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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THE ANTIMICROBIAL SUBSTANCES FROM *STREPTOMYCES VIRIDODIASTATICUS* (NRC1)

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ABSTRACT

Streptomyces viridodiastaticus isolated from Qalubiya soil of Egypt is capable to produce antibacterial and antifungal compounds. It showed the highest level of antimicrobial activity in the shaken culture broth after 7 days of incubation at neutral pH value using starch, potassium nitrate and dipotassium hydrogen phosphate amended medium. Two active fractions from the antimicrobial substance were extracted by diethyl ether at acidic pH and purified by Sephadex G-100 column chromatography. Elementary analysis indicated the absence of nitrogen from the two fractions. The empirical formulas for the two fractions (A) and (B) were $C_6H_{13}O$ and C_5H_7O while the molecular formulas were $C_{30}H_{65}O_5$ and $C_{20}H_{28}O_4$ respectively. The ultraviolet, infrared and mass spectra of the purified substance (A) indicated the presence of hydrogen bond, methyl group, diketones, aliphatic compound, alkenes and hydroxyl group. The purified substance (B) contained all the groups present in substance (A) except hydroxyl group.

Keywords: Antimicrobial substances, characterization, production, *Streptomyces.*

INTRODUCTION

The actinomycetes represent a large and heterogeneous group of microorganisms comprising several families and numerous species (Waksman, 1959 and Krassilinikov, 1970). Streptomycetes (Class Actinobacteria, Order *Actinomycetales*, Suborder *Streptomycineae*, Family *Streptomycetaceae*) are Gram-positive, filamentous soil bacteria that undergo morphological differentiation during their life cycle (Dworkin *et al*., 2006). They are considered to be one of the major groups of soil bacteria. *Streptomyces* produce a wide range of secondary metabolites, including antibiotics, many of which are of clinical importance in the treatment of infectious diseases or diseases caused by the proliferation of malignant cells (Pelczar *et al*., 1986; Innes and Allan, 2001). They are noteworthy as antibiotic producers making three quarter of all known products; the *Streptomyces* species are especially prolific and can produce many antibiotics and other classes of biologically active secondary metabolites (Waksman, 1959; Demain, 1999).

The evolution and spread of antibiotic-resistant pathogens remains a major clinical problem (Silver and Bostian, 1993). Although the discovery of new antimicrobial agents has become increasingly more difficult, the search for unique metabolites from microorganisms remains an attractive venture (Bull *et al*., 1992; Omura, 1992). The isolation of antibiotic from microorganisms is relatively easy as compared to chemical synthesis of antimicrobial agents (Ahmed, 2007). It could improve the discovery of novel antibiotics that act as better chemotherapeutic agent (Kulkorni and Anyicojri, 1995).

Antibiotic production is influenced by several physico-chemical factors including nutrient supply, oxygenation, temperature and pH (Gesheva *et al*., 2005).

The present study describes the production of anti-microbial substances by a local isolate *Streptomyces viridodiastaticus*. Improvement of antibiotic production was achieved by optimization of the cultural conditions. Moreover, isolation, purification and characterization of the antimicrobial substances were studied.

MATERIALS AND METHODS

Organism

Streptomyces viridodiastaticus (NRC1) was isolated from Qalubiya soil of Egypt (Rifaat *et al*., 2006-2007). The strain was identified according to the International *Streptomyces* Project Scheme (Shirling and Gottlieb, 1966) and diagnostic key of Szabo *et al*. (1975).

Media

Streptomyces viridodiastaticus (NRC1) was cultivated on the basal salt liquid medium of Waksman (1961), adjusted to pH 7.0 for the production of antimicrobial substance. The medium contained the following components (g/l): 20.0 starch, 2.0 potassium nitrate, 1.0 dipotassium hydrogen phosphate, 0.5 magnesium sulphate, 0.5 sodium chloride, 3.0 calcium carbonate and 0.01 ferrous sulphate. The fermentation was carried out in 250 ml triple-baffled Erlenmeyer flasks containing 50 ml of medium and

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incubated at 28ºC for 7 days on a rotary shaker at 200 rpm.

Antimicrobial activity

The antimicrobial spectrum of *Streptomyces viridodiastaticus* was determined against test organisms obtained from Faculty of Agriculture, Cairo University namely Gram +ve bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus* and *Bacillus subtilis*), a Gram – ve bacterium (*Escherichia coli*), yeasts (*Candida albicans*, *Saccharomyces cerevisiae*, *Candida pseudotropicalis*) and filamentous fungi (*Macrophomina phaseoli*, *Helminthosporium turcicum*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus terreus*, *Fusarium oxysporum* and *Botrytis allii*). The antimicrobial activity was determined by a conventional agar diffusion method (Wu, 1984) using nutrient, yeast-malt extract and Czapek's Dox agar for bacteria, yeasts and fungi respectively (Waksman, 1961). The diameter of inhibition zone was measured after incubation for one day for bacteria and two days for yeasts and fungi at 28ºC.

Optimization conditions for antimicrobial production

Carbohydrates (D-glucose, D-galactose, D-fructose, D-mannitol, L-arabinose, L-rhamnose, xylose, sucrose, maltose, lactose, mannose, starch and cellulose) were tested for their ability to support the production of the antimicrobial substance from *Streptomyces viridodiastaticus*.

The addition of various compounds of nitrogen sources was also studied. Potassium nitrate in the medium was replaced by various nitrogen sources with equimolar amounts such as sodium nitrate, ammonium nitrate, ammonium oxalate, ammonium sulphate, ammonium dihydrogen phosphate, diammonium hydrogen phosphate or triammonium phosphate.

The phosphate in the medium was also replaced by various phosphate sources with equimolar amounts. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium dihydrogen phosphate, diammonium hydrogen phosphate, triammonium phosphate, sodium dihydrogen phosphate, disodium hydrogen phosphate and trisodium phosphate were tested.

The *Streptomyces viridodiastaticus* (NRC1) was also incubated at different pH values from 5.0 to 9.0 to determine the optimum pH for highest antimicrobial production.

The time course of antibiotic production from 1 to 10 days was tested in shake flasks to determine the appropriate period of incubation for optimum antimicrobial production.

Extraction and purification of the antimicrobial substance

Three different methods were used to extract the antimicrobial substances namely, solvent-solvent extraction, adsorption and precipitation (Edwards, 1969). In solvent-solvent extraction method, the use of different solvents with different polarities was tested for their ability to extract the antimicrobial substance from the supernatant at different pH values 3, 7 and 9. Diethyl ether, ethyl acetate, butyl acetate, butanol, chloroform, petroleum ether and benzene were added to the concentrated broth (v/v) and shaken in separating funnel.

The adsorption of the antimicrobial substance from the supernatant of the culture broth was tested by shaking with alumina, silica gel or charcoal (2.5 % w/v).

Aqueous solution such as ethyl alcohol, methyl alcohol, acetone, calcium chloride and ammonium sulphate were tested for their ability to precipitate the antimicrobial substances from the supernatant at different pH values 3, 7 and 9.

In order to purify the antimicrobial substance, 20 litres culture broth was centrifuged to remove the cells. The supernatant was bioassayed by the agar diffusion method using test organism *Streptococcus pyogenes* and the active substance was extracted with equal volume of acidic diethyl ether three times. The diethyl ether layer was bioassayed. The extract was concentrated under vacuum and then performed on a Sephadex G-100 chromatographic column (25 x 400 mm) and eluted using 0.1-0.5 M NaCl. The high active fractions of the antimicrobial substances were collected and mixed with equal volume of acidic diethyl ether for further studies.

Physico-chemical properties of the antimicrobial substance

The retention factor (R_f) of the extracted antibiotic was calculated according to the method of Blinov and Khokhlov (1970). The solvent systems used were petroleum ether, benzene, chloroform, carbon tetrachloride, acetone, diethyl ether, ethyl acetate, butyl acetate, amyl alcohol, n-butanol, water saturated with n-butanol, n-butanol-acetic acid-water (2:1:1), n-butanol-pyridine-water (2:0.6:1) and 3% ammonium chloride in water.

Elemental analysis of carbon, hydrogen, nitrogen and oxygen as well as Infrared (I.R.), U.V. and mass spectra of the partially purified antibiotic were estimated using Vario Elementar, Fourier Transform 300 E infrared spectrophotometer, U.V./Vis/NIR 570v spectro-photometer and Finnigan Mat- SSQ 7000 respectively at the National Research Centre, Cairo, Egypt.

RESULTS AND DISCUSSION

Antimicrobial activity

Streptomycetes have been recognized as the potential producers of metabolites such as antibiotics. The antimicrobial substances produced by *Streptomyces viridodiastaticus* (NRC1) (Table 1) showed activity against Gram +ve bacteria (*Streptococcus pyogenes* and *Bacillus subtilis*) as well as fungi (*Aspergillus niger* and *Botrytis allii*). Bioxalomycins, which is a complex of a broad spectrum antibiotic, were isolated from fermentations of *Streptomyces viridodiastaticus*, exhibit excellent activity against Gram +ve bacteria and less active against Gram –ve bacteria (Singh *et al*., 1994). The obtained results suggest that *Streptomyces viridodiastaticus* (NRC1) could produce different antimicrobial substances.

Table 1. Antimicrobial spectrum of *Streptomyces viridodiastaticus*

Test organisms	Inhibition zone (mm)
Staphylococcus aureus	0
Streptococcus pyogenes	24
Bacillus cereus	0
Bacillus subtilis	20
Escherichia coli	0
Candida albicans	0
Saccharomyces cerevisiae	0
Candida pseudotropicalis	0
Macrophomina phaseoli	0
Helminthosporium turcicum	0
Aspergillus niger	28
Aspergillus flavus	0
Aspergillus terreus	0
Fusarium oxysporum	0
Botrytis allii	22

Factors affecting the production of antibiotic substance

Antibiotics production varies with the constituents of the media. The secondary metabolites accumulate only after the growth phase, i.e. when the culture attains a specific growth rate.

The antibiotic substance was detected on the second day of incubation and increased gradually to reach a maximum on the 7th day after which it decreased till the 10th day (Fig. 1). This observation was in agreement with Hassan *et al*. (2001**)** who found that antibiotic production by *Streptomyces violatus* in synthetic media reached the maximum on the 7th day. They mentioned that the highest yield of antibiotic production was obtained in the late exponential and the stationary phases.

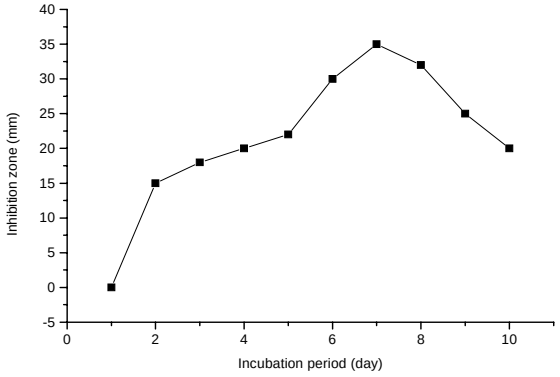

Fig. 1. Effect of incubation period on the production of the antimicrobial substances produced by *Streptomyces viridodiastaticus*.

The pH values of the culture showed a significant influence on the maximum productivity of antibiotic (Haque *et al*., 1995). The highest level of production of the antimicrobial substance produced by *Streptomyces viridodiastaticus* was detected with pH 7.0 (Fig. 2). Similarly, actinorhodin, a blue pigment antibiotic was produced in *Streptomyces coelicolar* culture at pH value around 7 (Bystrykh *et al*., 1996).

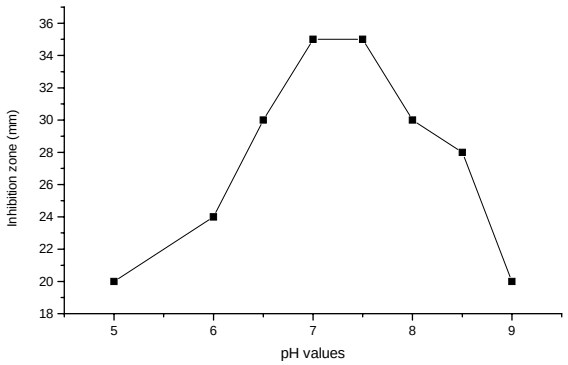

Fig. 2. Effect of different pH values on the production of the antimicrobial substance produced by *Streptomyces viridodiastaticus*

In an earlier study, the medium constituents and the process parameters were optimized by single factor optimization keeping the other factors constant (Elibol and Mavituna, 1998).

Starch supported the highest level of antibiotic production followed by L-arabinose and glucose (Fig. 3). Lower level of production was recorded with mannose, maltose, fructose, mannitol, lactose and sucrose. No production was detected with galactose, rhamnose, xylose and cellulose. Similarly, Sengupta and Paul (1992) detected a high level of antimicrobial substance produced by *Streptomyces* species on starch. The utilization of starch

by *Streptomyces viridodiastaticus* for production of antibiotic indicates the presence of an active uptake system for their substances.

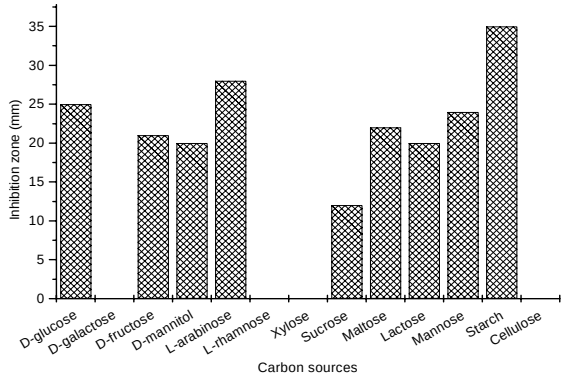

Fig. 3. Effect of different carbon sources on the production of antimicrobial substance produced by *Streptomyces viridiastaticus*

Potassium nitrate encouraged the production of antibiotic followed by sodium nitrate and ammonium sulphate (Fig. 4). Ammonium oxalate showed the lowest level of antibiotic production. The results revealed that the level of antibiotic production may be influenced by the type of nitrogen source supplied in the culture medium. Similar observations have been reported by Khaoua *et al.* (1991) and Mansour *et al*. (1996). It was noted by El-Tayeb *et al.* (2004) that potassium nitrate are superior to sodium nitrate for rifamycin production. For rapamycin, ammonium sulphate was the best nitrogen source (Lee *et al*., 1997).

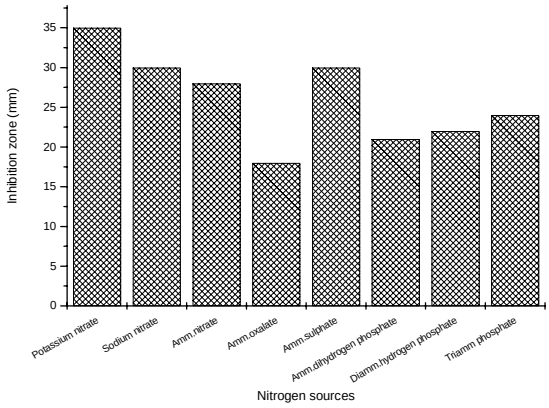

Fig. 4. Effect of different nitrogen sources on the production of antimicrobial substance produced by *Streptomyces viridiastaticus*

The level of production of the antimicrobial substance was greatly affected by the type of the used phosphate sources. In general, dibasic potassium, sodium or ammonium phosphates were more favourable than the mono- or tri-basic ones. Dipotassium hydrogen phosphate was the most favourable salt for antibiotic production (Fig. 5). These results are in agreement with those reported by other investigators (Harold, 1966 and Kishimoto *et al*., 1996). Phosphate was considered as a factor in the synthesis of a wide range of antibiotic (Martin and Demain, 1980).

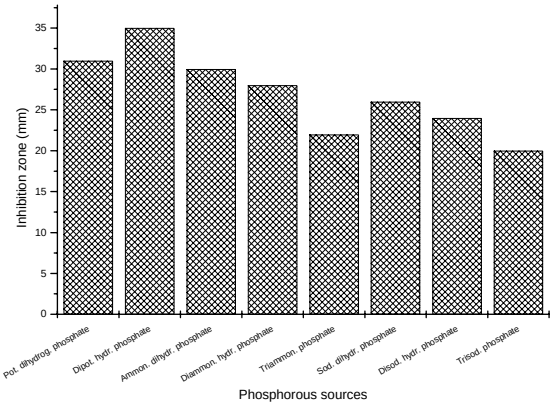

Fig. 5. Effect of different phosphorous sources on the production of antimicrobial substance produced by *Streptomyces viridiastaticus*

In this respect, Young *et al.* (1985) found that while nitrogen and phosphate salts were required for growth, they had negative effects on antibiotic synthesis while Farid *et al*. (2000) confirmed that only ammonium sulphate, sodium nitrate or beef extract were the suitable nitrogen sources in supporting natamycin production by *Streptomyces natalensis*.

Extraction of the antimicrobial substances

The antimicrobial substances produced by *Streptomyces viridodiastaticus* were best extracted by diethyl ether at acidic pH value (pH 3), followed by acidic ethyl acetate. Petroleum ether and benzene failed to extract the antimicrobial substance (Fig. 6). The same method of extraction for antimicrobial substances was performed by some workers (Hosokawa *et al*., 1996 and Kimura *et al*., 1997) however, most antimicrobial substances are extracted using ethyl acetate (Franco and Countinho, 1991).

The antimicrobial substance was completely adsorbed on alumina, silica gel and charcoal at various pH values except alkaline alumina (Table 2).

Results in Figure 7 indicate that the antimicrobial substance was highly precipitated by ethyl alcohol at acidic pH value, followed by acidic acetone and methanol. Calcium chloride and ammonium sulphate showed minor precipitate of the antimicrobial substance.

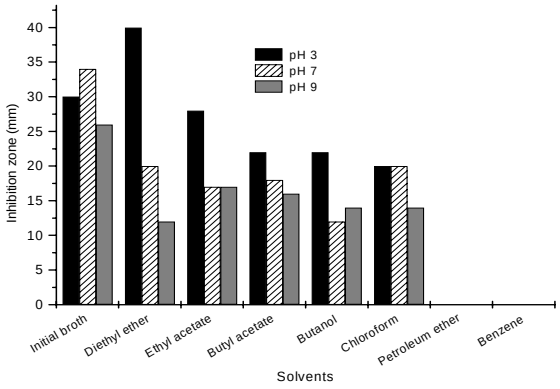

Fig. 6. Effect of different solvents on the extraction of antimicrobial substance produced by *Streptomyces viridodiastaticus* at different pH values

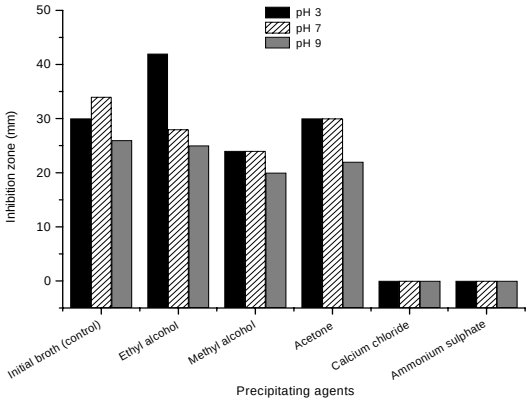

Fig. 7. Suitability to different precipitating agents at various pH values for the precipitation of the antimicrobial substance produced by *Streptomyces viridodiastaticus.*

Table 2. Suitability of alumina, silica gel and charcoal to adsorb antimicrobial substances produced by *Streptomyces viridodiastaticus* at various pH values

Adsorbent	Inhibition zone (mm)		
	pH 3	pH 7	pH 9
Initial broth	30	34	26
Alumina	00	00	20
Silica gel	00	00	00
Charcoal	00	00	00

Characterization of the antimicrobial substances

The antimicrobial substances showed high R_f values with n-butanol-pyridine-water, n-butanol-acetic acid-water, ethyl acetate, acetone, 3% ammonium chloride in water, diethyl ether and chloroform. The rest of solvents used showed lower R_f values (Fig. 8).

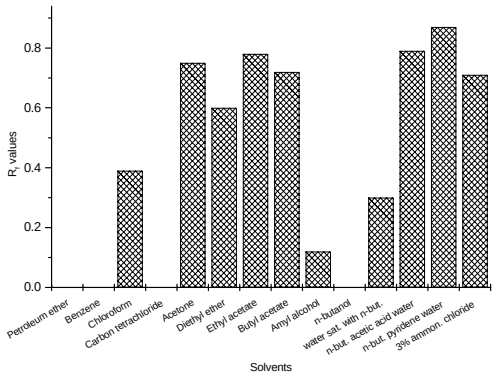

Fig. 8. R_f values of the antimicrobial substance produced by *Streptomyces viridodiastaticus* in different solvent systems

Structure of the antimicrobial substances

From Sephadex G-100 column separation, two fractions of the active antimicrobial substances were obtained (A and B). The substance (A) has yellowish brown colour, while substance (B) was colourless.

The elemental analysis of the two fractions reveals absence of nitrogen. The first fraction contained 72.76% carbon, 13.37% hydrogen and 13.87% oxygen. The second fraction contained 66.62% carbon, 7.81% hydrogen and 25.57% oxygen.

The I.R. spectrum of fraction A showed bands at 3432, 2925, 2855, 1719, 1589, 1459, 1266 and 1381 cm^{-1} which indicate the presence of hydrogen bond, methyl group, carbon hydrogen bond of methyl group, carbonyl group, diketones, aliphatic compound, alkene and hydroxyl group. However, fraction B showed bands at 3406, 2922, 2852, 1709, 1602, 1514, 1496 and 1456 cm^{-1} which indicate the presence of hydrogen bond, carbon hydrogen bond of methyl group, carbonyl group, diketones, aliphatic compound, alkene, and methyl group.

The U.V. absorption of the two fractions of the purified antimicrobial substances was detected at 278.5 and 280.5 nm. The mass spectra of fractions A and B showed M^+ ion at 460 and 402 m/z respectively.

The empirical formulas are $C_6H_{13}O$ and C_5H_7O and molecular formulas may be $C_{30}H_{65}O_5$ and $C_{20}H_{28}O_4$ for the antimicrobial substances A and B respectively.

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