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Identification and study of cultural conditions affecting Neomycin antibiotic produced by *Streptomyces* sp. N

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Abstract:-

Cultural conditions affecting antibiotic production by local isolate of *Streptomyces* sp. N were studied. Cultural conditions included growth medium, growth temperature, pH, and ion effect. The study showed that this local isolate was able to produce neomycin antibiotic. This compound showed a biological activity against gram – positive, and gram – negative bacteria. This effect was variable depending on the factors studied. Media of antibiotic production, R2 medium, and complete medium were better for *Streptomyces* growth. Growth of the organism for 5 days gave the largest inhibition zones against test bacteria. The organism showed better growth and antibiotic production when cultivated at 37^0C. The pH of the medium showed a dramatic effect on antibiotic production since low pH had increased antibiotic release to the medium resulting in observed increased in the biological activity. Ions like phosphate and citrate showed different effect on the antibiotic production. The phosphate had shown an enhancing effect where as citrate inhibit antibiotic production and lowered the growth of the organism on cultivation media.

دراسة الظروف الزرعية المؤثرة على انتاج المضاد الحيوي النيومايسين المنتج من قبل العزلة المحلية ستربتومايسيس N

المستخلص:-

تم دراسة الظروف الزرعية المؤثرة على انتاج المضاد الحيوي النيومايسين المنتج من قبل العزلة المحلية ستربتومايسيس N. اذ تم دراسة نوعية الوسط الزرعي ودرجة حرارة النمو والاس الهيدروجيني وتاثير الايونات.اظهرت الفحوصات الكيميائية والتشخيصية عن طريق الترحيل الكروماتوغرافي والطيف بالاشعة تحت الحمراء ان المضاد المنتج من نوع النيومايسين وله فعالية ضد بكتيرية للبكتريا الموجبة والسالبة لصبغة كرام.ان الظروف المثلى لانتاج المضاد كانت هي وسط الانتاج ووسط 2 عندما نميت البكتريا لفترة 5 ايام ودرجة حرارة 37 م. اما الاس الهيدروجيني فكان له التاثير الاكبر في انتاج المضاد ظهر عندما درست الفعالية الحيوية مرة اخرى بعد تنمية العزلة في اس هيدروجيني منخفض.والسبب في ذلك هو زيادة السماحية للجدار الخلوي للبكتريا في الاطلاق المضاد الى الوسط الزرعي. اظهر ايون الفوسفات تاثيرا منشطا لانتاج المضاد عندما اضيف للوسط الزرعي بينما ثبط ايون السترات الانتاج لهذا المضاد.

Introduction:-

Gram positive obligatory aerobic *Streptomyces* bacteria are abundant in most soils. For large part of their nutrition, they utilize insoluble organic debris by the production of variety of extracellular hydrolytic enzymes such as cellulase, hemicellulase, amylase, protease, and nuclease. They are morphologically adapted to this way of life [2].

Antibiotics are compounds that inhibit the growth of other microorganisms by some specific interference in their normal biochemistry. The specificity of action of antibiotics for particular targets, and hence for particular groups of organisms, is the reason why many of them are so valuable in medical, veterinary, and agricultural practice, and therefore, as industrial products [13].

Biochemical analysis of the end product had revealed that many streptomycetes might produce more than one type of antibiotics during their growth and development [16].

Biochemical analysis had shown that these antibiotics are derivatives of less complex molecules obtained by bacterial nutrition. Chemical modification and dimerization – polymerization may lead to produce more complex compounds with different function [1].

Such biochemical reactions obtain their energy from specific molecules that affect specifically not in antibiotic production only, but also in *Streptomyces* differentiation. These molecules were identified as guanosine $5'$ – diphosphate $3'$ – diphosphate (ppGpp) and guanosine $5'$ – triphosphate $3'$ – diphosphate (pppGpp).

Other studies found that other molecules may have an adverse effect in antibiotic production. The A – factor (2 – *s* – isocapryloyl – 3 – *s* – hydroxymethyl – γ – butyrolacton) is a potent autoregulatory factor essential for antibiotic production and spore formation in *Streptomyces* [9].

In *Streptomyces*, antibiotic production nearly always takes place only after rapid growth has ceased. When nutrients are abundant and readily available, microorganisms grow fast. In mixed communities, rapid conversion of nutrients to biomass is the overriding theme of the metabolism and its efficiency is maximized by the well – known regulatory system that governs such assimilation. Considering this fact, *Streptomyces* grown in a liquid media generally produce antibiotics during stationary phase or at low growth rate. This may reflect production by cell inside mycelial pellets that may be nutritionally limited and that have, therefore, entered stationary phase [14].Genes specifically involved in the production of particular antibiotic are invariably found clustered together, and only one set for methylenomycin production is known to be plasmid – located rather than chromosomal, while most of other antibiotics were found to be chromosomally determined [5].

The biosynthetic clusters usually also contain one or more genes that confer immunity to the antibiotic which vary considerably in their mode of action. Many genes have been identified that pleiotropically affecting antibiotic production in *Streptomyces*, and several of these are likely to play a global role in regulation of antibiotic production. Mutants in about half of these pleiotropic genes also showed deficiencies in morphological differentiation [8].

Neomycin has been described by Waksman and Lechevalier as an antibiotic substance produced by *Streptomyces fradiae*. It is very active against variety of gram – positive and gram – negative bacteria including streptomycin resistant strains. Early studies on neomycin concentrated by Swart *et al.*, indicated the presence of several antibacterial components for which the term "neomycin complex" was introduced [12].

Neomycin is extremely stable toward alkali since it withstand 18 hours of heating with barium hydroxide. Neomycin is stable at pH 2 at room temperature for at least 24 hours, but heating with 1 N or 6 N mineral acids results in extensive degradation with charring, particularly when 6N acid is used. Acute toxicity tests in mice indicated an LD_{50} of approximately 80 mg / kg intravenously, and 400 mg / kg subcutaneously. Thus, on a weight basis, neomycin is more toxic than streptomycin [15]. The aim of this work is to ascertain cultural conditions in production of neomycin – like antibiotic by the local isolate *Streptomyces* sp N.

Materials and Methods:-

Bacterial species:

Streptomyces sp. N isolate was a kind gift from Professor Dr. Mohammad A-K Ibrahim , test organisms *Bacillus thuringiensis*, *Bacillus sphaericus* were obtained from department of Biotechnology, Al-Nahrain University as a standard isolates imported from Pasteur Institute of France, *E. coli, Proteus mirabilis, Pseumonas aeruginosa, Candida albicans* were a standard isolates obtained from Central Clinical Laboratory , Ministry of Health.

Culture media:

The following culture media were used for cultivation of *Streptomyces* sp N.

Antibiotic production medium (Leach *et al.*, 1951), contain (Glucose 25 g, Yeast Extract 2.5 g, $CaCO_3$ 8 g, Kcl 4 g, KH_2PO_4 0.4 g, Soya bean meal 25 g, Tap water 1000 ml), R2 Medium (Hopwood *et al.*, 1985) (Sucrose 103 g, K_2SO_4 0.25 g, $MgCl_2.6H_2O$ 10.12 g, Glucose 10 g, Casamino acids 0.1 g, Distilled water 800 ml), 2.2 g of agar was placed in each 250 ml flask and poured in 80 ml of the solution. The flasks were closed and autoclaved. At the time of use, the media were melted and a solution of the following components was added to each flask: KH_2PO_4 (0.5 %) 1 ml, $CaCl_2.2H_2O$ (3.68 %) 8 ml, L – proline (20 %) 1.5 ml, TES buffer (5.73 % pH 7.2) 10 ml, Trace element solution 0.2 ml, NaOH (1 N) 0.5 ml,

Trace element solution ($ZnCl_2$ 40 mg, $FeCl_3.6H_2O$ 200 mg, $CuCl_2.2H_2O$ 10 mg, $MnCl_2.4H_2O$ 10 mg, $Na_2B_4O_7.10H_2O$ 10 mg, $(NH_4)_6Mo_7O_{24}.4H_2O$ 10 mg) TES buffer (Tris – base pH 8 0.05 M, NaCl 0.05 M, EDTA pH 8 5 mM) Gauza agar (Komagata, 1986) contain (Soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, K_2HPO_4 0.5 g, Agar20 g, Distilled water 1000 ml), Complete medium for streptomycetes (Hopwood *et al.*, 1985) contain (Agar 10 g, K_2HPO_4 5 g, NaCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, Bacto – pepton 2 g, Yeast extract 1 g, Casaminoacids 1.5 g, L – Histidine 50 mg, L – Proline 50 mg, Yeast nucleic acid hydrolysate 5 ml, Vitamin solution 1 ml, Glucose 25 g, Distilled water 1000 ml).

Extraction and identification of Neomycin:

Extraction and TLC chromatography of antibiotic was done according to Weinstein and Wgman, (1978), the method was modified to obtain sterile, and debris free extract from the broth of *Streptomyces* cultivated for 5 days in antibiotic production medium. This was done by filtration through 0.22 µm membrane. IR spectroscopy was made by Schimadzo spectrophotometer to identify functional groups in the compound extracted from chromatography paper.

Results and Discussion:-

Antibiotic production is a common feature in *Streptomyces*. It is so rare to find a bacterium of these genera deficient in production of such secondary metabolite in nature, only but those deficient in aerial mycelium production as a result of mutation. This proposed a complex network connecting antibiotic production with differentiation.This proposal led to the discovery of plieotropic genes that affect differentiation as well as antibiotic production [3].In normal isolates of streptomycetes, production of antibiotic may depend mostly on physiological and nutritional conditions. The organism was first grown on media mentioned previously to detect the best growth medium. Results showed R2 Medium, antibiotic production medium, and complete medium were more suitable for the growth of the organism, since they allowed fast growth and differentiation that was complete in 7 days, where as other media allowed growth and spore formation in 14 days. The reason may be attributed to the presence of nitrogen and carbon sources that are easily to be consumed by the organism and enhance differentiation in shorter time. These media were used for further research.

The isolate showed an antibacterial activity against gram – positive, and gram – negative bacteria that may indicate the presence of an antibacterial agent produced (table 1).

Table (1): Biological activity of the local *Streptomyces* sp N against gram positive and gram negative bacteria.

***Streptomyces* strain**	*Inhibition zone (mm)*						
	Staph. aureus	***Bacillus thuringiensis***	***B. sphaericus***	***E. coli***	***Candida albicans***	***Proteus mirabilis***	Pseudomonas ***aeruginosa***
N	**20**	—	**10**	—	—	**25**	—

The mark (---) =no inhibition zone.

To identify the antibacterial agent, the IR spectroscopy was performed. Figure (1) shows a great resemblance with the scheme obtained from neomycin that may refer to the presence to such antibiotic produced by this bacterium.

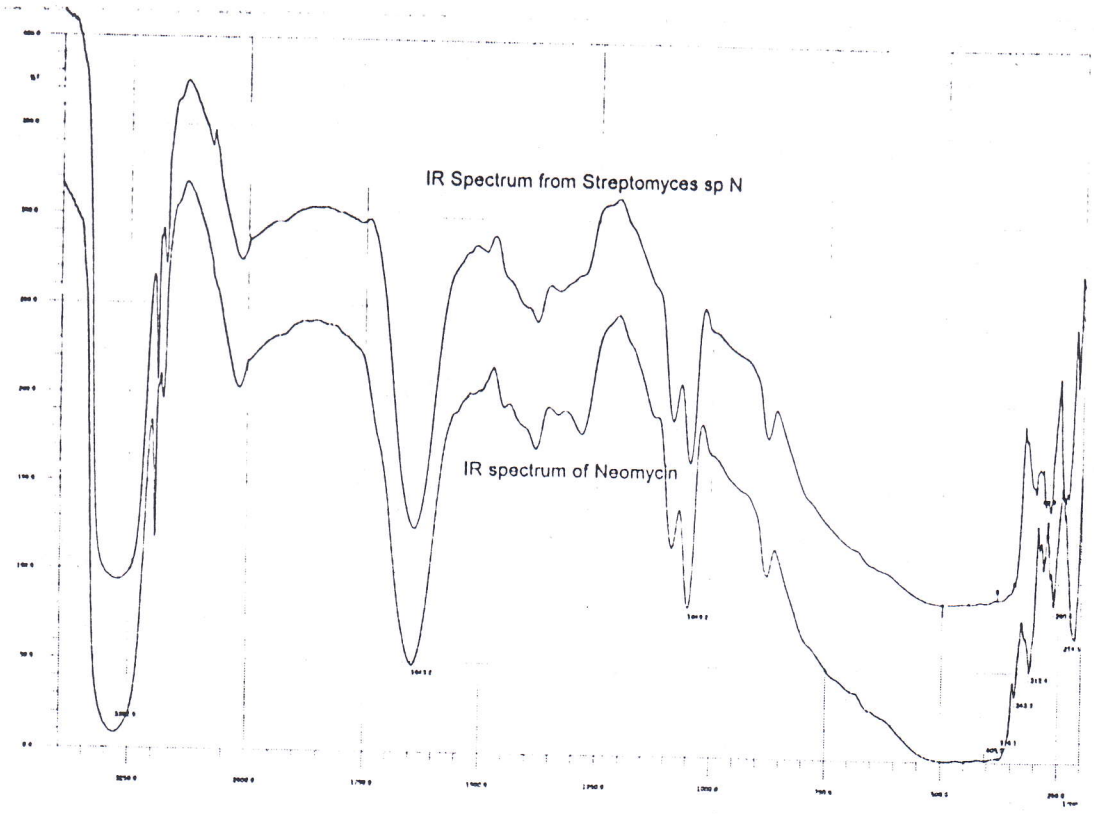

Figure (1): IR spectroscopy of *Streptomyces* sp N in comparison with the standard neomycin. The figure shows a great similarity between the two spectrums that indicates a similarity in structure of the two compounds.

Thin layer chromatography for the extract of the medium in which this bacterium was grown was done using neomycin as a standard to calculate the R_f value. Result showed that both compounds had the same R_{ff} of 0.33 Weinstein and Wgman, (1978) which may confirm the previous results. The result is shown in figure (2).

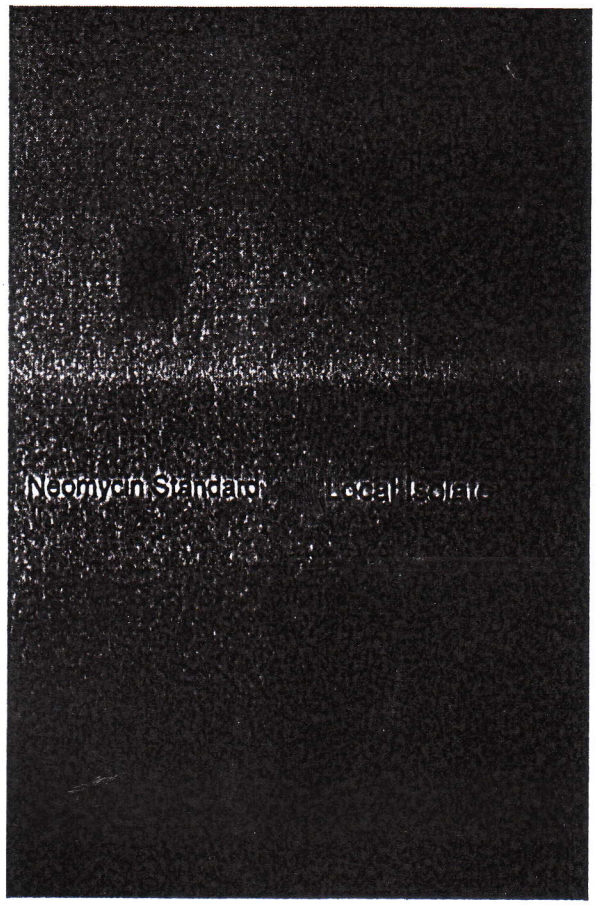

Figure (2): Thin layer chromatography of the extract obtained from the growth medium of the local Streptomyces isolate. The R_F of 0.33 was the same for the two compounds that may indicate the similarity between them which is Neomycin.

Factors affecting production of antibacterial agent were also studied. The first factor was the incubation period. Results showed that incubation of the bacterium for 5 days gave increased inhibition zone against test bacteria when biological activity was performed. The reason for this may be associated with the hydrolytic enzymes produced by the bacterium that may degrade the antibiotic [4].

PH of growth medium may play an important role in antibiotic production. Results showed when the bacterium was grown in acidic medium (pH 5), the biological activity had increased dramatically after 5 days of incubation. This may as a result of the change of permeability of the cell wall enhancing the release of increased amount of the antibiotic to the medium (table 2) that shows a dramatic increase in antibiotic production as a result of change of cell wall permeability.

Table (2): Effect of pH on the production of antibiotic when biological activity was performed against test organisms.

Streptomyces strain	***Inhibition zone in mm against test organisms***						
	Staph. aureus	*Bacillus thuringiensis*	*B. sphaericus*	*E. coli*	*Candida albicans*	*Proteus*	**Pseudomonas**
N	30	10	20	10	—	50	20

Growth temperature was tested as a factor affecting antibiotic production from the bacterium. Results showed that 37^0C represent the best growth temperature and antibiotic production, since no biological activity was observed when the local isolate was grown at 27^0C, and it fail to grow at 45^0C.

The effect of ions was also investigated as a factor controlling antibiotic production. Two types of ions referred to by Hopwood and Chater (1994) were tested phosphate and citrate.

When those ions were added to the medium they showed different effects. The citrate ion showed an inhibition effect while the phosphate showed an enhancing effect on antibiotic production.

This may be attributed to the interference of these ions with the primary metabolic pathway producing the precursors needed for the antibacterial agent production [4].

Conclusions:-

This study had focused on identification of Neomycin antibiotic produced by local *Streptomyces* isolate and studying factors affecting this antibiotic production that included growth condition, physical, and chemical factors. Among these factors, pH showed the most drastic effect on antibiotic production not only as an effect on cell wall permeability but it may interfere with other biological activities of the bacterium that need more study.

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