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Growth Phases of Some Antibiotics Producing *Streptomyces* and their Identification

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ABSTRACT. The growth phases of five cultures of antibiotic producing *Streptomyces* isolated from western region, Saudi Arabia were studied special reference to their potentiality to excrete antibiotics. It was found that these organisms grew exponentially during the first 4 to 7 days of incubation. The highest specific growth rate was observed in the case of *Streptomyces* MY18 being 0.48 day^{-1} (1.44 days doubling time). This isolate also showed the highest inhibition zone against *Staph. aureus* as compared with the other four *Streptomyces* isolates. This antagonistic effect was more pronounced at the last five days of incubation (stationary phase). Morphological, cultural and physiological properties of most active antibiotic producers revealed that *Streptomyces* MY18 and *Streptomyces* MR13 are strains of *S. aureofaciens* and *S. reseviolaceus* respectively.

Introduction

Antibiotics are a special kind of chemotherapeutic agents usually obtained from microorganisms. The word antibiotic has come to refer to a metabolic produce of one microorganism that in very small amounts is detrimental or inhibitory to other microorganisms[1]. Streptomycin was the first example of an antibiotic possessing a broad spectrum of activity, effective against many Gram-positive and Gram-negative bacteria[2]. Other antibiotics with even broader spectra of activity (for example, the tetracyclines) have been subsequently discovered. They also reported that *Streptomyces erytheus, S. halstiddi, S. griseus, S. fradiae* and *S. aureofaciens* produce erythromycin, carbomycin, streptomycin, neomycin and tetracycline respectively. It was also found that 46% and 0.6% *Streptomyces* isolates had an inhibitory effect on *Staph. aureus* and *E. coli* respectively. Welsch and Kalyuzhnaya *et al.* observed that

70-90%, 23-24% and 52-62% of actinomycetes cultures inhibited Gram-positive bacteria, *E. coli* and *Enterococci* respectively. Two phases are observed during the propagation of antibiotic producers[5,6].

The aim of this work was conducted to study the growth curves and growth parameters of the most active antibiotic producing *Streptomyces*. The antibiotic productivity as influenced by the different growth phases and elapsing of time was also observed.

Material and Methods

Five strains of *Streptomyces* used throughout this work were detected from Dr. Malibari*, A.A. who described them as the most active antibiotic producing strains isolated from western region soils of Saudi Arabia. These strains are *Streptomyces* MR10, MR13 (red series), MY16, MY18 and KY24 (gray series). *Straphylococcus aureus* 209P was used as a test organism for the first four *Streptomyces* strains while *Candida albicans* was used for the fifth *Streptomyces* strain (KY24).

Growth Phases and Antibiotic Production

In this investigation, the most active antibiotic producing *Streptomyces* isolates were grown in shake flasks as batch cultures for 11 days to study their growth kinetics and the efficiency of antibiotic production as influenced by elapsing of time.

Conical flasks (250 ml volume) containing 100 ml glycerol casein medium[7] were inoculated with *Streptomyces* isolates (MR10, MR13, MY16, MY18 and KY24) using 10ml of standard inoculum (5 to 7 mg dried biomass) from each isolate. The flasks were incubated on rotary shaker (180 rpm) at 30°C for 11 days. Five milliliters of the culture were taken daily and filtrated. The pellets were washed two times with distilled water and left for 10 minutes on filter paper to determine the fresh weight, then were heated at 70°C for three days to determine the dry weight (five replicates from each isolate). The culture filtrate was used to determine the effectiveness of the excreted antibiotic on test organisms using culture filtrate technique. *Staphylococcus* and Sabourad dextrose media[8] were used for propagation of *Staph. aureus* and *Candida albicans* as sensitive organisms.

The growth as indicated by dried biomass was plotted against time on semilog paper. Different growth phases were determined and growth kinetics was calculated from the exponential growth phase according to as follows[9,10].

- Specific growth rate (u) = $(\ln A - \ln A_o)\ (t - t_o)^{-1}$ where :

  $\ln A_o$ = Napierian log. of growth at the beginning of exponential phase (t_o time).

  $\ln A$ = Napierian log. of growth at the end of exponential phase (t = time).

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Doubling time $t_d = \ln 2 (u)^{-1}$
Effective yield (Y_E) = Dried biomass × initial substrate concentration

Examination of Streptomyces Isolates with Scanning Electron Microscopy

The surface of mycelium and conidiospores was examined by scanning electron microscopy after coating the specimen with carbon, gold and pallidium[11], in the Electron Microscopy Lab., Facultyof Science, King Abdulaziz University using JEOL JSM 35 Scanning EM.

Maintenance of Microbial Cultures

The microbial cultures used throughout this investigation were maintained by layphylization at −50°C using freeze drier (Labconco). These organisms were also maintained at 4-6°C after their propagation on the specific medium for each group as mentioned before.

Identification of the Most Active Antibiotic Producers

Two isolates of *Streptomyces* MY18 and MR13 were subjected to complete identification according to the morphological and physiological characteristics described in Bergey's Manual of Determinative Bacteriology[12]. The characteristic used in this investigation included cultural, morphological and physiological properties as follows :

A – Morphological and Cultural Characteristics. Shape of conidiophore, conidiospore surface, color of aerial and substrate mycelium were investigated for the cultures growing on the surface of starch-nitrate agar medium[13] malt extract agar[8] and glycerol asparagine agar[14] at 30°C for 10 days incubation.

The production of melanin pigment was observed by cultivation of tested isolates on the surface of glycerol-tyrosine agar[15] for 10 days at 30°C. The positive melanin producers were noticed by the production of deep brown soluble pigments.

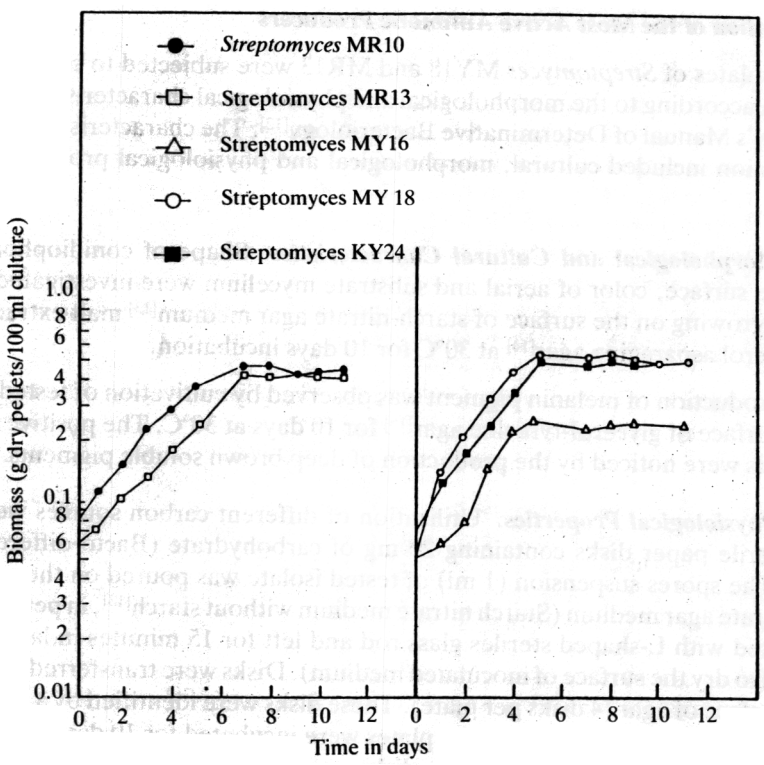
B – Physiological Properties. Utilization of different carbon sources were done using sterile paper disks containing 20 mg of carbohydrate (Bacto-differentiation disks). The spores suspension (1 ml) of tested isolate was poured on the surface of basal nitrate agar medium (Starch nitrate medium without starch[13], in petri-dish and distributed with L-shaped steriles glass rod and left for 15 minutes in laminar flow cabinet (to dry the surface of inoculated medium). Disks were transferred aseptically to the surface of agar (4 disks per plate). These disks were identified by a printed letter code for each carbohydrate. The plates were incubated for 10 days at 30°C. The growth of tested organism around the disks was used as an indication for utilization of different carbon sources (positive result). The tested carbohydrates were adonitol, arabinose, dextrose, dulcitol, galactose, inositol, inulin, lactose, levulose, maltose, sucrose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, trehalose and xylose.

Gelatin liquefaction (on gelatin-peptone medium, coagulation of litmus milk)[8] and decomposition of cellulose (on basal nitrate medium containing strips of Whattman No. 1 filter paper) were also observed.

Results and Discussion

Growth Curves and Antibiotic Production

It is clear from Tables (1 and 2) and Fig. (1) that the *Streptomyces* isolates grew exponentially during the first 4 to 7 days of incubation. The highest biomass (dry weight of pellets, Fig. 1) was observed on the seventh day of incubation i.e. the end of exponential phase (logarithmic phase) for *Streptomyces* MR10 and *Streptomyces* MR13 being 0.47 and 0.44 g 100^{-1} culture respectively (L.S.D. at 5% = 0.43 and at 1% = 0.058). On the fifth day of incubation, *Streptomyces* MY18 and *Streptomyces* KY24 reached to the end of exponential phase showing 0.55 and 0.53 g dry biomass 100^{-1}



L.S.D. for	*at 5%*	*at 1%*
1. Fifth day of incubation	0.056	0.077
2. Seventh day of incubation	0.043	0.059

FIG. 1. Growth curves of the most efficient antibiotic producing *Streptomyces* isolates

culture respectively (L.S.D. at 5% = 0.056, at 1% = 0.077). On the contrary, *Streptomyces* MY16 showed the end of exponential phase at early stage of growth (on the fourth day) where 0.24 g dry biomass was obtained. Specific growth rate (u) during the exponential phases of *Streptomyces* isolates (MR10, MR13, MY16, MY18 and KY24) varied from one isolate to another being 0.2530, 0.2846, 0.3922, 0.4800 and 0.4049 day^{-1} respectively. After the exponential phase, the growth slightly increased (phase of accelerating growth) or slightly decreased to be more constant at the end of four days of incubation (from 8 to 11 days). The maximum specific growth rate (0.48 day^{-1}) and the lowest doubling time (1.44 days) were observed in the case of *Streptomyces* MY18. These isolates also showed the maximal inhibition zone against *Staph. aureus.*

TABLE 1. Growth parameters of *Streptomyces* isolates and their maximal inhibition zone (mm).

Streptomyces isolates	u day^{-1}	t_d day	Effective yield %	Maximal inh. zone (mm)
MR10	0.2530	2.74	4.7	16
MR13	0.2846	2.44	4.4	22
MY16	0.3922	1.77	2.4	13
MY18	0.4800	1.44	5.5	30
KY24	0.4049	1.71	5.3	16

With respect to the antimicrobial effect of the culture filtrate during 11 days of incubation (Table 2), it was found that the sensitivity of test organisms (*Staph. aureus* for *Streptomyces* MR10, MR13, MY16 and MY18, *C. albican* for KY24) started on the 2*nd* of incubation for *Streptomyces* MR13 and MY18, on the 3*rd* day for *Streptomyces* MR10 and KY24 and on the 4*th* day for *Streptomyces* MY16. Thereafter, the inhibition zone increased gradually with elapsing of time to become more stable at the last 3 days of incubation. The highest inhibition zone was recorded on the 10*th* for *Streptomyces* MY18 being 30 mm diameter. This was followed by *Streptomyces* MR13 which showed 22 mm inhibition zone on the 10*th* day of incubation. *Streptomyces* MY16 gave the lowest antimicrobial effect (13 mm inhibition zone on the 9*th* day of incubation). The highest correlation coefficient between time of incubation and inhibition zone of different *Streptomyces* strains was recorded in the case of *Streptomyces* MY18 being 0.9186. It means that the increase of incubation time led to increase of inhibition zone.

Generally, it could be stated that the first 4 to 7 days of incubation are characterized by the formation of biomass whereas the maximal production of antibiotic was recorded in the stationary phase of growth. Many investigators came to the same conclusion[6].

Identification of the Most Active Antibiotic Producers

Streptomyces MY18 (gray series) and *Streptomyces* MR13 (red series) which showed higher activity in the antibiotic excreted in the culture than other isolates

TABLE 2. Antagonistic effect of *Streptomyces* isolates (Culture Filtrate) as influenced by time course of incubation.

Time in days	Zone of inhibition				
	MR10*	MR13*	MY16*	MY18*	KY24**
0	0	0	0	0	0
1	0	0	0	0	0
2	0	11	0	14	0
3	12	12	0	18	11
4	14	13	11	20	12
5	13	12	12	22	13
7	15	15	12	24	15
8	13	18	12	25	16
9	14	19	13	28	15
10	15	22	12	30	16
11	16	21	12	29	15

*Test Organism = *Staph. aureus* 209P
**Test Organism = *C. albicans* R17

Regression analysis for time and inhibition zone of :

1. *Streptomyces* MR10
   Y = 1.402 X + 2.355
   Correlation coefficient (R) = 0.8236
2. *Streptomyces* MR13
   Y= 1.483 X + 4.372
   Correlation coefficient (R) = 0.8946
3. *Streptomyces* MY16
   Y = 1.365 X + 0.191
   Correlation coefficient (R) = 0.8494
4. *Streptomyces* MY18
   Y = 2.802 X + 4.372
   Correlation coefficient (R) = 0.9186
5. *Streptomyces* KY24
   Y = 1.560 X + 1.764
   Correlation coefficient (R) = 0.8687

were subjected to complete identification using some morphological and physiological properties as described by Bergey's Manual of Determinative Bacteriology[12].

Streptomyces MR18 was characterized by the formation of spiral chains of conidiospores, smooth-surfaced conidiospore, gray aerial mycelium, yellow substrate mycelium and golden yellow soluble pigment (on starch nitrate, malt extract, glycerol nitrate, glycerol asparagine media), no melanin pigment (on glycerol tyrosine agar medium), and good growth on Czapek-Dox agar medium. This bacterium utilized glucose, galactose, levulose fructose, sucrose, arabinose and xylose as sole source of carbon. This strain also liquified gelatin (using gelatin-peptone medium), did not coagulate litmus milk medium and decomposed cellulose (using basal nitrate medium + strips of filter paper). This strain was highly inhibited by streptomycin and showed antimicrobial activities against *B. subtilis, Staphylococcus*

aureus, E. coli, Proteus mirabilis, G +ve *Bacillus* isolate No. 1 (from soil), and G –ve short rod isolate No. 2 (from soil).

It is clear from the morphological and physiological properties that the *Streptomyces* MY18 is considered to be a strain belonging to *Streptomyces aureofaciens.*

With respect to the second isolate MR13, it was found that this isolate form spiral chains of conidiospores, smooth-surfaced conidiospores, red aerial and substrate mycelium, red soluble pigment (on starch nitrate, glycerol nitrate and asparagine medium), no melanin pigment (on glycerol tyrosine agar medium) and poor growth on Czapek-Dox agar medium. This strain utilized glucose, xylose, arabinose, rhamnose, fructose, raffinose, mannitol, inositol and sucrose as sole sources of carbon. It liquified gelatin (using gelatin-peptone medium), did not coagulate litmus milk and no cellulolytic activity. This strain showed antimicrobial activity against *B. subtilis, Staph. aureus, E. coli, Proteus mirabilis,* G +ve *Bacillus* isolate No. 1, G –ve short rod isolate No. 2, *Saccharomyces cerevisiae, Aspergillus niger* and *Penicillium* sp. It means that this bacterium exhibits antibacterial and antifungal activity showing broad spectrum effect as compared with the first isolate which gave only antibacterial activity. The proposed name of this red series strain is *Streptomyces reseviolaceus.*

Generally, it could be concluded that all tested strains showed a progressive increase of biomass (dry weight) during the first 4-7 days of incubation. On the contrary, the highest antibiotic activity as recorded in the stationary phase of growth (the last five days of incubation). Although the accumulation of antibiotics started on the *2nd* or *3rd* day of incubation, the highest accumulation was observed on *9th* and *10th* day of incubation. It means that the organisms grew firstly to form a considerable amount of growth followed by the formation of antibiotic. It is expected that the production of antibiotics are usually biosynthesized as secondary metabolites. These results are in line with that observed by Pirt, S.J. and Righelato, R.C. and Lurie, L.M. *et al.*[5,6]. They reported that two phases are observed during the propagation of antibiotic producers. The first phase (trophophase) is characterized by a rapid growth (biomass production) and the second phase (idiophase) is characterized by a slow growth and maximal productivity of antibiotics. The most active organism among the five tested isolates was found to be *Streptomyces* MY18 (gray series). This bacterium gave a good growth which was accompanied by a high antibiotic activity. According to the morphological, cultural and physiological properties, this organism was found to be a strain of *Streptomyces aureofaciens.*

References

[1] **Pelczar, M.J., Chan, E.C.S.** and **Krieg, N.R.,** *Microbiology. 5th* ed. McGraw-Hill Book Company, New York, 917 p., (1986).

[2] **Stanier, R.Y., Adelberg, E.A.** and **Ingraham, J.,** *The Microbial World, 4th* ed., Prentice Hall, Inc., Englewood Cliffs, New Jersey, 871 p., (1976).

[3] **Welsch, M.,** Bactericidal substances from sterile culture media and bacterial cultures, with special reference to the bacteriolytic properties of actinomycetes, *J. Bacteriol.* **44,** 571-588, (1942).

[4] **Kalyuzhnaya, L.D., Bryanskaya, E.T, Litovchenko, E.T., Likach, I.G., Lysento, S.A., Maiko, I.I.** and **Protinnov, S.M.,** Isolation and study of actinomycetes having antagonistic properties from soil in several oblasts of Ukraine, *Microbiologia,* **31,** 654-661, (1961).

[5] **Pirt, S.J.** and **Righelato, R.C.,** Effect of growth rate on the synthesis of penicillin by *P. chrysogenum* in batch and chemostat culture, *Appl. Microbiol.* **15:** 1284-1290, (1967).

[6] **Lurie, L.M., Verkhotseva, T.P.** and **Levitov, M.M.,** Penicillin biosynthesis and two phase pattern of *Pencicillium chrosogenum* development, *Antibiotiki* **20,** 291-295, (1975).

[7] **Kuster, E.** and **Williams, S.T.,** Selection of media for isolation of streptomycetes, *Nature,* **202:** 928, (1964).

[8] *Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures, 9th* ed. Difco Laboratories Incorporated, Detroit, Michigan, (1977).

[9] **Painter, P.R.** and **Marr, A.G.,** Mathematics of microbial population, *Annu. Rev. Microbiol.* **22,** 519, (1968).

[10] **Doelle, H.W.,** *Bacterial Metabolism, 2nd* ed., Academic Press, New York, 738 p., (1975).

[11] **Grenhalgh, G.N.** and **Evans, L.V.,** Electron Microscopy. *In:* **Booth, C. (ed.),** *Methods in Microbiology,* Vol. **4,** Academic Press, London, (1971).

[12] **Buchanan, R.E.** and **Gibbons, N.E.,** *Bergey's Manual of Determinative Bacteriology, 8th* ed. Williams and Wilkins Co., Baltimore, pp. 599-881, (1974).

[13] **Waksman, S.A.,** *The actinomycetes,* Vol. III, Antibiotics of actinomycetes. The Williams and Wilkins Company, U.S.A., (1962).

[14] **Szabo, I.M.,** *Microbial communities in a forest Rendzina Ecosystem,* Akademia: Kiado-Budapest, (1974).

[15] **Shinobu, R.,** Physiological and cultural study for the identification of soil actinomycetes species, Mem. Osaka Univ. *B. Nat. Sci.* **7:** 1-76, (1958).

مـراحل النمو لبعض الاسـتربـتوميسس المنتجـة للمضادات الحيـويـة وتصنيـفها

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المستخلص . درست مراحل النمو لخمس مزارع من الاستربتوميسس المنتجة للمضادات الحيوية والمعزولة من المنطقة الغربية - المملكة العربية السعودية مع التركيز على جهدها في افراز المضادات الحيوية . ووجد أن هذه الكائنات تنمو أساسًا خلال الأيام الأربعة إلى السبعة الأولى من التحضين ، وقد لوحظ أعلى معدل نمو تخصص للسلالة ستربتوميسس إم. واي ١٨ وهو ٤٨ , ٠ /يوم (٤٤ , ١ يوم زمن تضاعف) . وقد أظهرت هذه السلالة أيضًا أعلى منطقة تضاد مع ميكروب ستافيلوكوكس أوريس إذا ماقورن بالعزلات الأربع الأخرى . وكان هذا التأثير المضاد أكثر وضوحا في الأيام الخمسة الأخيرة من التحضين (الطور الثابت من النمو) . وأوضحت الدراسات الظاهرية والمزرعية والفسيولوجية أن أنشط السلالات في إنتاج المضادات الحيوية تبعت النوعين ستربتوميسس أوروفيشنس ، ستربتوميسس روزيفيولاشيس .