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Extraction and identification of bioactive compounds produced from bacteria Streptomyces griseus by Gas chromatography - mass spectroscopy (GC – MS)

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Abstract:

Fourteen soil samples were collected from Al-Jadriya region, Baghdad, and about 10 of them contained Streptomyces griseus bacteria, and extracted and cultivated them on special medium SP2 broth with nystatin and azithromycin. The extracted 1 liter was to and was divided into two parts of 500ml and placed in the atoclave for the purpose of sterilization for 15 minutes at a temperature of 121C and then after cool the colonies were transferred from the dishes to the flask under sterile conditions. Only 7 to 10 colonies are transferred and placed at a temperature of 25C. We have covered the flask With a scarf and placed it in the laboratory room for a period of 10 days, and after the period ended, we noticed the presence of bacterial colonies growth, the Broth was transferred to drying by dividing the quantity and placing it in the centrifuge and taking the precipitate and placing it in the oven 45 C for 3-5 hours and placing it in a dish, the filtrate was then treated with ethyl acetate, the extract was taken to the Ministry of Industry and Minerals "Ibn Al-Bitar Center" and the analysis of GC Mass. It was concluded that there are 20 compounds were obtained from the Streptomyces griseus. In varying proportions. The highest concentration substance is Caryophyllene. This bacterial extract contained 20 compounds, including, Benzyl alcohol, Acetic acid, Carbonic acid, etc. The most present compound is "Caryophyllene" and the least compound is "4_Amino-5-imidazole carboxamide"

Key words:
Caryophyllene
Gas chromatography
mass
Streptomyces griseus

Introduction:

Actinomycetes are filamentous bacteria which produce two kinds of branching Mycelium, aerial mycelium and substrate mycelium. Actinomycetes constitute a Significant component of the microbial population in most soils and Streptomyces a count for 80% of the total Actinomycetes population (Poopal and Laxman, 2009). Actinomycetes produce a wide range of secondary metabolites and more than 70% of the naturally derived antibiotics that are currently in clinical use are Derived from soil actinomycetes (Elardo et al., 2009). The major group of Actinomycetes, Streptomyces spp. can produce an array of secondary metabolites having antibacterial or antifungal properties were applied for the human pharmaceutical use (Hughes et al. 2008). It has been reported that most of the actinomycetes are widely used in industries due to their ability to produce numerous antibiotics (Raja and Prabakarana, 2011), enzymes, vitamins, growth hormones and anti-cancerous agents (Berdy, 1995). Streptomyces genus can also produce valuable metabolites, enzyme inhibitors commercially valuable enzymes like lipases, cellulases, amylase and proteases (Ravel et al., 2000). High Performance Liquid Chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compound that have been dissolved in solution. HPCL is used to determine the amount of specific compound in a solution. In HPLC and liquid chromatography, where the sample solution is in contact with a second solid or liquid phase, the different solutes in the sample solution interact with the stationary phase. The difference in interaction with the column can help separate different sample components from each other (Maitland, 2010).

Materials and Methods:

Soil sample collection:

Collection of samples were collected from different region from (Baghdad, Jadriya) 14 sample were collected from 2022/ 10 November, 250

grams of soil Ranged from (5 to 15 cm), and they have been kept in polyethylene bags (20x 40 cm). The soil samples were exposed to the air for a week, also they pretreated with CaCO3 with a ratio of (10:1 soil: CaCO3) and kept at ambient temperature for a week, to enrich actinomycetes which usually prefer alkaline conditions and also to reduce the contamination. (Lechevalier, 1981; Abdulhameed, 2013; Lechevalier, 1981; Abdulhameed, 2013).

Isolation and Identification of Streptomyces from Soil:

One gram of dried and treated soil samples was used to make suspension, by Adding it in 99 ml of sterile distilled water (stock suspension) and they were Shacked in a shaker at 160 rpm for 30 minutes at room temperature., Serial Dilutions from 10^1To 10^3 were made from the stock suspension, and left for 10 Minutes. After shaking, 0.1 ml of each dilution were culture on Yeast Extract and Malt Extract agar (YEME) with Streptomycin 50 ug/ml, then spread by Sterile swab for making uniform distribution of the suspension on the surface of The media. The inoculated plates were incubated at 28°C for 7 to 10 days. These colonies were transferred from the mixed culture into separate agar plates and incubated at 28±1°C for 7 days. In order to obtain a pure growth of Streptomyces were re-streaked on International Streptomyces project (ISP2) to obtain pure colonies used for Identification (Nonoh et al., 2010 ; Oskay et al., 2004).

Streptomyces isolation and identification media:

International Streptomyces Project (ISP2) Medium (Shirling and Gottlieb, 1966)

Component	Quantity (g/l)
Yeast extract	4
Malt extract	10
Dextrose	4
Agar	4
glycerin	3
nystatin	3
azithromycin	1
Distilledwater	1

Table 1: *Components of ISP2 medium*

Identification of Bacterial Isolates

Suspected bacterial isolates were primarily identified by microscopic and cultural examinations،then by biochemical identification for final identification.

Extraction of metabolites from selected Streptomyces spp

The whole broth from fermentation media (500ml) of ISP2, the filtrate was then treated with ethyl acetate then ethyl acetate was washed with distilled water to get rid of the excess acids. The ethyl acetate extract was separated, and the aqueous phase was discarded. The precipitated complex was dissolved in the combined ethyl acetate extracts, and the solution was concentrated by evaporation to removal of ethyl acetate at 35˚C afforded a residue that yielded of crude complex

Gas chromatography - mass spectroscopy (GC-MS) Analysis of Streptomyces Ehtyl Acetate Extract

Ethyl acetate extract of Streptomyces isolates was analyzed by GC-MS for detecting the volatile compounds presented in the ethyl acetate extract, as well as, their quantity and quality ratios. The sample was reconstituted using 1ml Ethylacetate, and then one hundred microliters of the extract was transferred into auto sampler glass vials. GC-MS analysis was performed on GC-MS QP-2010; an inert cap pure wax capillary column used 30m x 0.25mm x 0.25µm film thickness with Helium as the carrier gas at a flow rate of 1.51 ml/minutes. The source was operated in positive ionization mode electron impact energy: 70eV and the detection were performed in a full-scan mode scan range: 35.00 – 700.00 m/z for a total run time of 27 minutes. The inlet and the transferline temperatures were maintained at 240˚C, while the ion source was kept at200˚C. Samples were injected in a split-less mode and separated by using a temperature gradient program as follows: 100˚Cfor 3minutes, to 150˚C at 10˚C/min and then maintained at 150˚C for 2minutes; then to 240˚C at10˚C/min and maintained at 240˚Cfor further 8 minutes. GC-MS spectra were evaluated by posting the software and searched in the National Institute of Standards and Technology (NIST), MS Search V2.0 browsers. The Sample was analyzed Ministry of Industry and Minerals Ibn Al-Bitar Center.

Results and Discussion:

Streptomyces isolation and Identification

From 14 soil samples, only 10 samples of soil were suspected to contain Streptomyces, mean 60% of them. The colonies suspect were selected basis on color that was ranged from gray, white and creamy, which were grown on yeast extract malt extract agar. The results were agreement with Mohamed et al.,(2015). The colonies size and morphology was in the range from 1 to 10 mm in diameter with relatively smooth surface at the growth beginning, whereas developed to a un aerial mycelium that appeared as powdery, soft and granular. Isolated Streptomyces colonies with chalky, slowly growing, aerobic, piled, as well as with different color of aerial and substrate mycelium. Also all colonies that were isolated possess earthy odors (Amin et al., 2016; Risan et al., 2017 a,b; Qasim et al., 2017).

Figure (1) : *Local isolates Streptomyces sp distinguished by color on ISP2 at 25C after 7-14 days.*

The local *Streptomyces* microscopic examination after gram staining that conducted the observations revealed that local *Streptomyces* is gram positive and rod shaped. It possessing branched mycelium in their morphology of cell as showed in (Aghighi *et al.*, 2004; Rana and Salam, 2014).

Test	Reaction	Biochemical Result
Citrate Utilization	Deep blue color	Positive
Cellulase	Clear zone	Positive
Urease	No change	Negative
Catalase	No bubbles	Negative
Melanin	Black to brown	Negative

Table (2): *Biochemical tests results of Streptomyces isolates*

Extraction of metabolites from selected *Streptomyces spp*

The total yield as the amount of the dry crud extract produced in 500 ml broth medium for isolates was 1.80 g\m. Secondary metabolite production began at 4th day after seeding build-up at the production level. The pH rose to a maximum of 7.2 and gradually decreased to pH 6.2 as the secondary metabolite production peaked. At 36 hours the dissolved oxygen dropped to zero and the rate of glucose consumption became 0.1% per hour. As in shake flasks, production closely followed glucose utilization. The rate of a production will increase (Khan and Patel, 2011). After complete the fermentations process addition of dilute sulfuric acid can lower the pH of the broth and left at 4°C for 96 hours and the precipitated complex was filtered off (Pandey *et al.*,1981).

The sulfate group content increased with acid concentration, acid-to-cellulose ratio, and hydrolysis time. (Roman and Winte, 2004). The filtrate was then extracted with ethyl acetate. Ethyl acetate an extremely weak basic (essentially neutral) compound and it evaporates quickly, leaving only the remains on the surface. The main point ethyl acetate has two chemical and biological characteristics including medium polarity and minimum toxicity on test strains. They can help to extract many biological compounds (polar and non-polar) and evaluate their activities. Also, because it has low toxicity, therefore its effect of biological cells cannot be considered (Hamedi, 2013).

(GC-MS) Analysis of Streptomyces Ehtyl Acetate Extract

The results presented in Figure (2) represent the GC-MS chromatogram of the ethyl acetate extract, 20 substances were obtained from the *Streptomyces* sample, in varying proportions. The highest concentration substance is Caryophyllene. This substance has many therapeutic and health benefits. It also works to stop the growth of cancer cells and relieve pain (Legault and Pichette, 2007).

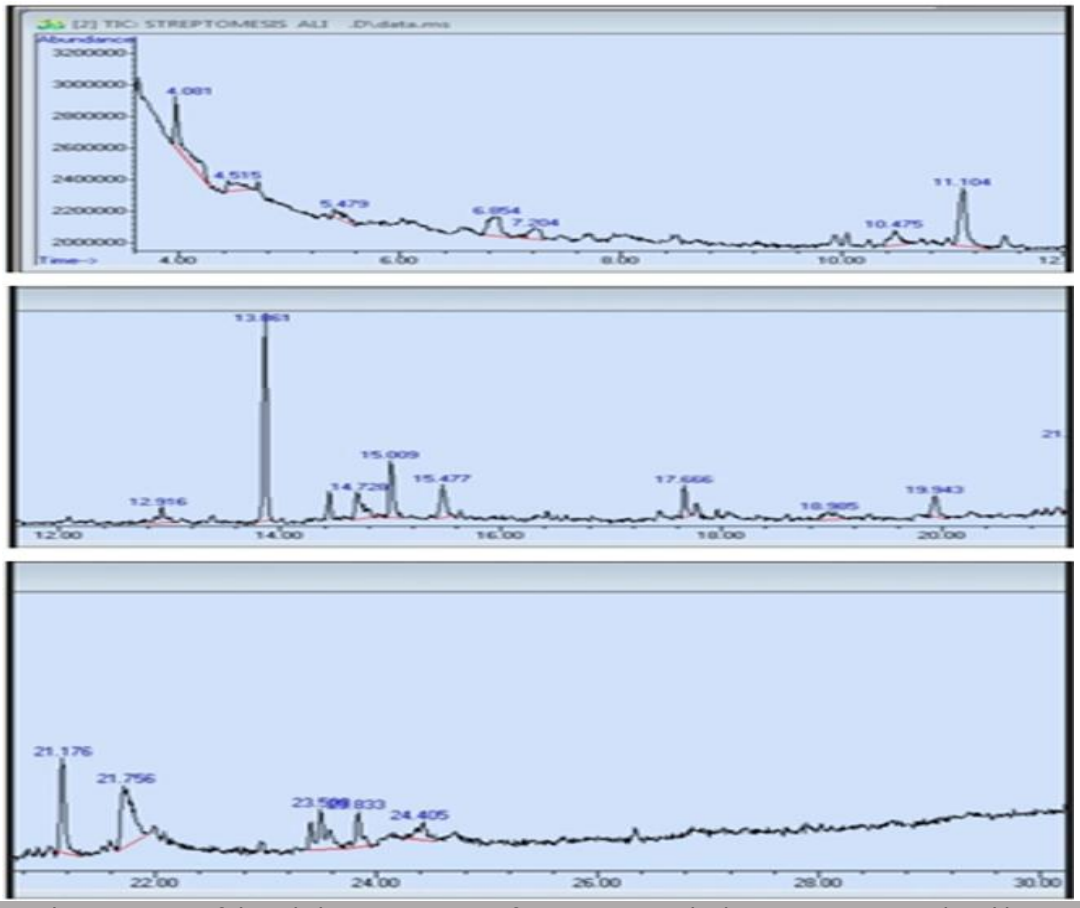

Figure (2): *GC-MS chromatogram of the ethyl acetate extract of Streptomyces Ehtyl Acetate Extract analyzed by GC-MS, with MS start time: 3.5 minutes and end time: 30 minutes.*

1 C:\GCMS\firmware\NIST11.L	4.080	8.14	Benzyl alcohol, benzyldimethylsily
2 C:\GCMS\firmware\NIST11.L	4.513	2.47	Acetic acid, hydroxy-, ethyl ester
3 C:\GCMS\firmware\NIST11.L	5.482	2.15	3-Butyn-1-ol
4 C:\GCMS\firmware\NIST11.L	6.858	4.99	No matches found
5 C:\GCMS\firmware\NIST11.L	7.205	2.66	Methyl 2,4,6-trichloro-3,5-dimetho
6 C:\GCMS\firmware\NIST11.L	10.476	2.95	4-Amino-5-imidazole carboxamide
7 C:\GCMS\firmware\NIST11.L	11.108	8.81	4-Amino-5-imidazole carboxamide
8 C:\GCMS\firmware\NIST11.L	12.917	2.48	No matches found
9 C:\GCMS\firmware\NIST11.L	13.861	13.91	**Caryophyllene**
10 C:\GCMS\firmware\NIST11.L	14.726	4.10	Hexa-t-butylthiatrisiletane
11 C:\GCMS\firmware\NIST11.L	15.012	5.17	2-[(p-Trimethylsilyloxy)phenyl]
12 C:\GCMS\firmware\NIST11.L	15.479	3.58	Hexa-t-butylthiatrisiletane
13 C:\GCMS\firmware\NIST11.L	17.669	2.55	2((trimethylsilyl)oxy)ethoxy)benz oate
14 C:\GCMS\firmware\NIST11.L	18.985	2.18	No matches found
15 C:\GCMS\firmware\NIST11.L	19.945	2.50	Trimethylsilyl 3-methoxy-2
16 C:\GCMS\firmware\NIST11.L	21.174	6.71	Hexadecanoic acid, methyl ester
17 C:\GCMS\firmware\NIST11.L	21.754	11.27	Octadecanoic acid
18 C:\GCMS\firmware\NIST11.L	23.511	7.19	Phenol, o-(isobutylsulfinyl)
19 C:\GCMS\firmware\NIST11.L	23.832	3.46	Methyl stearate
20 C:\GCMS\firmware\NIST11.L	24.403	2.74	1,2-Cyclobutanedicarboxylic acid

Table (3): GC-MS analysis profiles were applied for ethyl acetate extract of Streptomyces isolate.

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