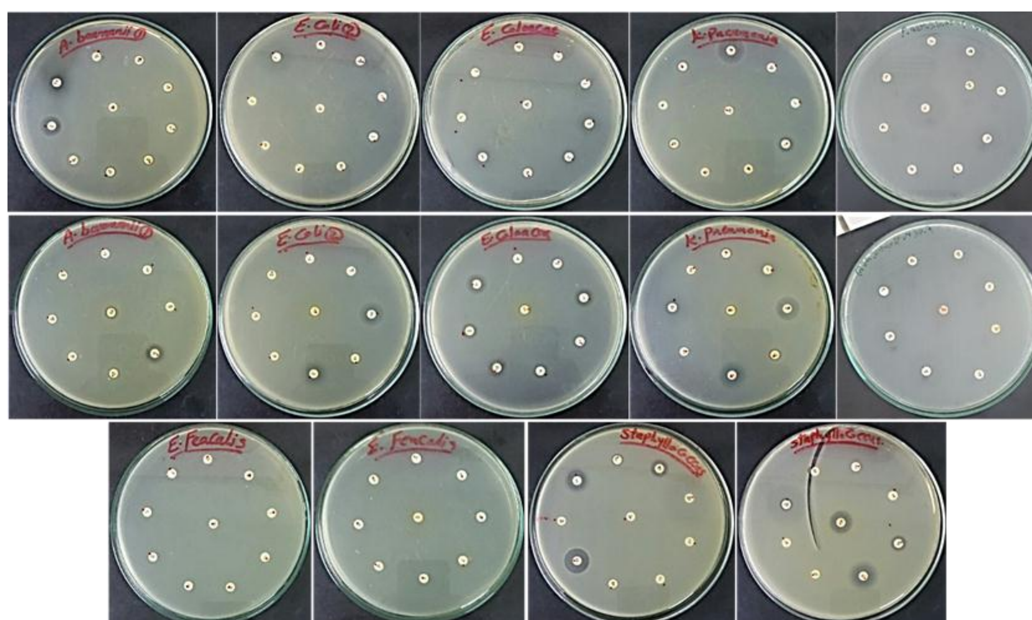

**Fig 1: Antibiotic profile of the selected multi-drug resistant bacteria.**

According to antibiotic susceptibility test of *Staphylococcus aureus,* it was considered methicillin resistant (MRSA), *Enterococcus feacalis* was considered vancomycin resistant (VRE). Also, all seven-bacterial isolate represent (ESKAPE) pathogens which refers to the microorganisms that are mainly involved in the resistance process (*Enterococcus* sp.*, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and enterobacteriaceae) emphasizing their capacity to "escape" from common antibacterial treatments.[35] ESKAPE – cause most nosocomial infections in both developed and developing countries and can easily spread by hospital personnel.[6] Karamese *et al*.[36] reported that the highest rates of nosocomial infections caused by ESKAPE are observed in the intensive care units (ICUs), which are also the units in which the most critically ill patients are treated and the highest mortality rates are detected. This may confirm that, the resistance of these isolates to most of used antibiotics. These MDR bacterial isolates were used for screening of different actinomycetes in order to discover new bioactive metabolites against these types of resistant bacteria.

***Isolation, purification and antibacterial screening of actinomycetes cultures against MDRB***

The present work involves the isolation of actinomycetes from five agriculture soil samples obtained from Empoli, Tuscany, Italy (latitude: 43º.43'0.63 N- Longitude:10º 53'57.70 E). A total of 37 actinomycetes were isolated and purified based on their morphology (powdery and leathery consistency and stick firmly to agar surface) and capability to grow on starch nitrate agar medium.

The preliminary screening of antibacterial activity against both MDRB and standard strains showed only 13 actinomycete isolates exhibited antagonistic property against at least one of the tested bacteria. Actinomycetes isolates coded MH-104, MH-126 and MH-133 were found to be active against at least five of the tested bacteria. So, these isolates were selected for second screening. Agar well diffusion results of the filtrate for the selected three actinomycetes showed that MH-133 isolate exhibited a considerable activity against all multidrug resistant bacterial isolates and the ATCC strains except *Enterobacter cloacae.* The antibacterial activity of filtrate for both of MH-104 and MH-126 isolates were active against most of tested bacteria except *Acinetobacter baumanii, Pseudomonus aeruginosa* and *Enterobacter cloacae* (Table 2). Depend on these results, the isolate MH-133 was selected as the most potent bioactive isolate.

**Table 2: Antibacterial Screening of the most potent isolates against MDRB.**

| No. | Indicator strain | Mean of inhibition zone diameter (mm) | | |
|---|---|---|---|---|
| | | MH-104 | MH-126 | MH-133 |
| 1 | MRSA | 10 | 12 | 16 |
| 2 | *E. faecalis* | 0 | 12 | 16 |
| 3 | *Bacillus subtilis-ATCC-6633* | 11 | 17 | 18 |
| 4 | *Micrococcus luteus-ATCC-4698* | 15 | 18 | 18 |
| 5 | *E. coli* | 18 | 16 | 12 |
| 6 | *Klebsiella pneumonia* | 17 | 18 | 13 |
| 7 | *Acinetobacter baumanii* | 0 | 0 | 14 |
| 8 | *Pseudomonus aeruginosa* | 0 | 0 | 12 |
| 9 | *Enterobacter cloacae* | 0 | 0 | 0 |
| 10 | *Salmonella typhi-ATCC-6539* | 19 | 19 | 20 |

Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. But now, due to the emergencies of multidrug resistant pathogens, there are basic challenges for effective treatment of infectious diseases.[13] So, the screening and isolation of promising actinomycetes with potential antibiotics is still an important area of research and urgent to counter the threats posed by the fast-emerging phenomenon of antibiotic resistance.[37] The most frequent multidrug resistant (MDR) bacteria are *Enterococcus faecalis*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp.[6] Traditionally, soil-derived actinomycetes have been most frequently screened for bioactive compounds. Unfortunately, the frequency of finding new compounds from normal soil-derived actinomycetes is declining because of the repeating in the isolation of known actinomycetes and antibiotics.[38] Alternatively, in this study we tried to get a novel antibacterial metabolite(s) from the screened actinomycete isolates against MDRB. Due to the resistance of indicator bacterial strains, out of the 37 actinomycetes isolates subjected to the preliminary and secondary antibacterial screening, only three isolates (8.1 %) were exhibited a broad-spectrum activity against most of tested bacterial strains.

***Identification of the actinomycete MH-133 isolate***

***Morphological characteristics***

Microscopic examination of the most potent actinomycete isolate MH-133 revealed that; aerial mycelium was long straight to rectiflexibile with spore

chains that confirmed with Scanning electron microscopy examination and produce extensively branched substrate and aerial hyphae. The spores were arranged in chains of cylindrical spores and smooth surface (Fig. 2: A&B).

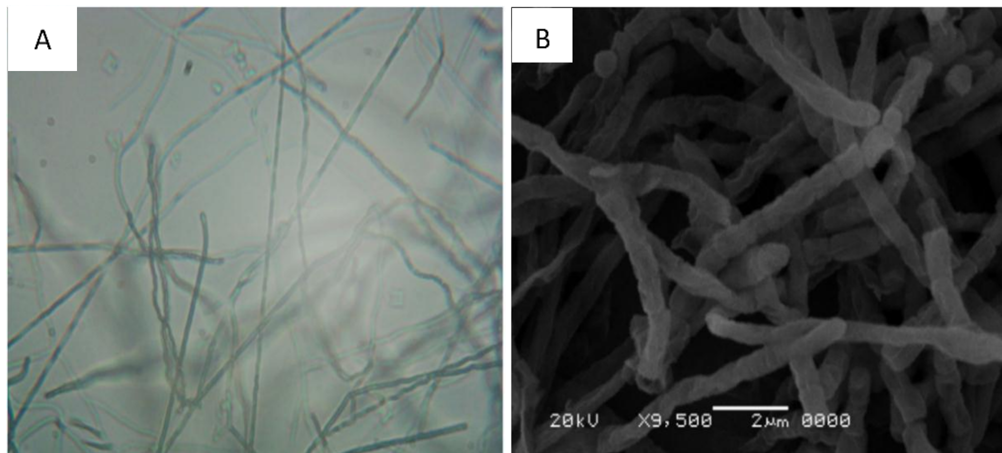


**Fig. 2: (A) Spore chain bearing hyphae of the isolate MH-133 by light microscopy (X600), (B) Spore chain structure of the isolate, MH-133 by scanning electron microscopy; (X9500).**

***Culture characteristics***

Culture characteristics of MH-133 isolate were observed on different ISP media. This isolate was grown well on ISP1, ISP3, ISP4 and ISP7 media and moderate growth was observed on ISP2, ISP5 and ISP6. It was produced white powdery colonies on the surface of agar while reversed side was yellow to pale brown without diffusible pigment on all ISP media (Table 3) and (Fig.3).

**Table 3: Culture characteristics of MH-133 isolate on different ISP media.**

| Medium | Growth | Aerial mycelium | Substrate mycelium | Diffusible pigment |
|---|---|---|---|---|
| Tryptone yeast extract broth (ISP-1) | Good | White | Yellow | No |
| Yeast extract malt extract agar medium (ISP-2) | Moderate | White | Yellow | No |
| Oatmeal agar medium (ISP-3) | Good | White | Light yellow | No |
| Inorganic salts starch agar medium (ISP-4) | Good | White | Light yellow | No |
| Glycerol – asparagine agar medium (ISP-5) | Moderate | White | Yellow | No |
| Peptone yeast extract iron agar medium (ISP-6 | Moderate | White | Yellow | No |
| Tyrosine agar medium (ISP-7) | Good | Greenish white | Pale brown | No |

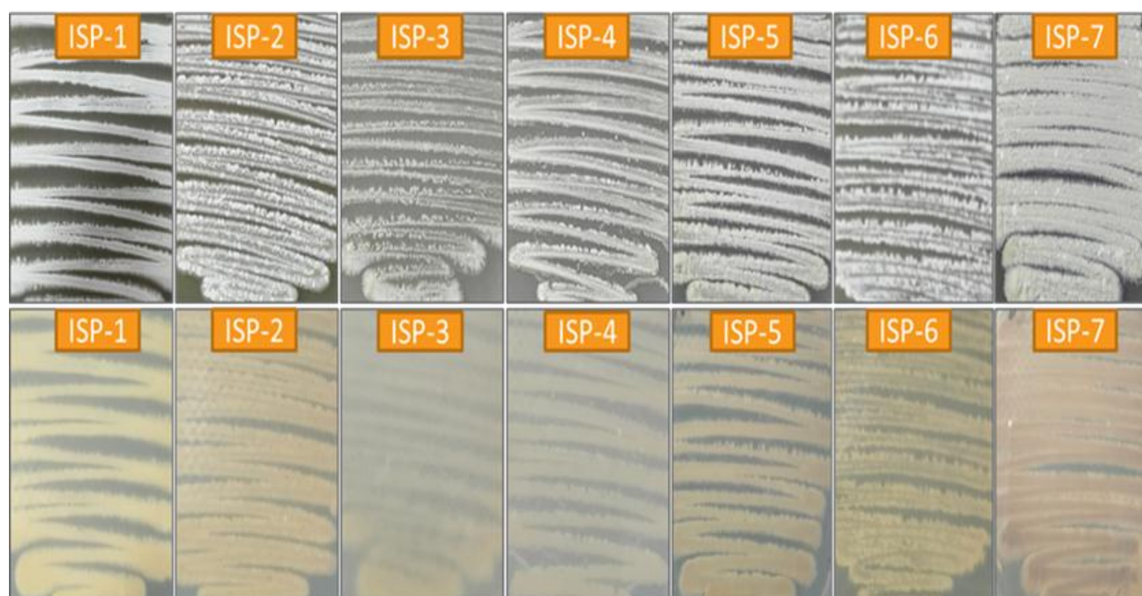


**Fig. 3: Photograph of culture characteristics of MH-133 isolate on different ISP media.**

***Physiological and biochemical characteristics***

The isolate MH-133 grown wide range of sugars and amino acids added to ISP-9 medium as a sole carbon and nitrogen sources respectively. Growth of isolate MH-133 was observed at a range of pH (6-9) but the best growth was exhibited at pH (7-8). Also, the temperature range was (15°C – 45°C). NaCl concentrations suppressed the growth starting from 7% and above. The growth was inhibited by each of sodium azide and phenol at the used concentrations but the growth was tolerated to crystal violet at 0.001% (w/v). The isolate was succeeded to grow on czapek's medium as shown in Table 4.

**Table 4: Physiological characteristics of MH-133 isolate.**

| Characteristics | Results | Characteristics | Results |
|---|---|---|---|
| **Utilization of carbon sources** | | **Growth at different temperatures (°C)** | |
| D- Glucose | ++ | **15** | + |
| L-Rhamnose | ++ | **20** | ++ |
| D-Xylose | ± | **25** | ++ |
| Mannitol | ++ | **30** | ++ |
| Inositol | + | **35** | ++ |
| Sucrose | + | **40** | + |
| Arabinose | + | **45** | **-** |
| Cellulose | + | **Growth at different concentration of NaCl (%)** | |
| Fructose | + | **1%** | ++ |
| Starch | ++ | **2%** | ++ |
| **Utilization of amino acids** | | **3%** | ++ |
| L-Asparagine | + | **4%** | ++ |
| L-Cysteine | - | **5%** | ++ |
| L-Valine | + | **6%** | + |
| L-Therionine | ++ | **7%** | **-** |
| L-Phenylalanine | ± | **8%** | **-** |
| L-Methionine | + | **9%** | **-** |
| L-Histidine | ++ | **10%** | **-** |
| L-Arginine | + | | |
| **Growth at different pH values** | | **Tolerance to toxic substances** | |
| **5** | **-** | Sodium azide 0.01 %(w/v) | **-** |
| **6** | + | Sodium azide 0.02 %(w/v) | **-** |
| **7** | ++ | Phenol 0.1%(w/v) | **-** |
| **8** | ++ | Crystal violet 0.001%(w/v) | + |
| **9** | + | Growth on czapek's medium | + |

"+" indicate Positive, "- " Negative, "±" doubtful results, "+" moderate growth "++" good growth.

***Biochemical characteristics***

Table 5 explained the behavior of the actinomycete isolate MH-133 toward different substrates where, it could hydrolyze starch, gelatin, lipid, pectin but not hydrolyze lecithin, casein and urea. Citrate utilization, Motility and $H_2S$ production are negative. Degradation of esculin was positive whereas tyrosine degradation was negative.

**Table 5: Biochemical characteristics of MH-133 isolate.**

| No. | Test | Results |
|---|---|---|
| 1 | Lipid hydrolysis | + |
| 2 | Starch hydrolysis | + |
| 3 | Gelatin hydrolysis | + |
| 4 | Casein hydrolysis | - |
| 5 | Tyrosine degradation | - |
| 6 | Urea degradation | - |
| 7 | Pectin degradation | + |
| 8 | Esculin degradation | + |
| 9 | Citrate utilization | - |
| 10 | Motility test | - |
| 11 | $H_2S$ production | - |
| 12 | Lecithin degradation | - |
| 13 | Catalase | + |
| 14 | Oxidase | + |

"+" Positive "- " Negative

***Molecular characterization***

The partial 16S rDNA sequence (934 nucleotides) of MH-133 isolate was determined and deposited in GenBank under the accession number KY698030. This sequence was aligned with those of *Streptomyces* reference species available in the GenBank database, which confirmed the identification of MH-133 isolate at the genus level. The similarity level was 95% with *Streptomyces baarnensis* strain NRRL B-1902 16S and *Streptomyces anulatus* strain NBRC 13369 the most closely related species as shown in Fig. 4.

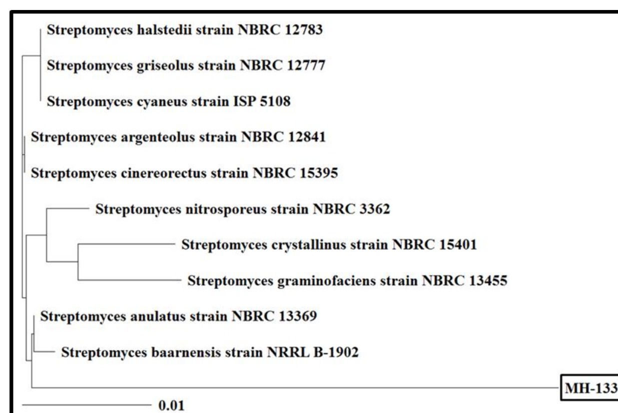


**Fig 4: Neighbor-joining tree, based on 16S rDNA sequences showing the relations between MH-133 isolate and the most closely related type strains of Streptomyces.**

The morphological characteristics and microscopic examination indicated that the spore chain is rectiflexibile. Spore mass is white and greenish white; while spore surface is smooth, substrate mycelium is Light yellow to yellowish brown without diffusible pigment on ISP-6 and 7 media. The results of physiological and biochemical characteristics of MH-133 isolate emphasized that this isolate related to a group of *Streptomyces*. The resulted sequence was aligned with available almost compete sequence of type strains of family streptomycetacae. It formed separate clade that was closely related to *Streptomyces baarnensis* strain NRRL B-1902 16S and *Streptomyces anulatus* strain NBRC 13369, sharing 16s rRNA gene similarity matrix is 95% but *Streptomyces anulatus* has different phenotypic characteristics such as, spore chain is rectiflexibile with open spirals type of two to several turns, hooks and loops of small diameter. So, *Streptomyces anulatus* can therefore be placed in Section Spirals or Retinaculum-Apertum also, aerial mass of *Streptomyces anulatus* on the most of ISP media is yellow and are not able to use iso-inositol, sucrose and raffinose as a sole carbon source[17,39] It is evident from the genotypic and phenotypic data MH-133 isolate was suggestive of being *Streptomyces baarnensis* MH-133. The genus *Streptomyces* provide a wide variety of new antibiotics more than any other genus; hence, it is of great importance for both industrial applications and human health care.[40]

***Fermentation studies***

***Effect of different media***

Along the course of screening the best culture media for antibacterial metabolites production, the result showed that the filtrate of *Streptomyces baarnensis* MH-133 was inactive when used with glycerol casien medium and soybean meal glucose medium. The filtrate obtained from fermentation media of starch casein medium, potato dextrose medium and riched medium were active only against Gram-positive MDRB. The filtrates of remaining media were active against both Gram-positive and Gram-negative MDRB with different degrees. modified marine medium was the best medium exhibiting the highest significant antibacterial activity (inhibition zone ranged from 14.6 – 17.6 mm) against all tested MDR bacteria (Fig. 5). So, this medium was used to determine the optimal conditions to enhance antibacterial productivity by *Streptomyces baarnensis* MH-133.

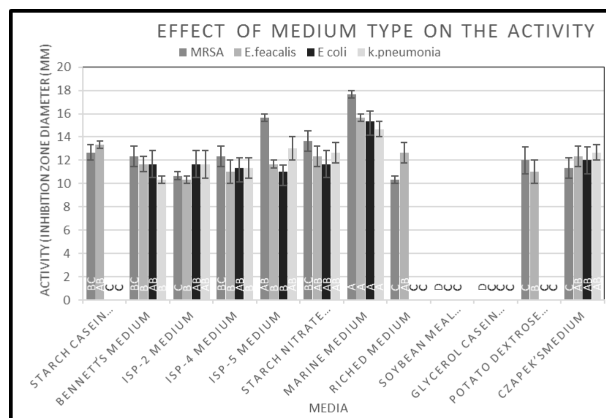


**Fig. 5: Effect of different media on the antibacterial activity of *Streptomyces baarnensis* MH-133.**

In fact, it has been shown that the nature of carbon and nitrogen sources strongly affect antibiotic production in different organisms[41] The results showed that antimicrobial activity was higher in modified marine medium having starch and peptone as carbon and nitrogen source respectively. The high yield rate from these media is not surprising because these organic substrates are the classic nutrients in cultivating *Streptomyces,*[42] Oskay,[31] also reported that the activity of actinomycete isolates could be increased or decreased remarkably under different nutritional cultural conditions.

***Effect of cultivation methods***

Effect of different cultivation methods on the production of bioactive metabolite(s) by *Streptomyces baarnensis* MH-133 was studied throughout their cultivation in both static and shaking (submerged) conditions. It was found that the submerged cultivation was shown significant increasing the antibacterial activity (inhibition zone ranged from 14-16 mm) than that of static condition (inhibition zone ranged from 10.6 – 12 mm) (Fig. 6).

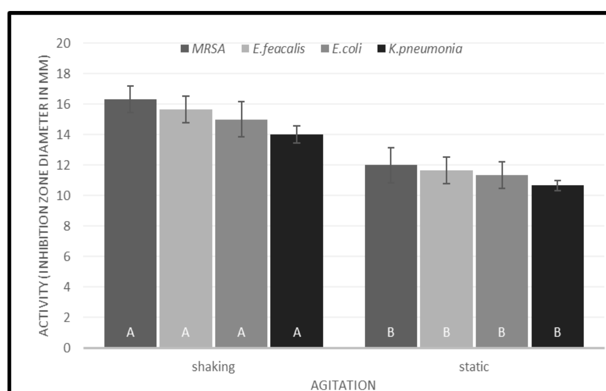


**Fig. 6: Effect of different cultivation condition on the antibacterial activity of *Streptomyces baarnensis* MH-133.**

Culturing condition of *Streptomyces baarnensis* MH-133 revealed that shaked culture enhance the antibacterial activity than static culture, it means that both aeration and nutrient availability performed by shaking is very important for metabolic activity of this strain. Our results agreed with the findings of many researchers that found

the antimicrobial productivity increased in shaking culture comparing with the static one. This is due to the fact that streptomycetes are obligate aerobic organisms.[43-45,30]

### *Effect of initial pH*

*Streptomyces baarnensis* MH-133 was seemed to produce antibacterial metabolite(s) at wide range of pH (6 -9) while the organism was failed to exhibit any inhibitory effect against all tested bacterial strains at initial pH 5. The optimum initial pH value capable of promoting active metabolite biosynthesis was found to be significantly at pH 8 where the inhibition activity (15 – 18 mm). also, initial pH 7 exhibited considerable activity (13-17mm) (Fig. 7).

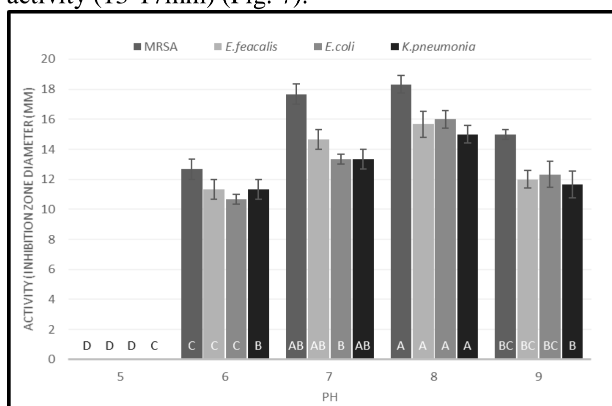


**Fig 7: Effect of initial pH on the antibacterial activity of *Streptomyces baarnensis* MH-133.**

The optimum initial pH of the medium is one of the key factors affecting growth, product formation of microorganisms and characters of their metabolism. soil Actinomycetes are marked by relative intolerance of acidity usually preferring neutral or slightly alkaline pH for the growth and antagonistic activity.[7] The results obtained from this study revealed that the antibacterial activity of *Streptomyces baarnensis* MH-133 found in pH range from 6 to 9 with optimum activity against all MDR bacteria at pH 8. This result is similar to that published by Bashir *et al.* [3] that reported the antimicrobial activity increased and attained maximum with gradual increase of pH from 6 to 8 and began to decrease at pH 9. No activity was observed at pH 5 against both indicator strains and the best activity was observed at pH 8.

### *Effect of incubation temperature*

Generally, the antibacterial activity of *Streptomyces baarnensis* MH-133 was appeared at wide range of temperature from 15 to 40°C while the activity disappeared at 45°C. Data represented graphically in Fig. 8 showed that, the optimum temperature able to produce antibacterial activity was significantly at 30°C, whereas, the diameter of inhibition zone ranged from 15 to 18mm against all tested bacteria. Also, the antibacterial activity at 25°C was insignificantly different than 30°C except the activity against *k. pneumonia* was higher at 30°C.

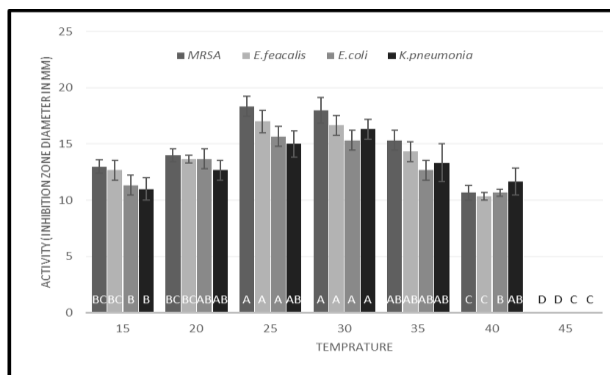


**Fig 8: Effect of incubation temperature on the antibacterial activity of *Streptomyces baarnensis* MH-133.**

Temperature has direct effect on the physiology, morphology, biochemistry and metabolites production of organisms.[48] The results obtained from incubation temperature study showed that, the antibacterial activity of *Streptomyces baarnensis* MH-133 lies in the range from 15 to 40°C with highest activity at 30°C. These results are in agreement with Abdelwahed *et al.*[49] who showed that The maximum antimicrobial secondary metabolites production was obtained at incubation temperature 30°C for isolates, (*S. cyaneus* DN. 37 and *S. lavendulae* DN. 7), then obvious decline in the productivity was occurred at 45°C.

### *Effect of inoculum size*

The inoculum size appeared to have visible effect on the antibacterial activity caused by *Streptomyces baarnensis* MH-133. The optimum inoculum size was 6% (v/v) that gives significant increase in the activity against all tested MDR bacteria (Fig. 9).

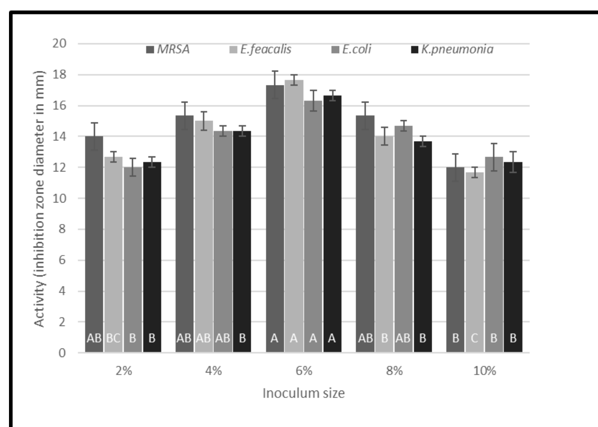


**Fig 9: Effect inoculum size on the antibacterial activity of *Streptomyces baarnensis* MH-133.**

Adequate inoculum can initiate fast mycelium growth and product formation, thereby reducing the growth of contaminants and antibiotic production attains its peak when sufficient nutrients are available to the biomass.[50] Our results showed that maximum inhibition zone, corresponding to maximum antibacterial activity, was observed at inoculum size 6% (v/v) and decreased gradually with increasing in inoculum size, this may

have attributed to the Conditions with a misbalance between nutrients and proliferating biomass result in decreased antibiotic synthesis.[51]

***Fermentation kinetic study***

The time course of antibacterial production, growth and carbohydrate consumption in modified marine broth are shown in Fig.10. It was found that the activity was started from the second day of incubation and increased gradually until the end of incubation (the eighth day). The biomass that expressed in term of cell dry weight (CDW) was represent a typical growth curve where, a significant increase in the biomass was observed from the first day till the fourth day, after that it the increase was insignificant. Total carbohydrates were estimated daily and it was significantly decreased gradually from the first day to the fifth day, then still constant in the last three days.

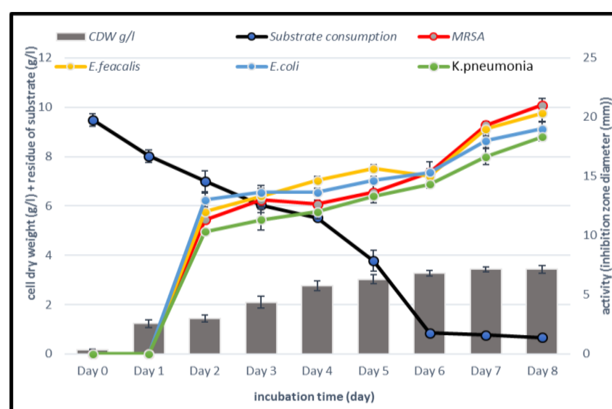


**Fig 10: Time course of *Streptomyces baarnensis* MH-133 on modified marine broth medium.**

In our case, there was strong correlation between carbohydrate consumption and biomass production. Also, antibacterial metabolite production was started in the log phase and reached the maximum value in the stationary phase indicating that metabolite production may be directly proportional to the growth rate. Mustafa,[52] reported that, the condition of incubation influenced quantitatively the biosynthesis of antibiotics as well as biomass. It has been observed that accumulation of antibiotics, cephalomycin C and clavulanic acid has been occurring in parallel with growth of defined medium.

## CONCLUSION

The current study revealed that, *Streptomyces baarnensis* MH-133 is a powerful soil isolate having a broad spectrum of activity against the most resistant types of pathogenic bacteria (ESKAPE). Studies on the separation, purification of bioactive compound and their identification would be rewarding.

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