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Biochemical studies on antibiotic production from Streptomyces griseus

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ABSTRACT

The present study was aimed for identify *Streptomyces griseus* by biochemical tests.All *Streptomyces* spp. isolates (MH1, MH7, MH3, MH17, MH25) were screened for their antibacterial activity using scross-streak technique against Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus*). Screening was performed by agar-well diffusion method and growth inhibition zones were measured in millimeters for each of the *Streptomyces*spp. isolatesMH1, MH7, MH3, MH25. Tested isolates have shown potent *in vitro* antibacterial activities against all tested pathogens. The highest activities were shown by isolate MH7 against *Staphylococcus aureus* (21 mm),*Pseudomonas aeruginosa* (17.8 mm), *Escherichia coli* (22.5 mm). *Streptomyces*spp isolates (MH1, MH7, MH3, MH25)were selected for biochemicalstudies. All isolates hydrolyzed starch. All isolates produced Catalase, Gelatinase, Protease, Urease, Amylase, Cellulase, Chitinase and Lipase. All *Streptomyces*spp. isolates have ability to reduce nitrate, Tyrosine degradation and Casein hydrolysis. Hydrogen sulphide (H2S) production, Oxidase production, Indole production and Melanine reaction were studied for *Streptomyces*spp. isolates(MH1, MH7, MH3, MH25). Most of the isolates were not H2S producer, Oxidase, Indole and Melanine reaction except one isolate(MH3). All *Streptomyces*spp.isolates could Citrate utilization and Pectindegradation except MH7. *Streptomyces*spp.isolates 4 isolate utilizes eight carbon sources (Carbohydrates utilization) such as the Glucose, Galactose, Fructose, Sucrose, Xylose, Maltose, Lactose and Mannitol). Results of *Streptomyces*spp. isolates by Utilization of nitrogen (amino acid) sources (L- arginine ,L-isoleucine, L- cysteine, L-glycine,L-tyrosine and L-alanine). Four *Streptomyces* spp. isolates were selected, characterized based and identified by biochemical examination according to Bergey's Manual of determinative bacteriology. The bacteria was described to genus *Streptomyces* and species *griseus*. Given 4 isolates are presumptively the same or similar species (e.g., *Streptomyces griseus*). All isolates to have possessed similar biochemical tests results as the ones that were *Streptomyces griseus.*

Keywords: *Streptomyces griseus*, antibiotics, Biochemical studies

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INTRODUCTION

Actinomycetes usually defined as Gram-positive bacteria that have high G+C (>55%) content in their DNA (Embley and Stackebrandt, 1994). Most soil actinomycetes are neutrophils, growing between pH 5.0 and 9.0 with an optimum close to neutrality. (Goodfellow and Williams, 1983). Actinomycetes produce about two-thirds of the known antibiotics and among them 80% are made by members of the genus *Streptomyces*, with other genera trailing numerically. Actinomycetes also account for 60% of secondary metabolites with biological activities other than antimicrobial, and again *Streptomyces* species account for 80% of these (Kieser*et al.*, 2000; Amin *et al.*, 2016; Risan *et al.*, 2016; Risan*et al.*, 2017; Risan*et al.*, 2018; Al-Rubaye*et al.*, 2018a, b; Risan*et al.*, 2019; Al-Rubaye*et al.*, 2020). *Streptomyces* is a genus represented by a large number of species and varieties. Over 500 species of Streptomyces are recognized by "Bergey's Manual of Determinative Bacteriology (Keiser *et al.*, 2000; Madigan and Martinko, 2005). *Streptomyces* are Gram positive aerobic bacteria belonging to the phylumActinobacteria (Stackebrandt*et al.*, 1997). About 61% of all the bioactive microbial metabolites were isolated from actinomycetes especially from Streptomycetes (Monchev*aet al.*, 2002). The productivity of *Streptomyces* strains, as antibiotic producers, remains unique amongst actinomycetes strains. The members of the genus *Streptomyces* constitute the major group in actinomycetes. Of 12,000 secondary metabolites with antibiotic activity, 55% are produced by *Streptomyces* and additional 11% by other actinomycetes (Paradkar*et al.*, 2001; Weber *et al.*, 2003).*Streptomyces griseus* strains are well known producers of antibiotics and other such commercially significant secondary metabolites. These strains are known to be producers of 32 different structural types of bioactive compounds.(Amano *et al.*, 2008). The present study was aimed for identify *Streptomyces griseus*bybiochemical tests

MATERIALS AND METHODS

The bacteriaused for all studies were*Streptomyces* spp.(MH1,MH7, MH3,MH17,MH25). This bacteriawasobtained from Al-Nahrain university, College of Biotechnology, Baghdad, Iraq.

Screening of Antibiotic Producing *Streptomyces* spp.

Screening of antibiotic producing *Streptomyces*spp. (MH1, MH7, MH3, MH17, MH25). Primary screening for antimicrobial activities was, according to (Kumar *et al.*, 2012; Amin *et al.*, 2016; Risan *et al.*,2016; Qasim and Risan 2017; Risan*et al.*, 2017a, b; Risan*et al.*, 2018; Al-Rubaye*et al.*, 2018a, b; Risan*et al.*, 2019), by using cross-streak technique, in which the *Streptomyces*spp.isolates(MH1, MH7, MH3, MH17, MH25)were used against three pathogenic bacteria. The *Streptomyces*spp.isolates were streaked at the center of Yeast extract-Malt extract agar (YEMEA) plates,and inoculated plates were incubated at 28°C for 7 days to secrete antibiotics into the medium. Each streaking was started near the edge of the plates and streaked toward the *Streptomyces* spp. growth line. The positive results were observed by the naked eye. Antimicrobial activity of *Streptomyces* spp. isolates were determined to carry out after the positive results were obtained from the primary screening by Agar-Well Diffusion method (Murray *et al.*, 1995; Amin *et al.*, 2016; Risan *et al.*, 2016; Qasim and Risan 2017), to test the antibiotic activity of the isolates.Three pathogenic bacteria, (All the tested pathogenic bacteria were obtained from Laboratory Microbiology / Mycology – college of Biotechnology), including Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus*), were used as test microorganisms for evaluation the antibacterial activity of *Streptomyces* spp. The bacteriawere maintained in nutrient agar at 4°C and sub-cultured before use. The selected isolates were streaked as parallel line on nutrient agar plates and incubated at 37°C for 2 days. After observing a good ribbon- like growth of the *Streptomyces*spp.(MH1, MH7, MH3, MH17, MH25) on the Petri plates, the pathogens were streaked at right angles to the original streak of *Streptomyces*spp. and incubated at 37°C for 24 hours.

The no appearance of growth or a less dense growth of tested microbial near the *Streptomyces*spp. isolates were considered as a positive results for production and secretion of antimicrobial metabolite by the isolates as described by (Risan *et al.,* 2016; AL-Samarraie,et al.,2019). Secondary screening (fermentation), for production of antimicrobial metabolites were carried out by inoculating 150ml of media (yeast extract, malt extract agar ISP2) in Erlenmeyer flask with 1.5 ml of prepared stock suspension cultures were incubated at 29±1°C, 150 rpm for 7days in shaking incubator, the broth was filtered with sterile Whatman No. 1 filter paper, and treated as extracellular crude extract (Risan *et al.,* 2016). To determination antimicrobial activities of*Streptomyces* spp. (MH1, MH7, MH3, MH17, MH25), were usingAgar well diffusion method was done to screen antimicrobial activities for best media and solvent extraction of antimicrobial metabolites, against tested microbial pathogens. Using sterile swabs, Mueller Hinton agar plates inoculated with microbial pathogens then dug wells of 6mm diameter using Pasteur pipette 60 ul of the extracts were loaded into wells and the plates were incubated at 37^{o}C for 24 hours. The plates were observed for zone of inhibition which recorded by metric ruler (Risan *et al.,* 2016). Agar well diffusion method was done to screen antimicrobial activities against tested microbial pathogens. Using sterile swabs, Mueller Hinton agar plates inoculated with microbial pathogens, and dug wells of 6mm diameter using Pasteur pipette, 60 ul of the extracts were loaded into the wells and the plates were incubated at 37^{o}C for 24 hours. The plates were observed for the inhibition zone in mili meter (mm), were measured after 24 and 48 hours by a metric ruler. The inhibition zones in millimeter (mm) were measured after 24 and 48 hours using an antibiotic zone reader. *Streptomyces*spp isolates (MH1, MH7, MH3, MH25)were selected for biochemicalstudies.

Citrate utilization tests

Sterile Simmon's Citrate agar slants were streaked with the *Streptomyces*spp. cultures and incubated at 28ºC for 4 days. Change in colour from green to blue indicated positive reaction. No colour change indicated negative result.

Nitrate reduction test

Nitrate broth was prepared and dispensed into test tubes. The test tubes weresterilized and one loop full of cultures were inoculated and incubated at 28±2ºC for days. After incubation,

Biochemical Characteristics

*Streptomyces*spp. isolates (MH1, MH7, MH3, MH25)were selected for biochemical characterization, various biochemical tests were studied. Many characteristic were studied. Different carbon and nitrogen sources were carried out according to (Lechevalier and Lechevalier 1967; Cowan, 1974; Gordon *et al.*, 1974; Elwan*et al.*, 1977; Rowbotham and Cross, 1977; Collins*et al*., 1995). These tests, including Hydrogen sulphideproduction, Nitrate reduction, Amylase, Cellulase, Gelatinase, Citrate utilization, Tyrosine degradation, Pectin degradation, Protease production, Chitinase, Lipase, Urease production, Catalase, Oxidase production, Casein hydrolysis, Indole production, Melanine reaction and Starch.

Melanin pigment production test (Shirling and Gottlieb, 1966)

Melanin production was considered to cause browning of organic media containing tyrosine and it was carried out with tyrosine agar medium. The agar medium was transferred in to test tube, sterilized and made into slants. The slants were inoculated with active cultures and incubated at 28ºC. After 2-4 days, the production of soluble pigments and the colour of the vegetative and aerial mycelium in the slants were observed.

Indole test

Peptone broth was prepared and the *Streptomyces*spp. cultures were inoculated. After incubation the indole production was tested with Kovac's reagent. Red colour ring formation indicated positive reaction where yellow colour ring indicated negative result**.**

few drops of alpha naphthalamine and sulphanilic acid wereadded. The red colour formation indicated positive result.

Urease test: Sterile Christensen's urea slants were streaked with the *Streptomyces*spp.culturesand incubated at 28ºC for 4 days. Chang in colour from yellow to pink indicatedpositive reaction**.**

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Catalase test: A clear slide was taken and drop of culture suspension was placed on it. Fewdrops of hydrogen peroxide were added to the cultures. The evolution of air bubblesfrom the suspension indicated a positive reaction.

Oxidase test: The cultures were rubbed over the filter paper containing a reagent N-N tetramethyl paraphenylene diaminedihydrochloride. Purple colour indicated positive result.

Starch hydrolysis: Nutrient starch agar plates were prepared and sterilized. The plates were inoculated with the *Streptomyces*spp.isolates as a single line streaks and incubated at 28ºC for 7 days. A positive reaction was indicated by the formation of zone of clearance of the medium around the colonies, which was further visualized by adding Lugol's iodine.

Casein hydrolysis: Skim milk agar plates were prepared and sterilized. Then the medium waspoured in to petriplates. After solidification, the cultures were streaked as a single line and incubated. The formation of zone of clearance around the colonies indicated the positive result.

Lipid hydrolysis: Spirit blue agar was prepared and tributyrin was added as the substrate forlipase activity. The substrate mixture was homogenized in the magnetic thermal stirrer and sterilized. The medium was then inoculated with the *Streptomyces*spp.isolates in a zigzag manner and incubated. A positive lipase activity was determined by the reduction of dye around the colonies.

RESULTS AND DISCUSSION

Screening of antibiotic producing*Streptomyces* spp.

All *Streptomyces*spp.isolates (MH1, MH7, MH3, MH17, MH25) were screened for their antibacterial activity on malt extract yeast extract agar medium using scross-streak techniqueagainst Gram-negative (*Pseudomonas aeruginosa*and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus*).A broad spectrum of antibacterial activity was observed in 80% (4 out of 5) (MH1, MH7, MH3, MH25) of the total *Streptomyces*spp. isolates (Table 1).Among four *Streptomyces*sppisolates, one isolates (MH17) didn't show any antibacterial activity against any type of pathogenic bacteria (Gram-negative and Gram-positive bacteria), while fourof *Streptomyces*spp.isolates (MH1, MH7, MH3, MH25) showed antibacterial activity Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus*).

TABLE 1: Primary screening of *Streptomyces*spp. isolates using scross-streak technique on malt extract yeast extract agar medium

Isolates	Gram-positive	Gram-negative		Note
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Escherichia coli***	
MH1	+	+	+	Selected
MH7	+	+	+	Selected
MH3	+	+	+	Selected
MH17	-	-	-	Neglected
MH25	+	+	+	Selected

Screening was performed by agar-well diffusion method and growth inhibition zones were measured in millimeters for each of the *Streptomyces*spp. isolatesMH1, MH7, MH3, MH25, the results are shown in Table 2. Tested isolates have shown potent *in vitro* antibacterial activities against all tested pathogens. The highest activities were shown by isolate MH7 against *Staphylococcus aureus*(21 mm),*Pseudomonasaeruginosa* (17.8 mm), *Escherichia coli* (22.5 mm). It is alsoevident in Table 2 that isolates MH1and MH25 have shown strong activities against all pathogenic bacteria with inhibitionzone diameters ranging between 14.7 and 17 mm.Isolate MH3 have shown moderate inhibitory effect against pathogenic bacteria with inhibition zones diameters in the ranging between 10 and 12.5 mm.

TABLE 2: Inhibition zones (mm) by different *Streptomycesgriseus*isolates againstpathogenic bacteria

Isolates	**Zone of inhibition (mm)**		
	Staphylococcus aureus	***Pseudomonas aeruginosa***	***Escherichia coli***
MH1	14.7	15	17
MH7	21	17.8	22.5
MH3	10	10	12.5
MH25	15.5	16.5	15

Streptomyces is the most prolific drug producing genus, *Streptomyces* species have shown an outstanding capacity for the production of secondary metabolites, many of which effectively can treat human diseases. (Challis and Hopwood, 2003). As *Streptomyces* have shown an outstanding capacity for drug production, different campaigns in geographically distant locations currently aim to isolate new antibiotic producers. (Sottorff*et al.*, 2019). Actinobacteria have shown to be an exceptional source of new antibiotics and pharmaceuticals in general (Barka *et al*., 2016). Al-Ansari *et al.*, (2019). Ten Actinomycetes were isolated and tested for its antibac-terial efficacy. These, five actinomycetes were selected forthe production of antimicrobial agent against *E. coli*, *P. aeruginosa*, *B. subtilis*, *E. aerogenes* and *P. mirabilis*. Ethyl acetate extract of the selected five actinomycetes showed broad spectrum antibacterial activity.

Actinomycetes are production of about half of the secondary metabolites, antibiotics, antitumour, immunosuppressive agents and enzymes (Berdy, 2005; Bull, and Stach, 2007; Olano*et al.*, 2009)

Biochemical Characteristics of *Streptomycesgriseus* isolates

*Streptomyces*spp. isolates (MH1, MH7, MH3, MH25)were selected for biochemicalstudies. Result of the biochemical results were tabulated in table3, biochemical testes (Catalase production,Hydrogen sulfide production, Nitrate reduction, Citrate utilization, Oxidase production,Casein hydrolysis,Indole production, Melanine reaction,Starch,Amylase,Cellulase,Gelatinase, Tyrosine degradation,Pectin degradation,Protease production, Chitinase,Lipase,Urease production, nitrogen and carbon sources)of *Streptomyces*spp. isolates (MH1, MH7, MH3, MH25).Show in (Table 3). Ability of *Streptomyces*spp.isolates to produce enzymes were as varied.

All isolates hydrolyzed starch. All isolates produced Catalase, Gelatinase,Protease, Urease, Amylase, Cellulase, Chitinase and Lipase. All *Streptomyces*spp.isolates have ability to reduce nitrate, Tyrosine degradation and Casein hydrolysis.Hydrogen sulphide (H2S) production, Oxidase production, Indole production and Melanine reactionwere studied for *Streptomyces*spp.isolates(MH1, MH7, MH3, MH25). Most of the isolates were not H2S producer, Oxidase, Indole and Melanine reaction except oneisolate (MH3).All *Streptomyces*spp.isolates could Citrate utilization and Pectindegradation except MH7. *Streptomyces*spp.isolates 4 isolate utilizes eight carbon sources(Carbohydrates utilization) such as the Glucose, Galactose, Fructose, Sucrose, Xylose, Maltose, Lactose and Mannitol). Results of *Streptomyces*spp. isolates by Utilization of nitrogen (amino acid) sources (L- arginine ,L-isoleucine , L- cysteine, L-glycine ,L-tyrosine and L-alanine).

Table 3: Biochemical characteristics of *Streptomycesgriseus* isolates after 5 days growth on ISP 2 medium

No.	Characteristic	MH1	MH3	MH7	MH25
1	Catalase production	+	+	+	+
2	Hydrogen sulfide production	-	+	-	-
3	Nitrate reduction	-	-	-	-
4	Citrate utilization	+	+	-	+
5	Oxidase production	-	+	-	-
6	Casein hydrolysis	-	-	-	-
7	Indole production	-	+	-	-
8	Melanine reaction	-	+	-	-
9	Starch	+	+	+	+
10	Amylase	+	+	+	+
11	Cellulase	+	+	+	+
12	Gelatinase	+	+	+	+
13	Tyrosine degradation	-	-	-	-
14	Pectin degradation	+	+	-	+
15	Protease production	+	+	+	+
16	Chitinase	+	+	+	+
17	Lipase	+	+	+	+
18	Urease production	+	+	+	+
19	Carbohydrates utilization (Glucose, Galactose, Fructose, Sucrose, Xylose, Maltose, Lactose and Mannitol)	+	+	+	+
20	Utilization of nitrogen sources				
	L- arginine +	+	-	+	+
	L-isoleucine	-	-	-	-
	L- cysteine	-	-	-	-
	L-glycine	+	+	-	+
	L-tyrosine	+	+	+	+
	L-alanine	+	-	+	+

Four *Streptomyces* spp. isolates were selected, characterized based and identified by biochemicalexamination according to Bergey's Manual of determinative bacteriology (Locci 1989). The bacteria was described to genus *Streptomyces* and species *griseus* (Table3)Given 4 isolates are presumptively the same or similar species (e.g., *Streptomyces griseus*) . All isolates to have possessed similar biochemical tests results as the ones that were *Streptomyces griseus*. *Streptomyces griseus* is a spore-forming, alkaliphilic bacterium known to produce many types of secondary metabolites, including the antibiotic streptomycin (Liu *et al.*, 2005). Our resultes agreement with Ram(2014). Many bacterial genera including Streptomcyes are not only morphologically and microscopically identical but yielded colonies which are not clearly distinguishable. The biochemical activities of such pure cultures frequently allow genera and species characterization and identification(Gillies, 1984). Biochemical characterization of the isolates recovered in this study involved 20 diagnostic characters that are recommended by International *Streptomycete* Project (ISP), and were successfully utilized by various investigators in the field (Anderson and Wellington, 2001; Oskay*et al.*, 2004; Risan*et al.*, 2016; Risan*et al.*, 2017; Al-Rubaye*et al.*, 2018a, b).Yang and Wang (1999) reported the production of amylase and protease enzymes by different species of *Streptomyces*. Results ofOskay*et al*. (2004) and Anderson and Willington (2001). Showed that starch hydrolysis was observed by twelve of the *Streptomyces* isolates while the left four isolates do not have the ability to hydrolyze starch.

Although it is generally considered that amylase and protease are mainly fungal and eubacterial products, the results of starch hydrolysis and protease expression. The production of citrase, urease, catalase and oxidase are considered for characterizing*Streptomyces* (Nitsch and Kutzner, 1969; Gotoh*et al.*, 1982).The hydrogen sulphideand melanin production has been considered as the other important charactersfor the identification of actinomycetes (Shirling and Gottlieb, 1966).Espinoza *et al.*, 2013 study biochemical (API) profiles of *Streptomyces* bacteria from the Laguna Madre as β-galactosidase activity; Arginine Dihydrolase; Lysine Decarboxylase; Ornithine Decarboxylase; Citrate Utilization; Hydrogen Sulfide Production;Urease; Tryptophan Deaminase;Indole Production;Acetoin Production (Voges-Proskaur); Gelatinase; Glucose; Mannitol; Inositol; Sorbitol; Rhamnose; Sucrose; Melibiose; Amygdalin; Arabinose; Nitrate Reduction to Nitrite; Nitrate Reduction to Nitrogen Gas.

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