The efficiency of Soran M Medium in Selective Isolation of Antibiotic Producing Actinomycetes

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Abstract

To assess the role of enriched constituents forming Soran M medium in selective isolation of antibiotic producing actinomycetes, six soil samples were collected in November 2016 from different sites in Soran, Erbil, Iraq. To culture soil samples spread-plate technique was used on Soran M and standard medium such as Arginine Glycerol Salt agar. Preliminary screening was done using cross-streak method against four bacterial pathogenic strains and *Candida albicans*. Nine isolates from total 40 actinomycetes isolates showed antimicrobial activities against pathogens. The morphological and cultural characterization of these isolates was performed and showed that 3 isolates belonged to the genus *Nocardia* while the others belonged to *Streptomyces*. The potential strain MB3 with both broad spectrum antibacterial and antifungal activity was isolated on both media but showed different abilities to produce antibiotics. It was identified as *Streptomyces* sp. Findings from this investigation revealed that nutrient media with different composition affect the isolation of antibiotic producing actinomycetes with different activities.

Keywords: Actinomycetes, Antifungal activity, antibacterial activity, Soran M medium.

كفاءة وسط " سوران أم" الغذائي في العزل الإنتقائي للبكتريا الخيطية المنتجة للمضادات الحيوية

الخلاصة

لتقييم دور المكونات الغنية المكونة لوسط "سوران أم" الغذائي في العزل الانتقائي للبكتريا الخيطية المنتجة للمضادات الحيوية، تم جمع ست عينات تربة في شهر تشرين الثاني 2016 من أماكن مختلفة في مدينة سوران ، أربيل، العراق. وقد استخدمت تقنية النشر في الطبق Spread- plate technique لزرع عينات التربة على وسط "سوران أم" الغذائي ووسط أكار الارجنين والكليسرول الملحي القياسي. تم الكشف الاولي عن العزلات المنتجة للمضادات الحيوية باستخدام طريقة التخطيط المتقاطع Cross-streak method مع أربع من العزلات البكتيرية الممرضة وعزلة واحدة من الخمائر الممرضة هي النوع *Candida albicans*. أظهرت تسع عزلات قدرتها على إنتاج المضادات الحيوية من أصل 40 عزلة من البكتريا الخيطية التي تم عزلها على كلا الوسطين. تمت دراسة الخصائص والصفات الشكلية والمزرعية لتلك العزلات وقد تبين عائدية ثلاث عزلات منها الى الجنس *Nocardia* في حين كانت البقية تتبع الجنس *Streptomyces*. كانت العزلة MB3 هي الأقوى في قدرتها على إنتاج مضادات حيوية مضادة للبكتريا واسعة الطيف ومضادات فطرية والتي تم عزلها على كلا الوسطين ولكن بقدرات متباينة على انتاج المضادات الحيوية، وقد أظهر التشخيص عائدية تلك العزلة للجنس *Streptomyces*. كشفت الدراسة الحالية أن المكونات الغذائية المختلفة للاوساط الزرعية المستخدمة في عزل البكتريا الخيطية المنتجة للمضادات الحيوية تؤثر على قدرتها على إنتاج تلك المضادات.

Introduction

Actinomycetes are classified as a group of gram positive, aerobic and filamentous bacteria, which compress a group of branching unicellular microorganisms that unique for their spore forming abilities and formation of mycelium structure which maybe of two kinds; substrate and aerial mycelium. The colonies have pastel color, soil-like odor, hard and stick into agar [1, 2]. Actinomycetes were first classified as fungi because they have true aerial hyphae which were considered to be fungal characteristic, also actinomycetes are now recognized as true bacteria because of their cell wall and internal structures are typically of bacteria rather than fungi[3]. The major groups of order Actinomycetales are Actinoplanetes, Moduromycetes, Nocardiforms, Actinomycetes, and Streptomycetes [4]. The actinomycetes are free living saprophytic bacteria mostly found in soil, water, and colonizing plant. Also they were identified as one of major groups of soil populations [5]. Actinomycetes are important that they have the ability to produce bioactive substance which have been utilized in research series in numerous institutional and industrial laboratories. This has resulted in the isolation of certain agents combating a variety of human infections because most of natural occurring antibiotics have been isolated from different genera of actinomycetes [6, 7]. The antagonism is common phenomenon among the bacteria in the soil result from the antibiotics and toxins production. Actinomycetes are used in medicine as they have the ability to synthesize different biologically active secondary metabolites such as antibiotics, antitumors, herbicides, pesticides, and different enzymes such as xylanase and cellulase. In the medicines, antibiotics have a great therapeutic value and commercial importance [8]. The number of actinomycetes in the soil is intermediate between those of bacteria and fungi, so dilution is necessary for counting colonies and allow the isolation of large number of bacteria. Usually, the numbers of fungal colonies are lower than filamentous bacteria in such dilution, but with their large colonial spread still make interfering with the analysis of actinomycetes on the culture medium. If the soil contains large number of fungal colonies often enough to make analysis impossible, then to avoid this problem we can use selective nutrients on the medium, and incorporation of selective inhibitor into the medium. Several substances have been suggested as selective substrates for actinomycetes. The use of L-Arginine as a selective nitrogen source favoring actinomycetes over bacteria. Some of them has the ability to utilize dextrose, fructose, inositol, lactose, maltose, mannitol, rhamnose, starch, sucrose, xylose, as carbon source [9, 10]. Sprouts are very special, they are germinated seeds. They are easy to produce and nutritionally-rich making them an important part of the live food. They are very important source of nutrients and have many benefits. The beneficial changes in the nutrition, when seed become sprout are the starch in the seed converts to simple sugar, protein provides amino acid, and the fat breaks down to fatty acid, the minerals, vitamins, proteins, and organic compounds increase in sprout .The most common seed sprouts involved are Mung bean, Alfalfa, Fenugreek. The Mung sprouting is easy and common and has a fast growing and maturing. Mung

seeds are variable, may be dark brown, green, and golden in color. The mung bean contains linolenic, linoleic, and oleic acid, coumestrol, co-enzyme Q10, hemagglutinin, trypsin inhibitor, 25% protein with 8 essential amino acids, and a good source of arginine and histidine. Also contains many vitamins (like; A, B1, B2, B3, B12, B15, B17, C, E and K) and minerals like; Calcium, Copper, Iron, Magnesium, Manganese, Potassium, Sodium, Zinc, Selenium, Phosphorus and Molybdenum [11, 12]. Most of the members that belong to actinomycetes group can grow on poor nutritionally environment, while some of them are fastidious which need special amino acids for their growth and metabolism. Sprouts are excellent source of protein, amino acids, fatty acids, vitamins, and minerals. Soran M medium is a semi-synthetic medium was prepared by Noori and her collages in Soran University last year [13]. This medium had showed high selectivity for aerobic actinomycetes.

Aim of study

The current study aimed to asses and to compare the efficiency of Soran M medium with currently used medium arginine-glycerol salt medium in selective isolation of actinomycetes, detect and identify antibiotic producing strains growing on both media.

Materials and Methods

Collection of Samples

Six soil samples were collected from different locations in and around Soran city, Ruwandiz and Bapshtian, Erbil, Iraq. The sample were collected using clean and sterilized vials with sterilized spatula, samples were dried separately at 37 ˚C for 7 days [2].

Preparation of media

For the selective isolation of aerobic actinomycetes, two types of media were used. Soran M medium was prepared as described by Noori and her Coworkers, 2016 [13] from 50% of Mung sprouts extract added to base medium composed of 0.8% NaCl and 1.2% agar-agar. Then sterilized in autoclave at 121 ˚C, 15 psi for 20 min and arginine-glycerol salt medium was prepared as described by El-Naqeeb and Kechevalier [14] which have the following composition (in g/liter of D.W.): arginine monohydrochloride, 1.0; glycerol, 12.50; NaCl, 1.0; K_2HPO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; $CuSO_4$. $5H_2O$, 0.001; $ZnSO_4$. $7H_2O$, 0.001; $Fe_2 (SO_4)_3$. $6H_2O$, 0.010; $MnSO_4$. H_2O, 0.001; agar, 15.0 (pH 6.9 - 7.1).

Isolation of Actinomycetes

One gram of soil sample was suspended in 9 ml of normal saline (0.85 % NaCl, w/v) and serially six times diluted using decimal dilutions and cultured using spread-plate method [15]. 1ml inoculum from three highest dilutions (10^{-4}, 10^{-5} and 10^{-6}) were spread into surface of Soran M and AGS media and incubated at 37 ºC for 7-14 days [2].

Identification of Actinomycetes

Colonies grown on each type of media (Soran M and AGS) were identified using macroscopic and microscopic characteristics. All different colonies were subcultured on nutrient, blood and chocolate agar and incubated at 37 ˚C for 4 days to show their morphological characteristics and blood hemolysis. Gram stained smears were prepared from purified colonies to detect the morphology of actinomycetes microscopically [10].

Screening of antibiotic production

Antimicrobial activity of actinomycetes was detected by perpendicular streaking of each isolate on Mueller-Hinton agar along with culture of pathogenic microorganisms (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus* sp. and *Candida albicans*) [16].

Characterization of antibiotic producing Actinomycetes

According to the screening methods the most potent Actinomycete isolates were selected and characterized by morphological, biochemical and physiological methods.

Morphological Characteristics

The morphological method includes macroscopic and microscopic characterization. Actinomycetes isolates were macroscopically differentiated by their colonial characteristics, e.g. size, shape, color, consistency etc. For the microscopy, the isolates were grown by cover slip culture method. The cover slip culture technique was carried out by inserting the sterile coverslip at an angle of 45° in to solidified Casein agar plates. A loopful of inoculum of each isolate was streaked along the line, where the coverslip meets the agar and the plates were incubated at 37°C. When good culture obtained they were then observed for their mycelial structure, conidiospore and arthrospore arrangements on the mycelia under microscope (1000X). Cover slip in the medium facilitates the distinction between substrate mycelium and aerial mycelium [17]. The morphologies of the isolates was compared with the Actinomycetes morphological characteristics provided in Bergey's Manual for the presumptive identification of the isolates. Acid fast staining also was used for detecting partially stained cells of *Nocardia* spp. [10].

Biochemical Characteristics

Various biochemical tests were performed for each isolate with the ability to produce antibiotic. These included; catalase, oxidase, OPNG, casein hydrolysis. Each isolate was also tested for its ability to utilize specific carbon sources such as, Maltose, Mannitol, Fructose, Arabinose, Sucrose and Mannose. All isolates also were cultured on Sabouraud dextrose agar and incubated for 96hours at 37°C, the colony characteristics, diffusible pigmentation and odor were daily checked and carefully observed [10, 18].

Results

Occurrence of antibiotic producing Actinomycetes

A total of 40 actinomycetes were isolated from 6 soil samples collected from different sites in Soran soil included Bapshtian, around Ruwandiz, Ruwandiz. After drying the sample and preparation of dilutions poured on media, after 7 days of incubation at 37°C, all 40 actinomycetes were purified, subcultured and maintained for further use. Both media used, Standard Arginine Glycerol Salt agar and Soran M Medium, showed efficiency in selective isolation of actinomycetes from soil samples. Grey colored, orange and creamy chalky mycelial cultures were the predominant among the isolates obtained. Among the 40 isolates of actinomycetes in both media, about 10 isolates showed antibacterial activity and the remaining isolates had no activity. Of these, 6 isolates were isolated in AGS agar while the other 4 were isolated in Soran M medium. The

degree of Actinomycetes occurrence and their sources are shown in table 1.

Table (1):- Occurrence and isolation of antibiotic producing Actinomycetes on selective media

Sample code	Source	No. of isolates growing on Arginine Glycerol Salt agar	No. of isolates on growing Soran M agar
A.	Bapshtyan -1	2	0
B.	Bapshtyan -2	3	1
C.	Around Rwandoz -1	1	2
D.	Around Rwandoz -2	0	1
E.	Rwandoz -1	0	0
F.	Rwandoz -2	0	0

As later noted, approximately most of isolates grown on AGS agar were also grown on Soran media but they showed a little difference in growth characteristics in each medium type made us confused. After getting microscopic and biochemical tests for antibiotic producing isolates the complete separation and distinction between confusing isolates has occurred. Final results showed that one isolate was grown on both media. Among 10 antibiotics producing isolates, 2, 3 and 1 were isolated from Bapshtyan -1, Bapshtyan -2, and Around Rwandoz -1, respectively. All of these isolates were first isolated on AGS agar. While 1, 2, 1 were isolated from Bapshtyan -2, Around Rwandoz -1and Around Rwandoz -2, respectively. No actinomycetes isolates with antimicrobial activity were isolated from Rwandoz -1 or Rwandoz -2.

Antimicrobial activity of the actinomycetes isolated.

According on the primary screening of antimicrobial activity of the actinomycetes isolates shown in table 2, the results showed that a total of 7 actinomycetes have antifungal activity against *Candida albicans* out of 10 actinomycetes isolated.

Table (2):- Screening of antimicrobial activity of the actinomycetes isolates.

Isolate No.	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Bacillus* sp.	*Candida albicans*
SA2	+	-	-	-	-	+
SA4	-	-	+	-	-	-
SB3	-	+	-	-	-	-
SB3b*	-	+	-	-	+	+
SB6	-	-	-	-	+	+
SC1	-	-	-	-	-	+
MB3*	+	+	-	+	+	+
MC2	-	-	-	-	+	-

MC3	-	-	-	-	-	+
MD7	+	-	-	-	-	+
*	Same Strain from same source isolated on Soran M and AGS agar.					

The best result was shown to be recorded by the isolate MB3 that exhibited broad spectrum activity against both Gram negative and Gram positive bacteria in addition to antifungal activity. Narrow spectrum activity was shown by the isolate SB3b against Gram positive bacteria in addition to antifungal activity. In spite of different antibacterial activity which has been shown by both MB3 and SB3b, in dependence upon the microscopic and macroscopic characteristics, and the pattern of biochemical results for them, they were completely similar. Also, both of them have been isolated from Bapshtyan -2. Other isolates showed species specific activities, 2 were active against *Bacillus* sp., one against *Escherichia coli*, anther against *Klebsiella pneumoniae*, one against *Staphylococcus aureus* and anther isolate was active against *Pseudomonas aeruginosa*.

Identification and characterization of Actinomycetes

All the 9 isolates which showed antimicrobial activity were introduced for further studies and identified up to genus level. The results of the microscopic, macroscopic characteristics and the pattern of biochemical tests showed that the potential isolate MB3, which exhibited broad spectrum antibacterial and antifungal activity, showed creamy solid colonies with chalky white aerial top, produced soluble faint brown pigment on Nutrient agar. Microscopically appeared with straight sporophores or wavy but not spiral, flexous with elongated cylindrical spores, as illustrated in figure 1. These characteristics met the description of *Streptomyces* sp.

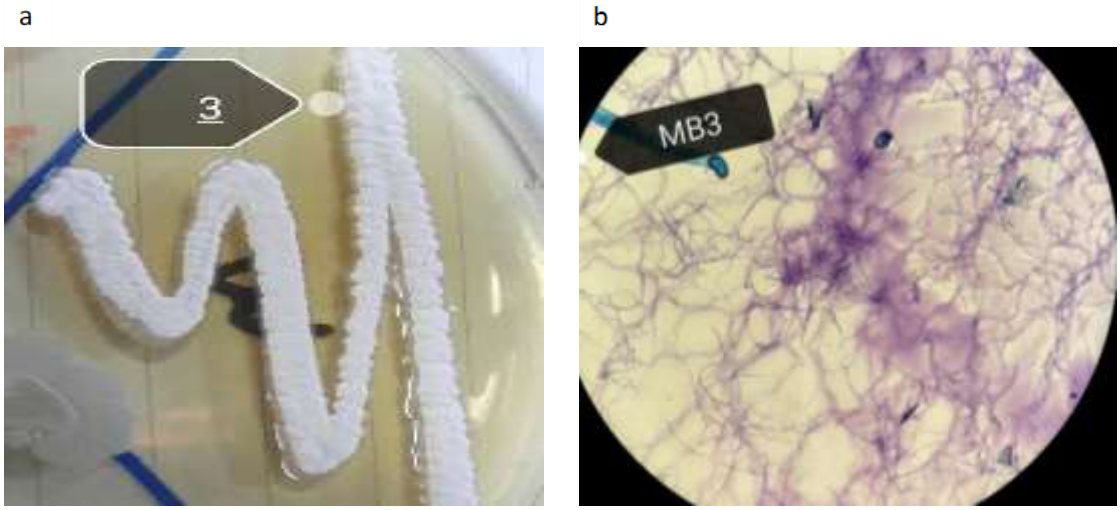

Figure 1: a) Cultural characteristics, b) Microscopic characteristics of MB3 (*Streptomyces* sp.).

The microscopical examination and the cultural characteristics on Casein and Sabouraud agar media revealed that the isolates; SA2, SB3, SC1, MC2 and MC3 also have similar characters as that of the genus *Streptomyces* and these are illustrated in Table 3.

Table (3):- Morphological.and Cultural.characteristics of *Streptomyces* isolates.

Isolate No.	Morphological characteristics		Cultural Characteristics		
	Spore bearing hyphae	Growth	Vegetative Mycelia Colour	Aerial Mycelia Colour	Soluble Pigment
SA2	Loops	Good	Yellow	White	Nil
SB3	Spirals	Abundant	Colorless	Brown	orange
SC1	Closed spirals	Weak	Orange	White	Light brown
MB3	Flexous	Abundant	Creamy	White	Light brown
MC2	Spirals	Abundant	Creamy	White	Reddish Brown
MC3	Fascicled	Good	Grey	White	Nil

The colonial characteristics, presence of nonseptate mycelium and soluble pigments secreted on medium were carefully observed. The morphological characters of the active isolates; Gram stained SA2, SB3, SC1, MC2 and MC3 were also studied for microscopic characteristics under oil-immersion (100x). The observations showed that all of them were gram positive with non-septate hyphae. Three isolates were identified as *Nocardia* spp. as they were partially acid fasts, oxidase positive, catalase positive and have a substrate mycelium with septae that fragmented into bacillary, coccoid and long slender bacilli these included the isolates; SA4, SB6 and MD7, respectively. Biochemical tests were conducted for oxidase, catalase, casein hydrolysis, Hemolysis, ONPG test and carbohydrates utilization tests. All the isolates namely SA2, SB3, MB3, SC1, MC2 and MC3 showed catalase positive (Table 4). Both isolates of *Nocardia*; SB6 and MD7 completely showed the same biochemical results and the same cultural morphology. Both isolates SB3 and SC1 couldn't utilize and grow on some carbohydrate containing media. Most of isolates grew but couldn't produce acids.

Table (4):- Physiological and Biochemical characteristics of actinomycetes isolates.

Isolate No.	Oxidase	Catalase	Casein hydrolysis	Hemolysis	ONPG	Acid fasting	Carbohydrate Utilization Test					
							Arabinose	Fructose	Maltose	Mannitol	Mannose	Sucrose
SA2	+	+	+	+	+	-	-	+	-	+	+	+
SA4	+	+	-	-	+	+	-	+	-	+	+	+
SB3	-	+	+	+	-	-	-	NG	-	NG	+	NG
SB3b*	-	+	+	+	+	-	-	-	-	-	-	-
SB6	+	+	-	+	+	+	-	-	-	-	-	-
SC1	+	+	-	-	+	-	-	NG	NG	-	-	NG
MB3	-	+	+	+	+	-	-	-	-	-	-	-
MC2	-	+	+	+	+	-	-	-	-	-	-	-
MC3	+	+	+	-	-	-	-	-	-	-	-	+
MD7	+	+	-	+	+	+	-	-	-	-	-	-

* Similar to MB3, NG: No growth.

Discussion

Actinomycetes are important flora in most soils in particular agricultural type as they share mutualistic relationship with most plants taking a benefit of their ability to produce antimicrobial agents that inhibit and kill harmful surrounding microbes in addition to their ability to decompose wide range of complex organic compounds [(2)]. In this study 40 actinomycetes soil isolates were evaluated for their antimicrobial activity. Out of 40, only nine isolates, exhibited different antimicrobial patterns against Gram positive and Gram negative bacteria and unicellular fungal species *Candida albicans*. Those 9 isolates were taken from agricultural soil samples collected from Bapshtyan and Around Rwandoz on Standard AGS agar and Soran M agar to assess the effect of nutrient composition on isolation and enhancing the ability to produce antibiotics. Most of these isolates belong to *Streptomyces* genus as it considered to be the most important and agricultural soil predominant producer of bioactive molecules, like antibiotics: antibacterials, antifungals, antitumoral, pesticides, etc. [(19)]. The nine isolates, except SA2 and MD7, showed different ability to inhibit bacterial and fungal pathogens from each other. Only one potential isolate named MB3 which identified as *Streptomyces* sp. exhibited wide spectrum of antimicrobial activity. This isolate was isolated on Soran M agar which composed of sprout mung extract highly enriched with nutrients especially minerals that support the growth and flourish of actinomycetes. Essential mineral elements, such as magnesium, potassium, sodium, calcium and phosphorus are as necessary as amino acids and vitamins in maintenance of life, wellbeing and

production [20]. These mineral are highly supplied in mung sprouts [12]. Same isolate from the same soil sample was first named SB3b as it was isolated on Standard AGS agar showed restricted antibacterial activity in spite of similar microscopic, cultural characteristic and biochemical characteristics. This result support the finding of Avignone-Rossa and his Coworkers, (2013) [19] that the wide diversity of the chemical products synthesized by *Streptomyces* is caused by the presence of a variety of metabolic pathways in their nucleoid collectively known as secondary metabolism. Mentioned secondary metabolic pathways are in charge of their highest activity when the microorganisms undergo a sequences of developmental changes related with the formation of aerial hyphae (in solid culture media) or to the start of the stationary phase (in liquid cultures). The setoff of those physiological states, and therefore of the associated synthesis of secondary metabolites, is related to the depletion of some growth nutrients, and subsequently notable decrease in growth rate may be the switch on for secondary metabolism. Enhancement in the antibiotic production was validated experimentally by several researches [20, 21, 22]. Also, this result agreed with what was found by Singh and his collegues (2012) [23], as they showed that the potentiality of antibiotics (antimicrobial activity) could be enhanced by the incorporation of exogenous metal ions and complex compounds based on sodium, potassium, magnesium, calcium and phosphorus. In conclusion, findings from this investigation revealed that nutrient media with different composition affect the isolation of antibiotic producing actinomycetes with different activities. Specific nutrients enhance and support the potentiality of antimicrobial activity of some actinomycetes. According to this result, we recommend to expertize the effect of specific nutrients singularly on the production and activity specific known antibiotics produced by Streptomyces.

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