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A Study on Actinorhodin-like Substance Production by *Streptomyces* IQ 45

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ABSTRACT

Production of pH-pigment (actinorhodin – like substance) was ascertained from ten *Streptomyses* isolates. *Streptomyses* IQ45 isolate was only isolated which produced pH-sensitive pigment. The production of pH-sensitive pigment was detected by fuming over ammonia. After extraction of this antibiotic, a number of physiocochemical characterizations were carried out which involved (IR, UV, MP, CHN-analysis, and solubility test). Indicated that this antibiotic is an actinorhodin-like substance. TLC of the extracted substance showed a single spot with Rf value equivalent to (0.26) which was close to that of actinorhodin.

These antibiotics showed inhibitory activity against *Staphylococcus* aureus similar to that of actinorhodin produced by *Streptomyces coelicolor* A3 (2). The productivity of this antibiotics was (45 mg/L) at pH 8.5 and (40 mg/L) at pH 7 from the mycelial mat and (10 mg/L) when extracted from the liquid medium at pH7.

KEYWORDS: Streptomyces; Actinorhodin; extraction.

الخلاصة

أظهرت العزلة **(IQ45)** القابلية على انتاج المضاد الملون الشبيه بالاكتينورودين الحساس للتغير في الأس الهيدروجيني من مجموع عزلات الستربتومايسيس العشر التي تم اختبارها بالاعتماد على التغير في لون المستعمرات بعد التعرض لبخار الأمونيا.

بعد استخلاص هذا المضاد أشارت الفحوصات الفيزيوكيميائية (Solubility test, CHN, MP, UV, IR) الى ان هذا المضاد هو شبيه للاكتينورودين. كما أظهرت تقنية كروموتوغرافيا الطبقه الرقيقه وجود بقعه واحده مع Rf بقيمة (٠,٢٦) مطابقة لمعدل السريان الخاص بالأكتينورودين.

أظهر هذا المضاد فعالية ضد بكتريا *Staphylococcus aureus* مشابهة لفعالية الاكتينورودين المنتج من قبل سلالة *Streptomyces coelicolor* A3 (2).

بلغت انتاجية هذا المضاد ٤٥ ملغم / لتر عند الأس الهيدروجيني (٨,٥) و ٤٠ ملغم / لتر عند الأس الهيدروجيني (٧) من حصيرة المايسيلا و ١٠ ملغم / لتر للمادة المستخلصة من الوسط السائل عند الأس الهيدروجيني (٨,٥)، بينما لم يتم الحصول على نتائج من الوسط السائل عند الأس الهيدروجيني (٧).

INTRODUCTION

Streptomyces is one of the most important of actinomycetes genera, represents member of aerobic, gram positive unicellular filamentous bacteria, characterized by a high genomic G+C content (mean 74 mol %) [1]; with cell walls containing LL-diaminopimelic acid [type I] or *Streptomyces* type according to [2]. This genus includes the largest number of species and variants that about 90-95% of actinomyces isolated from soils belong to the genus *Streptomyces* [3, 4]. Members of this genus characterized by morphological complexity and also exhibit a remarkable capacity for biochemical differentiation producing a wide variety of secondary metabolites that include about 65% of known antibiotics, whereas 15% from fungi and 10% from other bacteria.

The most important antibiotics produced by *Streptomyces* include tetracycline, chloramphenicol, erythromycin, and aminoglycosides (such as streptomucin, dihydrostreptomycin, gentamycin, kanamycin, tobramycin and neomycin) [5]. Many of the polyketide antibiotics produced by *Streptomyces* are also quinine antibiotics that related to the group of benzoquinone or anthraquinone or to the naphthoquinones [4, 6, 7].

Isochroman-quinone antibiotics represent an important group of quinine antibiotics that

include in addition to actinorhodin, kalafungin (Hoekesma & Krueger, 1976), nanomycins (Tanaka *et al.*, 1975), and the griseusin [8].

Some of polyketide quinine antibiotics are used as an antitumor agent such as daunomycin, adriamycin, streptoningrin and granaticin [9].

Polyketides represent perhaps the largest family of secondary metabolites especially abundant among actinomycetes. Numerous fungal polyketides are toxins, such as patulin and the aflatoxins; mammalian example include the prostaglandin and leukotriene hormones; and a large class of plant polyketides are flower pigments important for insect pollination [10].

Moreover, this genus also produces many enzymes such as β-lactamase, cellulose, amylase, protease, ligninase, xylanase and chitinase, vitamins and soluble pigments such as melanin.

In addition *Streptomyces* are good producer of growth promoters like indol-3-acetic acid which is produced by *Streptomyces*, *Atroolviaceus*, and *Streptomyces mutabillis* [11].

Member of the genus *Streptomyces* are characterized by producing many of pH-sensitive pigments like actinorhodin, granaticin and medermycin. These pigments could be considered as an acid-base indicator for example, actinorhodin is red in acid and blue in alkaline conditions; whereas granaticin is red in acid and purple in alkaline conditions while the medermycin is yellow in acid and brown in alkaline conditions [12].

The presence of such indicator pH-sensitive pigments or litmus like pigments was found in *Streptomyces coelicolor* (Muller) [13].

Some of the pH-sensitive pigments have antibacterial activity, notably, actinorhodin, granaticin and undecylprodigiosin (Wright Hopwood, 1976; Snipes *et al*.,1979; Hopwood *et al*.,1995), and some have antitumour activity e.g. granaticin [14].

The aim of the work is:

1. Production and chemical detection of actinorhodin – Like substance produced by *Streptomyces* (IQ45).
2. Study of antibacterial spectrum of actinorhodin-like substanc

MATERIALS & METHODOLOGIES

Bacterial Strains

The following microorganisms were obtained from culture collection of biotechnology department, college of science, Al-Nahrian University.

1. *Streptomyces* IQ45, Z1, SH-10, LMS 25-9, AE, 69B1, *S. lividans*, *S. erythreus*, *S. rochei*, *S. aurofaciens*.
2. Test microorganisms:

Gram positive bacteria: *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*.

Gram negative bacteria: *Escherichia coli*.

Yeast: *Candida albicans*.

Molds: *Aspergillus sp*.

Culture Media

The following media were used Glucose yeast extract malt extract agar, inorganic Salts-Starch agar, Gauza, glycerol-Asparagin agar, Starch-Yeast extract medium [15]. Extraction of Actinorhodin [16] Starch- Yeast extract medium using 250 ml with pH values adjusted to 7 and 8.5 were inoculated with 1ml of spore suspension, and then incubated at temperature 30 ˚C on a rotary shaker at 120 rpm for 7 days.

Actinorhodin-like substance were extracted from mycelia and filtrate

A. Mycelium (5.5 gm) obtained by filtration of liquid culture medium, was washed with 250 ml 0.1M-HCL and successively extracted for 2h with 27ml 2M-HCLfor 30 min with 27 ml acetone. After drying in vacuum for 4 to 5 hr., the powdered mycelium was ground with 2 vols sand and actinorhodin was extracted in 27 ml 2M-NaOH.

The supernatants after centrifugation was adjusted to pH 3 and the red precipitate of crude actinorhodin that formed was collected, dried and ground to powder then purified using dioxn.

B. The above method was used to extract actinorhodin-like substance from *Streptomyces* culture filtrate. Thin- like chromatography of Actinorhodin-like substance [16, 17].

Silica gel plates (5×20) cm with 0.25 mm thickness was washed with the solvents mixture [acetone/benzene (1:2, by vol.)]

Physicochemical examinations of purified Actinorhodin-Like is substance Ultraviolet spectral (UV) of Actinorhodin-like substance [18].

Infrared spectra (IR) of Actinorhodin-like substance [16]. Melting point (MP)[4]. Solubility Test Estimation of actinorhodin-like substance production spectrophotometrically [19].

Actinorhodin-like substance synthesis was estimated spectrophotometrically by measuring the optical density at 602 nm of cell free culture supernatants that had been adjusted to pH9.

Antibacterial spectrum of actinorhodin-like substance

The antibacterial activity of actinorhodin-like substance was detected in two ways:

1. Agar plug assay method
2. Wells assay method

RESULTS AND DISCUSSION

Members of the genus *Streptomyces* are known for their ability to produce a vast array of secondary metabolites. Many of these components are used as antibiotics; some of these are pH-sensitive pigments.

Ten *Streptomyces* isolates were selected for possible production of pH-sensitive pigments. Results indicated that one isolate (*Streptomyces* IQ45) could produce blue color as tested by exposure the culture to ammonia vapor figure (1) it is worth noting that ammonia vapor is usually used for detection of actinorhodin [12,15,16,20].

Thus it might possible to suggest that S. IQ45 is an actinorhodin-like substance producing isolate. The effect media on production of pH-sensitive pigment by *Streptomyces* IQ45 was studied. Five agar culture media (YEME, Inorganic salt starch, Gauza, Glycerol-asparagine and SY) were used for this purpose.

Fuming the colonies over ammonia showed blue color with various intensities Table 1. Dark blue color was observed when *Streptomyces* IQ45 was grown on SY medium. While moderate intensity of blue color was observed when this isolate was grown on (glycerol-asparagine and inorganic salt starch) media and lower intensity of the blue color was observed when *Streptomyces* was grown (gauza and YEME) media.

Variation in color intensities might due to the effect of media composition. In this experiment, pH has no effect on the color because it was the same for the five media. It has been reported that medium composition effects the formation of *Streptomyces* metabolic product e.g. antibiotic since actinorhodin is pH sensitive pigment, this might explain the variation in the intensity of blue colors on different media [21].

Table 1. Intensity of blue color pigment produced by *Streptomyces* IQ45 isolate.

	Agar culture media	Color intensity of Mycelia
1	YEME	Light blue
2	inorganic salt starch	Moderate blue
3	Gauza	Light blue
4	Glycerol-asparagine	Moderate blue
5	Starch-Yeast extract (SY)	Dark blue

Extraction of Actinorhodin

Grost – Allman (1981) method was followed to investigate actinorhodin-like substance production by IQ45 isolate which was grown in starch-yeast extract medium.

Actinorhodin was extracted from Streptomyces IQ45 from both mycelial mat and from liquid medium at two different pH values (7 and 8.5), the results were shown in Table 2. This table showed that actinorhodin-like substance production was localized to mycelial mat pH 7, but it may be released to liquid culture media at pH above 7 [22]. Moreover, the actinorhodin-like substance production in mycelia mat at pH 8.5 was relatively higher than at pH7.

Various studies showed that actinorhodin often produced at pH 7 [23, 24].

Grost-Allman (1981) reported that 65mg/L of actinorhodin could be obtained from *S. coelicolor* A3(2) at pH 7. While Wright and Hopwood 1976 were obtain a greater amount of actinorhodin (430 mg/L) from mycelial mat of *S. coelicolor* (1190) when grown at the same pH value.

Our study showed that there is no production of actinorhodin-like in liquid medium at pH7,

while 10mg/L were extracted from liquid media at pH 8.5.

Rudd & Hopwood 1979 reported that the actinorhodin is very soluble in polar solvents at pH values above 7 and poorly soluble below pH7. This might explain the presence of actinorhodin in liquid culture at pH8.5.

Wright and Hopwood 1976 reported that actinorhodin production start at pH about (6.7) and it is insoluble and remains localized in or on the mycelium.

Table 2. The effect of pH on actinorhodin production from mycelial mat and filtrate of *Streptomyces* IQ45 culture.

Actinorhodin production mg/ml	Extracted part	pH of Culture Media
40	Mycelial mat	7
0	Filtrate	
45	Mycelial mat	8.5
10	Filtrate	

Identification of actinorhodin-like substance by thin-layer chromatography

The possible production of actinorhodin-like substance by *Streptomyces* IQ45 was ascertained by thin-layer chromatography (TLC), aceton/benzene (1:2, by vol.) was used as a mobile phase.

The results of TLC were shown in table (3). In this experiment one spot was obtained from IQ45 mycelial mat when grown at pH7 with Rf value of 0.28. one spot was appeared on TLC plate with Rf value of 0.27 from IQ45 mycelial mat when grown at pH 8.5, and one spot of Rf value of 0.26 was obtained when extracted from liquid medium at pH 8.5. whereas no spot was detected at pH7 of extracted from liquid media. Rf values of various isochromane quinine antibiotics were in the range of 0.08 and 0.38 when using the solvent mixture [aceton/benzene, (1:2 by vol.)]. In this regard Rf values of actinorhodin and related compounds were (0.22-0.27) [6, 25, 26].

The results which shown in Table 3 indicated that the Rf value of actinorhodin-like substance that extracted from mycelia mat and liquid media pH 8.5 were 0.27 and 0.26.

Whereas Rf value of 0.28 was obtained in extract from mycelia mat at pH 7. These values were similar to the obtained by krone *et al*.,1982 [17].

Furthermore thin layer chromatography had been employed for detection of some isochromane quinone antibiotics using the solvent systems composed of tertiary butyl alcohol-acetic acid-water (72:3:25) or ethyl acetate-acetic acid-water (88:6:6) [27].

Table 3. Rf value of extracted substances from both mycelia mat and filtrate at pH7 and pH 8.

Rf value	pH of culture	Extracted part
0.28	7	Mycelial mat
0.27	8.5	
0	7	Filtrate
0.26	8.5	

Physico chemical properties of purified actinorhodin-like substance extraction from (*Streptomyces* IQ 45)

A number of physic chemical tests carried out of actionorhodin-like substance that involved IR spectra, U.V. spectra, MP, CHN-analysis and solubility test.

The results of IR spectra were shown in (Figure 2) referred to the presence of peaks located at 3200 cm^{-1} due to OH- groups at 3050 cm^{-1} due to two=CH groups, at 2850 cm^{-1} due to COOH-groups and, at 1725 cm^{-1} due to carboxylic acid-carbonyl groups and at 1650-1670 cm^{-1} due to the quinine absorption these IR peaks are characteristics of actinorhodin [24] UV spectrum (Figure 3) showed the presence of peak at 230 due to the presence of isochromane quinine antibiotics [16].

The melting point of purified actinorhodin in this experiment was 260˚ while actinorhodin extracted from *S. coelicolor* A3(2) had amelting point of 270˚ [27]. This difference might due to the impurities present in the sample, furthermore the accuracy of thermometer that was used in the experiment might influence the measurements. Moreover, one might suggest the differences in the resolution power between various recording eyes CHN-analysis of purified sample revealed the following composition, C=31.71%, H=25.58% and N=3% C and H values are close to the C and H values of actinorhodin from *S. coelicolor* A3(2). (%C=30, %H=28) [20]. The presence of small

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amount of nitrogen might be due to the impurities that might come from the remains of cell debris.

Solubility test of prepared actinorhodin showed its ability to dissolve in organic solvents (ethanol, acetone, dixoan) and in alkali like sodium hydroxide (NaOH) and in acid like hydrochloric acid and in distilled water.

The above results suggested that *Streptomyces* IQ45 is a producer of pH- sensitive or acid-base indicator (actinorhodin-like substance).

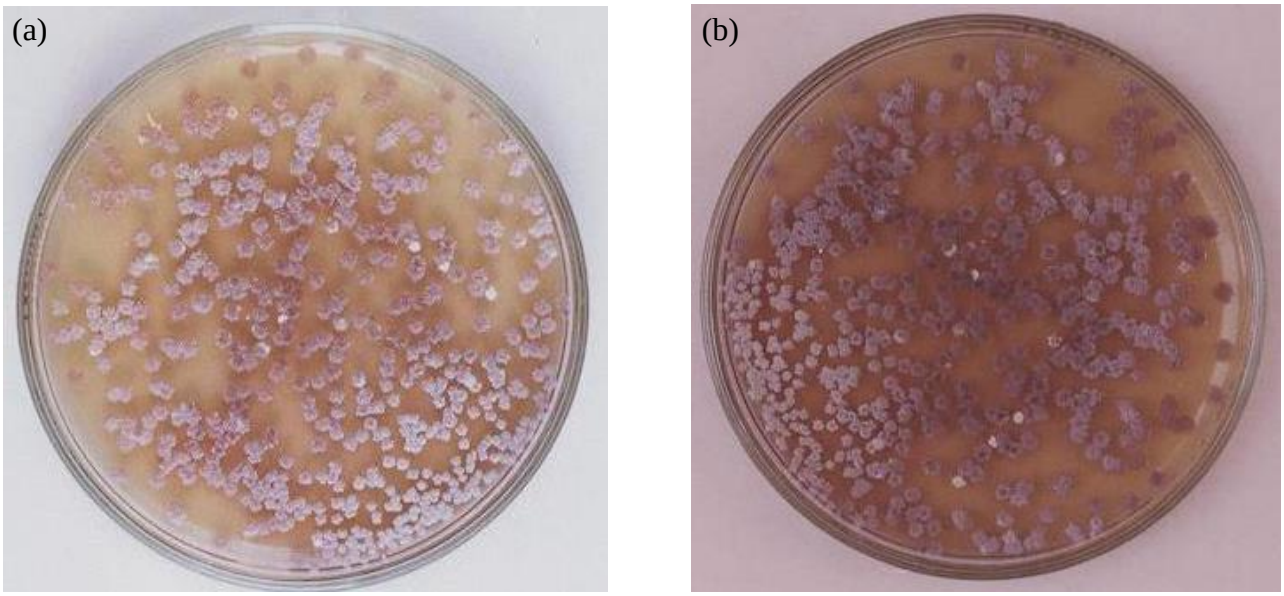

Figure 1. Ammonia fuming test of pH-sensitive pigment production by (*Streptomyces* IQ45) which was grown on (SY) media for five days at 37 ˚C, a) Before fuming, b) After fuming.

Antibacterial spectrum of actinorhodin-like substance

The biological activity of purified actinorhodin-like substance produced by *Streptomyces* IQ45 was determined using both agar plug assay method and wells method. The inhibitory activity of actinorhodin-like substance using agar plug assay method against *Staphylococcus aureus* showed that concentration in µgml-1 (25,50,75 and 100) produced inhibition zone of diameter in millimeter (14,17,24 and 27) respectively and (20,27,33 and 40) millimeters of inhibition zone diameter respectively when using wells method. So that the biological activity of actinorhodin-like substance extracted from this isolate showed high inhibitory activity against *Staph. aureus* even at low concentrations (25 μgml^{-1}). These results almost similar to those obtained for the activity of actinorhodin produced by *S. coelicolor* A3(2) (Wright and Hopwood, 1976). The obtained results indicated that the inhibitory activity as determined by using wells method was higher than plug assay method.

The inhibitory activity of actinorhodin-like substance related to the chemical structure of this antibiotic (isochromane quinine antibiotics) which has effect on the protein synthesis (ribozymal protein) of G+ve and G-ve bacteria [19]. The chemical detection and purification of this antibiotic was the major important in this research, so in the next step, we tried to concern with the inhibitory activity of this antibiotic against other test organisms.

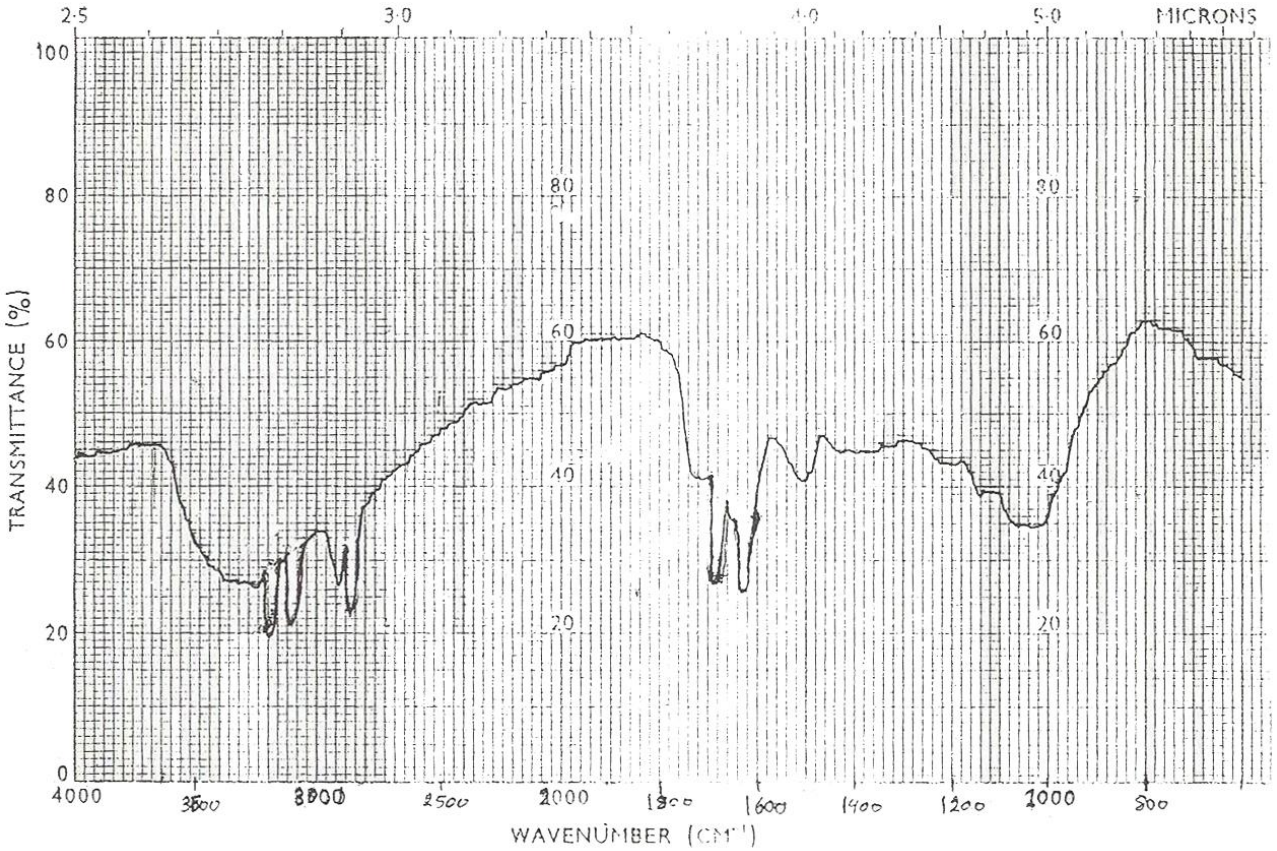

Figure 2. IR spectra of actinorhodin-like substance produced by *Streptomyces* IQ45 from mycelia mat at pH 8.5.

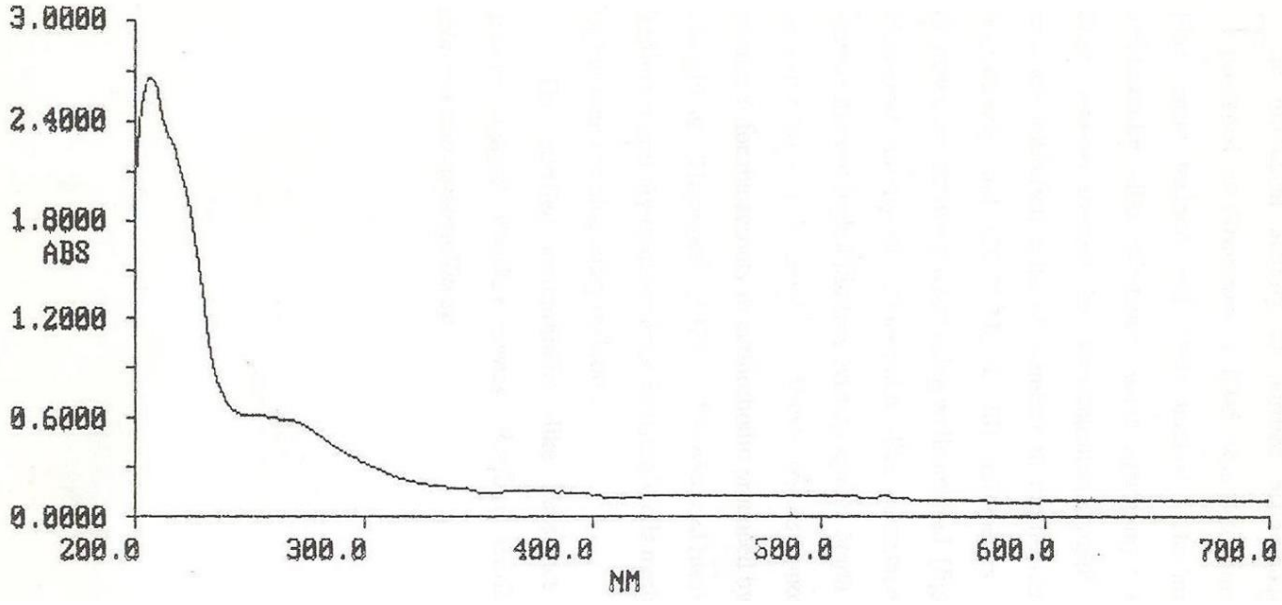

Figure 3. U.V. spectra of actinorhodin-like substance produced by *Streptomyces* IQ45 from mycelia mat at pH 8.5.

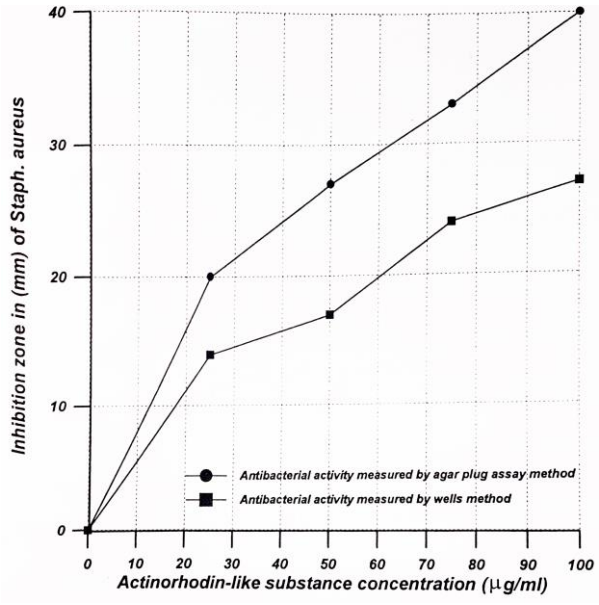

Figure 4. the inhibitory activity of actinorhodin-like substance against test bacteria (*Staph. aureus*).

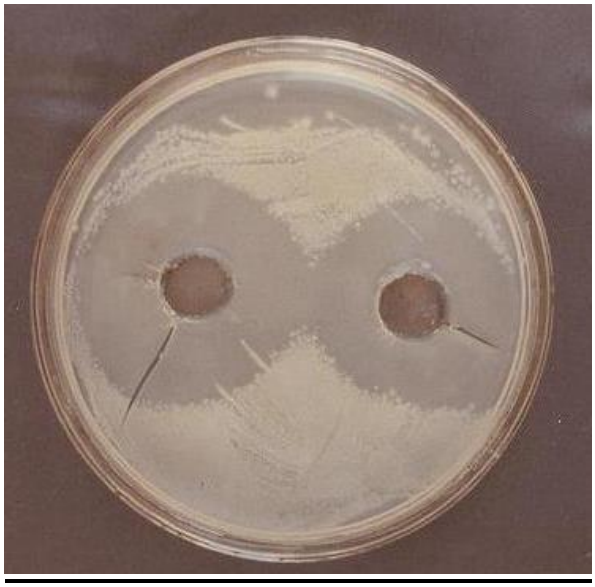

Figure 5. the inhibitory activity of actinorhodin-like substance extract from *Streptomyces* IQ45 against *Staphylococcus aureus* by wells method.

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