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IDENTIFICATION ACTIVE COMPOUNDS OF BACTERIA *STREPTOMYCES* USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Aim of the study assess optimum Growth of *Streptomyces* and identification active compounds of bacteria *Streptomyces* using HPLC. The biochemical and physiological testes of *Streptomyces* sp. Show that the *Streptomyces* produced extra cellular enzymes like amylase, urease, catalase, lipase, protease, pectinase, cellulase and phosphatase, no HCN production and negative for indole production. Estimating the stationary phase for isolated *Streptomyces* were growing on malt extract yeast extract broth (ISP2), and getting the optical density (OD) at 600nm during (1-7) days and optimal growth of them ranged (6.5 to 8), while were salt concentrations ranged from (1 to 7% NaCl). The best growth happened at 30 °C by measuring the absorbance of the growth and getting the optical density (OD) at 600nm. We watched the growth state activity of isolated strain which started after 72 hours of incubation at 30°C, when the OD was about (0.572). The HPLC analysis of extracellular crude extract was showed found five different antibiotic (Tetracycline, Streptomycin, Neomycin, Vancomycin and Kanamycin).

KEYWORDS: Compounds, Bacteria, *Streptomyces,* HPLC.

INTRODUCTION

Actinomycetes are a group of Gram-positive bacteria with high guanine and cytosine content in their DNA (Kumari *et al*., 2006; Khucharoenphaisan *et al*., 2012**)**. Actinomycetes was derived from Greek 'attack' means a ray and mykes means fungus and given to these organisms form initial observation of their morphology (Babalola *et al.,* 2009). Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants (Palavesam, 2010). 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; Risan *et al.,* 2016; Amin *et al.,* 2016), 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). *Streptomyces* are aerobes, chemoorganotrophic bacteria and they need organic carbon source, inorganic nitrogen sources, and mineral salts and don't need vitamins and growth factors (Lee and Demain 1997). Most of *Streptomyces* sp. are mesophile and grow in temperatures 10-37 (Deeble *et al.,* 2005), but three species *Streptomyces thermonitrificans, S. thermovulgaris* and *S. thermoflavus* are thermophile and grow in temperature 45-55 (Srivibool *et al.,* 2004). *Streptomyces* grow in pH 6.5-8.0 (Cabello *et al.,* 2003; Qasim and Risan, 2017). *Streptomyces* are not only more resistant to drought and form arthrospore but also require less moisture than other bacterial and are very sensitive to water logged conditions (Subbarao, 1999). This study aimed to identification some active Compounds of *Streptomyces* using High-Performance Liquid Chromatography.

MATERIALS AND METHODS

Isolation of *Streptomyces*

One gram of dried and treated soil samples were 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 10^1 to 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 (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 (Oskay *et al.*, 2004). Based on cultural characteristics, suspected colonies of *Streptomyces* were selected which are characterized as small, white, pin-point, rough, chalky and a clear zone of inhibition around them, these colonies was confirmed their identification by (types of Gram's stain, aerial and substrate mycelium color, pigment production and pigment color). 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 (ISP) to obtain pure colonies used for identification (Nonoh *et al.*, 2010).

Antimicrobial Production

Different media were used for isolation and identification of *Streptomyces* sp. According to Shirling and Göttlieb, (1966) International *Streptomyces* projects (ISP) and Khan and patel, (2011), in (Table 1) after 7days of incubation, antimicrobial metabolites extraction were carried.

Table 1: International *Streptomyces* projects (ISP) used for isolation and identification of *Streptomyces.*

No.	Medial name	Abbreviation
1	Tryptone-yeast extract broth	ISP1
2	Yeast extract-malt extract broth	ISP2
3	Inorganic salts-starch broth	ISP4
4	Glycerol-asparagine broth	ISP5
5	Peptone-Yeast Extract Iron agar	ISP6
6	Tyrosine Agar	ISP7
7	Glycerol yeast extract broth	GYE

Antimicrobial of *Streptomyces*

Extracellular crude extract of *Streptomyces* sp., were screened for their antibacterial activity *invitro* by well diffusion method (Bagamboula *et al.*, 2004). Using sterile swabs, Mueller Hinton agar plates inoculated with microbial pathogens, and dug wells of 6mm diameter using Pasteur pipette, the wells and the plates were incubated at 37^{o}C for 24 hours. The plates were observed for the inhibition zone, which recorded by a metric ruler.

Optimum conditions for production Secondary metabolite from *Streptomyces*

Different nutritional and growth factors were studied to determine the optimum conditions for secondary metabolite production by *Streptomyces*. These conditions include sugar utilization, organic acid fermentation, growth in different PH, growth in different temperature and effect of salt concentration.

Sugars Utilization Medium (Kuster, 1968)

Basal medium used for detection sugar utilization contain the following:

Component	Quantity(g/l)
$(NH_4)_2SO_4$	2.56
KH_2PO_4	2.38
K_2HPO_4	5.56
$MgSO_4.7H_2O$	1
$CuSO_4.5H_2O$	6.4
$FeSO_4.7H_2O$	1.1
$MnCl_2.4H_2O$	7.9
$ZnSO_4.7H_2O$	1.5
Agar	20
D.W	1L

Each sugar (D-glucose, D-mannitol, D-xylose, L-arabinose) was sterilized by filtration and added as a final concentration of 1% to basal medium, after adjusted the pH to 7.0 and autoclaved. After incubation for 10 day at 30°C positive result was indicated by observation growth in the medium.

Organic Acids Formation Medium (Kuster, 1968)

Suspension (A)

Component	Quantity(g/l)
Glucose	50
Yeast extract	3
$MgSO_4.7H_2O$	0.62
Bromocresol purple	0.3
D.W	1L

Suspension (B)

Component	Quantity
$Na_2HPO_4.2H_2O$	1.06
KH_2PO4	0.544
D.W	1L

Suspension (C)

Component	Quantity(g/l)
$CaCO_3$	10
D.W	1L

Tubes were labeled with 0.2 ml of suspension C and autoclaved. Solution A and B were autoclave separately, mixed, and 1.8 ml of the mixture was added to each test tube containing suspension C. Inoculated tubes were incubated for 10 days at 30°C. acid formation was observed by disappearance of $CaCO_3$ and color changing of bromocresol purple to yellow.

Optimum Growth in different pH

locally isolated *Streptomyces* were grown on yeast extract malt extract broth (ISP2) supplemented with different pH (4, 5, 6, 7, 8, 9 and 10) in order to obtain optimum pH for the isolates. Screw cap tube (50ml) occupied with 30ml of yeast extract malt extract broth (ISP2) in duplicates, one with 0.5ml stock culture

suspension and other as a negative control without culture suspension, then incubated at 28°Cfor 4 days and the growth was checked by reading their absorbance at 600nm (Khan and Patel, 2011)

Optimum Growth in different temperature

Growth in different temperatures was tested by incubating PDA slants inoculated with spore suspension of the test isolates at 20, 25, 28, 35, 40 and 50°C in an incubator for 8 days. (James *et al*., 1991).

Optimum effect of sodium chloride concentrations

Sodium chloride tolerance of locally isolated *Streptomyces* was evaluated by growing them in malt extract yeast extract broth (ISP2) medium supplemented with graded doses of sodium chloride (1, 2, 3, 4, 5, 6,7,8 and 9 % w/v), with the negative control without inoculate it with the isolate *Streptomyces*, finally after 4 days of incubation at 28°C, the results were observed by read their absorbance at 600nm. The strain which was survived in highest (maximum) NaCl concentration it was consider and remain viable, as the strain salt-tolerance (Tresner *et al*., 1968).

Preparation of Standards and Sample for HPLC Analysis

Standard Preparation

A weight of 10mg of standards were dissolved in 50ml of methanol (HPLC grade) to get 200ppm which was further diluted by dissolving 1ml of this solution in 50ml methanol (Mauricio *et al*., 2007).

Sample Preparation

A volume of 20 ml of sample were dissolved in 50ml of methanol (99%) (HPLC grade). Further dilution by adding 1ml of this solution to 50ml using (99%) methanol HPLC grade (Mauricio *et al*., 2007).

High-Performance Liquid Chromatography (HPLC) Analysis for extracellular extract of *Streptomyces*

A volume of 20 ml of the standard and 20ml of the sample were injected to HPLC and record the chromatogram, calculated the content of the sample in comparison with standard. The concentrations were calculated according to the following equation: **Concentration = [area of sample/area of standard] × sample concentration× dilution factor** (Rajaseka and Elango, 2011).

RESULTS AND DISCUSSION

Physiological and biochemical properties

The biochemical and physiological testes of *Streptomyces* sp. Show in (Table - 2). The *Streptomyces* produced extra cellular enzymes like amylase, urease, catalase, lipase, protease, pectinase, cellulose and phosphatase, utilization of citrate was positive, no HCN production or volatile toxicity was noticed, and negative for indole production.

Table 2: Biochemical and physiological testes of *Streptomyces* sp.

Reaction	Response	Result
1.Melanine reaction -Medium ISP.2	Brownish of medium	Negative
- Medium ISP.6	Brownish of medium	Negative
2. Soluble Pigmented - ISP4	NO Brown	Negative
- PDA	Dark Brown pigment	Positive
3. Urease	Red to deep pink	Positive
4. Catalase	Bubbles	Positive
5. Amylase	Clear zone	Positive
6.Protease	Clear zone	Positive
7. Gelatinase	Narrow zone	Positive
8.Pectinase	Clear zone	Positive
9.Cellulase	Clear zone	Positive
10.Phosphatase	Clear zone	Positive
11. Indole production	No color zone	Negative
12. Citrate Utilization	Deep blue color	Positive

Primary screened of *Streptomyces* as anti-microbial

Thirty five of locally isolated *Streptomyces* were obtained from three different region sources of soil that tested the activities of antimicrobial against human microbial pathogens (bacteria) by using the technique of cross streak, such, *Escherichia coli* for gram-negative bacteria, *Staphylococcus aureus* for gram positive bacteria. The result of the process for primary screening program against human microbial pathogens indicated the activity of antimicrobial for all potential isolates were streaked as a straight line on nutrient agar media separately and incubated for 7 days at 28 °C. After their growth were completing, many types of strains for microbial pathogens were streaked at right angle, without touching each other, in addition to incubated for 24 hours at 37 °C, (Rana and Salam, 2014). Concluded that microbial pathogens were not able to grow near the antimicrobial compound produced by the isolated actinomycetes. For all 45 locally isolated *Streptomyces* the process were done, most of the isolates *Streptomyces* have a power against tested pathogens, but the protocol of our designed was concentrated to select the most active and broad spectrum activities.

Antimicrobial Activities of *Streptomyces*

For testing the best media composition four different media broth (ISP1, ISP2, ISP4 and ISP5) used to evaluate the best composition of media that give the maximum antimicrobial activity against tested microbial pathogens (*Escherichia coli*, *Staphylococcus aureus*) by agar well diffusion assays. Each medium broth inoculated with isolated *Streptomyces* suspension incubated for 7 days at 28±1 °C in shaking incubator after completing the incubation period the broths were centrifuged for 5 minutes at 5000rpm, the supernatants

were collected and filtered through whatman No. 1, as well as used as extracellular crude extract alone. The obtained results from other research showed the bestinhibition zone on gram positive bacteria *Staphylococcus aureus* from that of Boudjelal *et al.*, (2011) got 24mm inhibition also Naine *et al.*, (2015) obtain 24 mm, so comparing these results with our results especially that obtained by (bag3) 22 mm inhibition when growing on ISP2, while the result show that media (ISP2) ues for production of antimicrobial metabolites against gram negative *Escherichia coli* which recording only 18mm whereas the other used media showed moderate efficacy against bacteria *Escherichia coli* (gram negative). So comparing our results against bacteria *Escherichia coli* (gram negative) were less than Gurung *et al.*, (2009) results, which obtained 17mm inhibition zone; also Attimarad *et al.*, (2012) obtained 25mm inhibition. The project results were similar to that obtained by Hozzein *et al.*, (2011) they obtained 15mm inhibition zone. Antony-Babu *et al.*, (2008) the secondary metabolites and antimicrobial products of isolated actinomycetes produce extracellular products in nature that yield a powerful antimicrobial metabolites activity were describe.

Estimating Growth Curve (incubation period) for *Streptomyces*

Estimating the stationary phase for isolated *Streptomyces* (bag3) were growing on malt extract yeast extract broth (ISP2), from recording the growth state which presented in fig(1) reveled the result that happened by measuring the absorbance of the growth and getting the optical density (OD) at 600nm. We watched the growth state activity of isolated strain (bag3) which starting after 24 hours of incubation, when the OD was about 0.082 (first day) then it reached 0.262-0,432 in second and third day, which indicated that the strain completed their lag and log phase in the growth curve only through three days, because at the 4rd day of incubation it was reaching the maximum OD 0.535, so its mean that the strain reached to the stationary phase, therefore, the strain will be able to start the production of secondary metabolites after four days of incubation.

Results were agreed with that obtained by Boudjelal *et al.*, (2011) recording the maximum growth at 4 days incubation, the study of Khan and patel, (2011) for isolated *Streptomyces* in 5th days it was reaching the stationary phase, also Abdul Wahab *et al.*, (2015) obtained the same results in which reach to the stationary phase at 5 days and ended at 6 days of incubation, when the strain started producing antimicrobial metabolites at log phase and the concentrated activity was found at stationary phase. Stationary phase considered as a phase which giving the highest amount of secondary metabolites as mentioned by (Khan and patel, 2011).

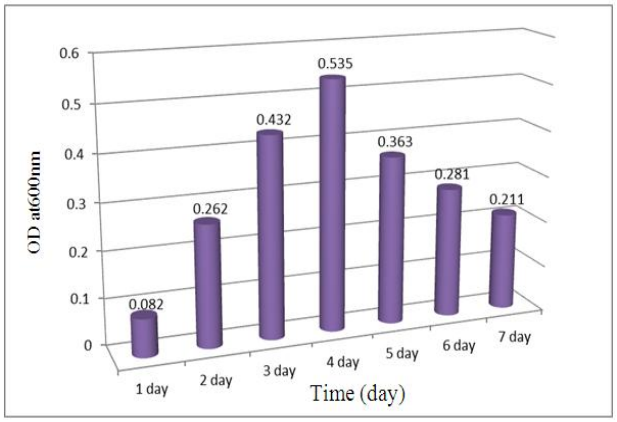

Figure 1: Growth curve of locally isolate *Streptomyces* (Bag3), growing on malt extract yeast extract broth for 7days at 28±1 °C, measuring the growth rate absorbance at 600nm.

Estimating the Optimum pH Value for Isolated Strain (bag3)

Streptomyces are well known species of actinomycetes which preferred neutral to alkaline environment pH, the optimal growth of them ranged (6.5 to 8) however, some actinomycetes (*Streptomyces*) have been found in acidophilic environment as described by Kontro *et al.*, (2005). For determining the optimum pH for isolation (bag3) *Streptomyces* was growing on yeast extract malt extract broth (ISP2) with different pH value ranged from (5, 6, 7, 8 and 9) (fig 2) their growth rate was measured spectrophotometric ally at 600 nm. The results presented in (Figure 2) summarized the determination of the optimal pH value for isolate (bag3), according to the result the highest amount of absorbance (0.468) was recorded at pH 7,5 which means the maximum amounts of growth was achieved after four days of growth of isolated strain (bag3) with pH 7,5 while the remaining pH values were also gave the high O.D values especially pH 8 and pH 6 about (0.267 and 0.223 respectively), this results indicated that the isolated strain having a wide range for growth in different pH values especially at alkaline ranges.

The optimum pH value for isolated strain of *Streptomyces* (bag3) was recorded optimum value at pH 7,5 this result was in between with the results obtained by previous published research, Boudjelal *et al.*, (2011) revealed that the pH was varied between 7.0 and 8.5 during the incubation of their isolated strain.

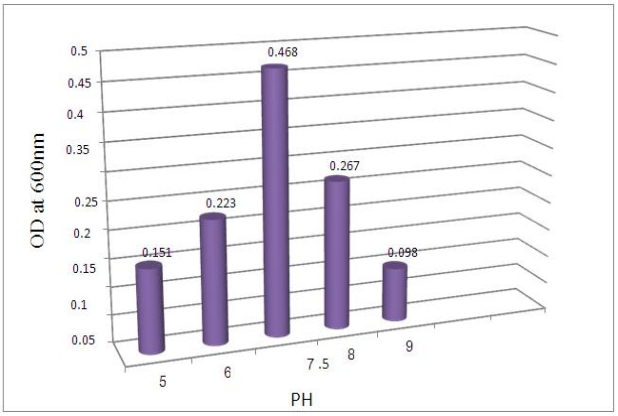

Figure 2: Estimating optimal pH value of locally isolated *Streptomyces* (Bag3), on malt extract yeast extract broth ISP2 after 4 days of incubation at 28±1 °C.

Estimating Salt-Tolerance (NaCl) for Isolated Strain (bag3)

The capability of isolated strain (bag3) for growing in malt extract yeast extract broth(ISP2) supplemented with different (NaCl) salt concentration ranged from (1 to 7% NaCl) was determined at the basis of salt tolerance against growth rate, and it was measured spectrophotometically through obtaining the amount of optical density at 600nm.

The result in (Figure 3) summarized the salt tolerance of isolate strain (bag3), which indicated that the isolated strain it grow till 4%, while above this concentration from (5% to 7%) we did not found any growth, because the absorbance became below the blank value which means that there was no growth. in the study of Singh *et al.*, (2014) on *Streptomyces sannanensis* SU118 they found salt tolerance of NaCl about 3%, this results were also obtained by Khan and patel (2011); Abdul Wahab *et al.*, (2015), further more, our results were in agreement with that obtained by Boudjelal *et al.*, (2011) and Gebreyohannes *et al.*, (2013) they isolated actinomycetes species that survive till 5% NaCl concentration.

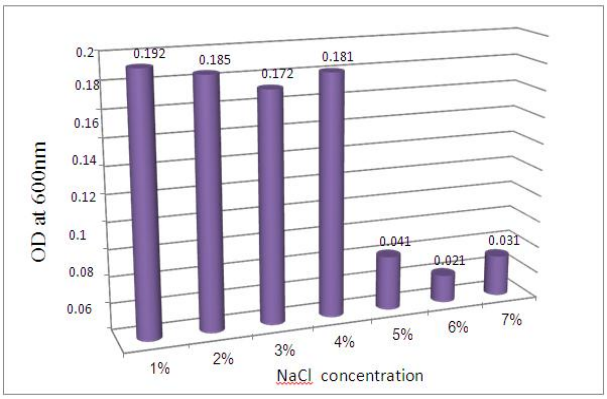

Figure 3: Estimating NaCl salt tolerance of isolated of *Streptomyces* (Bag3) growing on ISP2 supplemented with different NaCl concentration, incubated at 28±1 °C for 4days.

Estimating the best temperature for isolated *Streptomyces* (bag3)

After *Streptomyces* isolates were grown in yeast extract and malt extract (ISP2) and incubated at different temperatures (20, 25, 30, 35, 40, 45 and 50°C), results in figure (4) illustrate that the best growth happened at 30 °C by measuring the absorbance of the growth and getting the optical density (OD) at 600nm, We watched the growth state activity of isolated strain (bag3) which started after 72 hours of incubation at 30°C, when the OD was about (0.572). The Moderate growth occurs at temperature (25 and 40°C), less growth at 50°C our result were agreement with that obtained by Ventosa *et al.*, (1998) they getting good grow at 30°C.

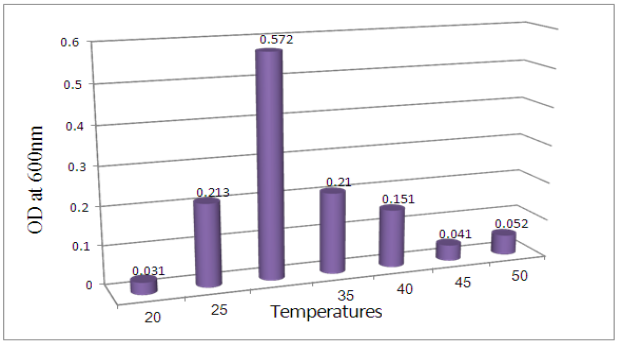

Figure 4: Estimating optimal temperature value of locally isolated *Streptomyces* (Bag3) on malt extract yeast extract broth ISP2 after 4 days.

Purification of Extracellular Crude Extract of Isolated Strain (bag3)

Partial purification of extracellular crude extract was carried out by extraction and determining the bioactive compounds with High Performance Liquid chromatography (HPLC) through carrying out bioautography in the presences of standard antibiotic.

Purification by High Performance Liquid Chromatography (HPLC)

HPLC analysis was done to detect the concentration of important active compounds present in *Streptomyces*. HPLC analysis of extracellular extract of *Streptomyces* indicated the presence five active compounds. Figure (5) revealed different peaks of antibiotic present in extracellular extracts of *Streptomyces* in same retention time in compare with a stander but with different area.

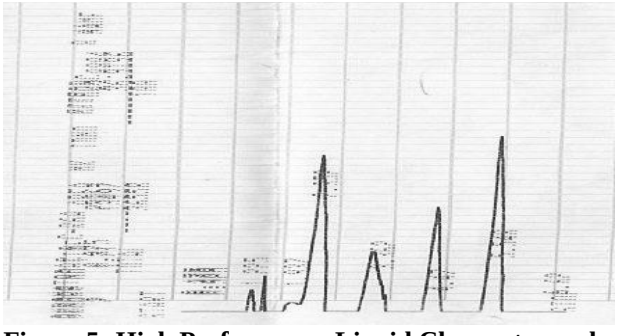

Figure 5: High Performance Liquid Chromatography of standard antibiotic.

The HPLC of extracellular crude extract was showed five different antibiotic (Tetracycline, Streptomycin, Neomycin, Vancomycin and Kanamycin,). figure (6) and (table 3), revealed that the same retention time of the sample in compare with a stander but with different area.

Table 3: Show the R_f values for each standard antibiotic and area also the concentration of each antibiotic found in *Streptomyces* sp.

SEQ	Subject	Retention Time minute	Area	Concentration µg/ml
1	Tetracycline	1.793	9380	3.7422
2	Streptomycin	2.273	26463	185569
3	Neomycin	3.592	33342	13.381
4	Vancomycin	4.567	33249	13.2639
5	Kanamycin	5.635	48510	19.3522

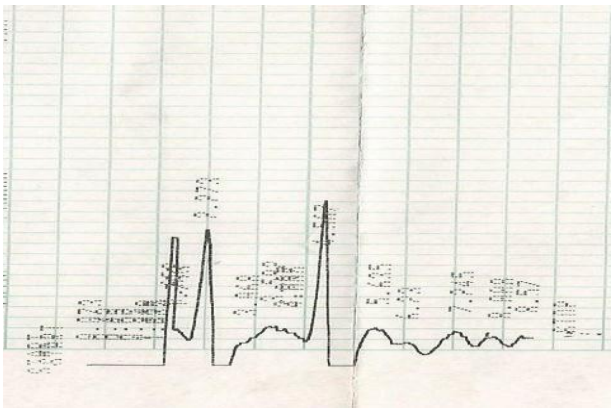

Figure 6: High Performance Liquid Chromatography for extracellular extract of *Streptomyces.*

Awais *et al.*, (2007), they described that thin High performance liquid chromatography are regularly used for analysis and characterization of antimicrobial compounds from producing microorganisms. In order to determining the R_f of the bioactive extract of extracellular with R_f of standard antibiotic the results presented in (Table 3) summarized all R_f values for each standard antibiotic and area also the concentration of each antibiotic found in *Streptomyces* sp.

CONCLUSION

The isolated *Streptomyces* appeared more susceptible to Gram-positive bacteria *Staphylococcus aureus* than Gram-negative bacteria *Escherichia coli.* Optimum condition for the production and extraction of the bioactive compounds were successfully completed, for the best media, optimum incubation days, optimum pH, NaCl salt tolerance and Temperature. Analyzing by High Performance Liquid Chromatography gave five active compound (Tetracycline, streptomycin, neomycin, vancomycin and kanamycin).

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