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THE PRODUCTION OF ANTIMICROBIAL AGENTS BY WILD & RADIO ACTIVATED *ACTINOBACTER SP.*

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Abstract

The production of several volatile substances like Geosmin responsible of the characteristic wet earth odor distributed in terrestrial and aquatic ecosystems, especially in soil. Members of this group are produce clinically useful antitumor drugs such as Anthracyclines, Antimetabolites, Arzinophilin, Mitomycins and Vancomycins .

DNA cleavage by topoisomerase I or II inhibition , Mitochondria permeabilization inhibition and inhibiting tumor-induced angiogenesis .

Isolation , Purification of Actinobacter from different sources and Determination of biological activities (Antimicrobial / Anti-tumor) .

Optimization of production media (natural and synthetic) of the promising bioactive compounds (Antimicrobial / Anti-tumor).

Finally, Determination of the anti-tumor activity of the bioactive compound (s) of Actinobacter against different tumors.

Introduction

At the beginning of the infectious diseases were the leading cause of death worldwide. Tuberculosis, pneumonia, and diarrheal diseases caused 30% of deaths, due to the high rate of infant mortality from childhood infections (Cohen, M. L. 2010). As the progressed, deaths due to infectious diseases decreased dramatically as a result of a number of public health initiatives: better nutrition and housing, safer food and water, improved hygiene and sanitation, vaccination, and the discovery and widespread use of antibiotics. Life expectancy continued to rise in reaching a record high of 77.8 years in 2004, and chronic diseases like heart disease, cancer, and stroke, replaced infectious diseases as major killers (Minino, A. M.,2011). Society had distanced itself from the threats of smallpox, paralytic poliomyelitis, and tuberculosis that had impacted previous generations. Unfortunately, that success contributed, in part, to major pharmaceutical companies' reducing antibiotic development programs and redirecting resources toward diseases (Culotta, E. 2009.). Isolation and identification of antimicrobial compounds from cultures of antimicrobial producing bacteria is problematic because of the low antibiotic yields (Gastaldo, L.,2009). In addition, fermentation broths may contain many compounds that could interfere with isolation and purification. A current strategy for simplifying and speeding the isolation and purification of known antibiotics is the use of adsorbent resins (Tsueng, G., and K. S. Lam. 2012). Resins

offer two advantages in antibiotic discovery projects involving antibiotic-producing bacteria. (1) Adsorbent resins selectively bind antibiotics in fermentation broths and (2) inclusion of adsorbent resins during growth may relieve feedback repression of antibiotic production (Tsueng, G., and K. S. Lam. 2012). Adsorbent resins have also been shown to bind and thus recover a variety of antibiotics from fermentation broths, including kirromycin (Gastaldo, L.,2009), teicoplanin (Lee, J. C.,2011), and glendamycin (Casey, J. T.,2012), but because not all compounds in fermentation broths are bound by the resins (ideally only the active antibiotics) the level of extracellular, non-antibiotic cellular products is reduced. Adsorbent resins have also been employed to sequentially with one resin removing non-antibiotic fermentation metabolites and impurities and a second resin to selectively bind and isolate the active antibiotic (Ghosh, A. C.,2012). Consequently, purification protocols deal with fewer competitors. A key requirement of adsorbent resins is that once the antimicrobial has bound, it must be eluted from the resin and isolated. As studies of antibiotic production genes have identified linked regulatory gene sequences, it has been expected that the synthesis of the novel metabolites (e.g., antibiotics) would be subject to regulation within the cellular machinery. In many instances this would be expected to involve negative feedback by the antibiotic itself. Binding of the antibiotic to adsorbent resins would remove the antibiotic from the culture medium and thus relieve feedback repression resulting in increased production of antibiotic. It has been suggested that increased levels of production could be due to the binding of antibiotics by the adsorbent resins, thus overcoming feedback inhibition or repression (Gastaldo, L.,2009). Although adsorbent resins have been used to increase production and enhance isolation of known antibiotics, they have not been employed with unknown antibiotics produced by bacteria. Quite possibly, resins could relieve feedback repression and selectively bind antibiotics to simplify and speed antibiotic discovery, thereby overcoming the challenge of the low yield of antibiotics in any type of natural product, whether microbial cells or plants.

The objectives of this project were to: (1) measure the ability of resins to bind the structurally or chemically unidentified antimicrobial agents in cultures, (2) elute unknown antibiotics from resins and measure their activity, and (3) incorporate resins into growing cultures to increase production of antibiotics.

Materials & Methods

Identification of Resin that Bind Antimicrobial Compounds. Two approaches have been used for measuring the binding of antibiotic activity in cultures. Approach 1. For each resin, 10 mL of each cell-free culture filtrate was transferred to four (4) sterile 16 x 150 mm screw capped tubes and no resin added or 0.02, 0.05 or 0.10 gm resin added and the tubes were agitated on a rotator at 120 rpm at 30ºC overnight. The tubes were removed from the rotator, the resin allowed to settle, 1 mL of the supernatant collected without resin, and 1 ml filter-sterilized by passage through a 0.22 μm pore size Spin Filter. The MIC of each filtrate was measured.

Approach 2. For each resin, four 125 ml flasks containing 10 ml of ¼-strength Tryptic Soy Broth with 0.2 % sucrose (TSB+S), and either no resin, 0.02 gm, 0.05 gm, or 0.10 gm of a particular resin, were inoculated with a single colony of the antibiotic-producing strain. The cultures were incubated on a shaker (60 rpm) at 30°C for 7 days. The purity of every culture was checked by streak plate. Cell-free supernatants were prepared from 1 ml of culture using a 0.22 µm pore size spin-filter and 10 µl spotted on a lawn of every target for the antibiotic. The spot plates were incubated at 30°C for two days and examined for evidence of clearing (zones of inhibition) to confirm antibiotic production. Presence of clearing in the resin-free control coupled with the absence of a cleared zone was taken as evidence of resin binding of the unknown antibiotics. In addition, the lowest resin amount resulting in complete 121 removal of antibiotic activity was identified.

Culture conditions : The strains were inoculated (1 cm^2) in liquid medium (30 ml/250 ml Erlenmeyer flasks) containing per liter: 0.5 g $(NH_4)_2 HPO_4$, 0.8 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4.7 H_2O$, 0.055 g $CaCl_2.2 H_2O$, 4.0 mg $ZnSO_4.6 H_2O$, 0.2 g yeast extract and 1.0 ml thiamine (2.0 mg/mL). The carbon sources naphthalene and phenanthrene were added either together with the inoculum and incubated for 6 days or on the 3 rd day of cultivation and incubated for 10 days. The strains causing the greatest degradation of PAHs were selected, inoculated 3 x 1 cm^2 in the medium described above, and incubated for 3, 6, 9, 12 and 15 days. 300 µL of a 5.0 % (w/v) naphthalene solution in acetone or 3.0 mL of a 0.5% phenanthrene in acetone was used per flask, and added together with the inoculum. The final pH of the culture media was 5.0. Following inoculation all cultures were incubated under stationary conditions at 30ºC. To correct for the loss of PAHs, controls were prepared with uninoculated flasks. All experiments were run in duplicates.

During systematic screening for pigmented Actinomycetes, a reddishbrown pigmented strain was isolated from the hills . The sample was serially diluted and plated on starch casein agar (composition: soluble starch 1.0%, casein 0.03%, KNO3 0.2%, NaCl 0.2%, K2HPO4 0.005%, CaCO3 0.002%, FeSO4·7H2O 0.001%, and agar 2.0%). The sample was incubated at 30°C for 7 days to allow sporulation and pigmentation, subsequently preserved in 20% glycerol at -80°C.

Lyophilization and Extraction of Cultures : Culture material was frozen and the entire culture was freeze-dried. The resultant powder was extracted by stirring overnight in 146 either methanol or chloroform: methanol (1:1). The resultant slurry was filtered to remove solids, and the solvent was removed in vacuo.

16S rRNA sequence and phylogenetic analysis : Extraction of genomic DNA and amplification of the 16S rRNA gene from strain was carried out as described by (Lin *et al.* 2010). The amplified product was sequenced, and the sequence obtained was compared with similar sequences retrieved from the GenBank nucleotide sequences database. A constructed using the neighbor-joining method (Sahin, N. 2011), and the

topology of the phylogenetic tree was built by bootstrap analysis (Dharmaraj, S. 2011).

High performance liquid chromatography (HPLC) : All HPLC analyses were performed with a Zorbax ODS (0.46 x 15 cm) C_{18} reverse phase column (Chromatography Products). Separation was achieved by isocratic elution in acetonitrile : water (70:30), with a flow rate of 0.8 mL/min and UV absorbance detector set at 450 nm for naphthalene and phenantrene was detected at 450nm at a flow rate of 1.0 ml/min.

Analytical LC-MS of pure extract : Liquid chromatography and mass spectroscopy (LC-MS) analysis was performed on a high mass accuracy Agilent Time of flight (TOF) mass spectrometer, which was interfaced to an Agilent 1100 series HPLC system equipped with a diode array detector (DAD). Mobile phases were methanol-water (80:20) in channel A and dichloromethane in channel B. Chromatographic separation was performed with a Zorbax RP C8 4.6×150 mm column operated with a flow rate of 1 ml/min. The following gradient was used: 0% B for 0-2 min, 0%-50% B from 2 to 20 min. The analyte was ionized using a chemical ionization source (APCI) with the following settings: 35°C dry temperature, 30°C vaporizer temperature, 50 psi nebulizer pressure, and 5.0 L/min dry gas. Reference ions for calibration of mass axis into the mobile phase after the column separation. This enabled routine accurate mass determination with over 3 ppm accuracy. The organic phase, which was separated during pigment extraction, was dried over anhydrous sodium sulfate and was concentrated under reduced pressure to yield 6.2 g crude extract. The ethyl acetate extract was subjected to silica gel (60-120 mesh) column chromatography, and was eluted with a linear gradient of hexane and ethyl acetate to yield 4 fractions. Protease (Chavira, R., 2009), lipase (Lonon, M. K., 2010), and alkaline phosphatase activities (Von tigerstrom, R. G. 2011) were measured in untreated and boiled cell-free culture filtrates and in organic and aqueous fractions of chloroform-methanol (1:1) extracts of representative *Actinobacter* isolates.

The pure active compound was performed through UV-vis spectra, infrared (IR), and nuclear magnetic resonance (NMR) analyses. UV-analysis was carried out using a Shimadzu UV-vis scanning spectrophotometer (UV-2100 PC). Scanning was performed using a wavelength range between 200 and 700 nm. The sample was maintained in a vacuum desiccator over KOH pellets for 48 h, followed by IR-spectral analysis with 1 mg of the sample in a transform infrared spectroscope (FITR; FT/IR-420 Jasco). NMR spectroscopy was performed for the compound dissolved in deuterated dimethyl sulfoxide (DMSO), and the spectra were recorded on a Bruker Avance 600 MHz instrument fitted with an inverse triple-resonance CryoProbe (TCI).

Anticancer property: The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Lin *et al.*, 2009) was employed to evaluate the anti-tumor effect of the purified compound on HepG2 (hepatic carcinoma) and HeLa

(cervical human carcinoma) cells *in vitro*. The absorbance was read on an enzyme linked immunosorbent assay (ELISA) reader at 544 nm, and the growth-inhibitory ratio was calculated using the formula (1−A/B)×100%, where A and B correspond to the mean absorbance of the treated and control wells, respectively. 5-Fluorouracil (5-FU) was used as a reference compound for the positive control.

Results & Discussion

Antimicrobial assay: Antimicrobial profiles of the bioactive compound were tested in terms of the minimum inhibitory concentration (MIC) against various pathogens by using the disc diffusion method Vijayabharathi *et al.* 2011 .

Table 1. Cultural characteristics of strain.

Culture media	Growth Aerial mycelium		Substrate mycelium	Pigment production
Inorganic salts-starch agar (ISP4)	Good	Grey	Dark brown	Reddish brown
Glycerol-asparagin -agar (ISP5)	Poor	Dull	white	Yellowish brown
Tyrosine-agar (ISP7)	Poor	Dull	grey Brownish	grey Melanin
Maltose tryptone agar	Poor	Reddish	grey	Pale yellow
Nutrient agar	Poor	Pale grey	brown	Pale yellowish

The microbial communities did change over the sampling period with some microorganisms reducing in number to the point of appearing absent in the banding pattern and others becoming numerous enough to identify bands (Table 1).

The strain utilized most of the sugars that were provided, indicating a wide pattern of carbon assimilation. These results were in close agreement with the findings of (Williams *et al.* 2010). The tests used in this study are indispensable tools for the classification of *Actinobacteria* (Kampfer *et al*., 2011). The biochemical characterization revealed that the strain can hydrolyze starch, casein, and cellulose, but that it cannot hydrolyze lipids or gelatin. The strain utilizes arabinose, cellulose, dextrose, fructose, galactose, mellibiose, mannitol, raffinose, and rhamnose. The optimum pH and temperature for growth of the strain were 7.0-8.0 and 37°C, respectively. The strain was able to tolerate NaCl concentrations of up to 6%. It showed a negative result in the Indole production, Methyl Red, Voges Proskaeur, and Citrate Utilization (IMViC) test and did not produce hydrogen sulphide. The *Streptomyces aurantiacus* based on 16S rRNA gene sequences. The morphological and biochemical characteristics also reflected those of *Streptomyces* genera. *S. aurantiacus* has previously been reported to produce only pamamycin and aurantimycin D (Grafe *et al*., 2010; Schlegel *et al*., 2011). To make the production of antibiotics feasible, it is necessary to optimize the production conditions. Changes in the nature, type, and concentration of carbon and nitrogen sources or in the mineral element components of the culture medium greatly affect antibiotic

synthesis in *Streptomycetes*. The utilization of starch by the strain for growth and the production of bioactive metabolites suggests the presence of an active uptake system for starch as substrate as previously reported for other *Streptomyces* sp. Lower concentrations of mineral ions were previously shown to support antibiotic production. In order to identify suitable fermentation conditions for mycelia and to promote bioactive metabolite production on fermentative medium, it is necessary to determine the effects of fermentation duration on biomass and metabolite production. We have demonstrated that biomass production reaches a maximum after 6 days of incubation, and that mycelia production decreases beyond this fermentation period. This may be due to nutrient depletion during prolonged fermentation periods.

Purification of the compound was carried out in a silica column, and the compound was chemically characterized by H- NMR and C- NMR . These results are consistent with previous screenings of *Streptomycetes* isolates, which showed strong activity against Gram-positive bacteria (Saadoun *et al*.,2010) and low activity against Gram-negative test microorganisms (Saadoun, 2010 and Sahin, 2011). The reason for the discrepancy in sensitivity is based on the morphological differences between Gram-positive and Gram-negative microorganisms. Whilst Gram-negative bacteria have an outer lipopolysaccharide membrane that makes the cell wall impermeable to lipophilic solutes, Gram-positive bacteria are more susceptible as they have a more permeable outer peptidoglycan layer (Yilmaz *et al*., 2012).

There are reports of similar, single compounds possessing such activity, which are produced by microorganisms belonging to the *Streptomyces* genus (Saha *et al*., 2010). The GI50 values were found to be minimum HL (0.006 µg/ml) and HepG2 (0.005 µg/ml) cell line. There was no significant difference found between the two cell lines. Thus, our compound was found to be more potent in killing cervical carcinoma and liver carcinoma cells. Furthermore, the compound was found to have stronger inhibitory activity against cancer cells at the lowest concentrations.

Similarly, Goragina *et al*. (2010) reported cytotoxicity in HMO2 and HepG2 cell lines with GI50 values of 0.007 and 0.010 µg/ml of resistoflavin respectively purified from *Streptomyces* strain B 8005. Morphological and physiological characterization and on 16S rRNA sequence analysis. It also exhibits cytotoxic activity against human hepatic carcinoma and cervical carcinoma cell lines *in vitro*. Further studies focusing on the mechanisms of cytotoxicity in human cancer cell lines are in progress. This study highlights potential strategies for the development of new antitumor compounds with improved therapeutic properties, and the utility of combinatorial biosynthesis approaches.

Table 2. Suspension, replicate plating highlighted cells are examples of variability among replicate plating as each bold corresponds to the same substrate in each plating.

	1	2	3	4	5	6
A	-0.01	**-0.005**	-0.014	0	0.778	**1.485**
B	0.198	-0.012	0.008	**0.001**	0.008	**1.075**

C	1.569	-0.008	-0.012	0.029	**0.123**	**0.525**
D	-0.011	-0.011	-0.009	0.796	**0.020**	**1.102**
E	0.679	-0.007	-0.013	**1.005**	0.003	**2.885**

Differences in substrate utilization are not clustered in the lower in each set wells; rather the variation appears to be substrate-specific.

Table 3. Illustration of substrate variability highlighted cells are examples of variability among replicate plating as each bold corresponds to the same substrate in each plating.

	1	2	3	4	5	6
A	**2.085**	2.255	2.675	**0.101**	-0.001	0.045
B	**0.105**	0.520	0.435	**1.250**	-0.012	0
C	0.054	**2.280**	0.105	0.220	**0.490**	0.010
D	**2.040**	0.580	-0.015	**-0.005**	-0.004	-0.012
E	1.700	**2.090**	-0.015	0	**0.045**	0.013

Variability in substrate utilization in replicates of a homogenized sample. **Bold** squares represent a positive reaction (>0.3 absorbance readings at 450nm).

These interactions could be due to the limited community size that was used in this study or may be specific to the antimicrobial-producer chosen. An antimicrobial-producer with a different range of active agents could have a different affect on the community. Community interactions would appear to have a larger effect in this study than the antimicrobials being produced. This may have misrepresented this aspect as artificial communities are not as robust as natural communities (Balser, T. C., 2012).

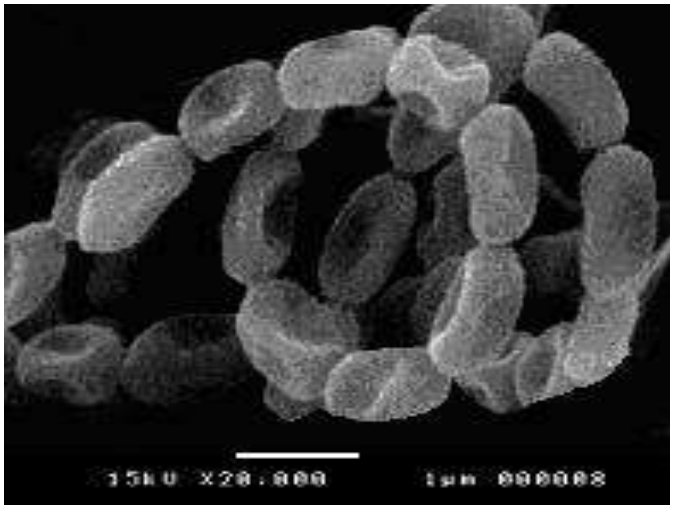

Fig. 1: Scanning electron micrographs of the *actinobacter sp*. isolate growing on starch nitrate agar medium, showing spore chain spiral and spore surfaces smooth (X20, 000) .

Table 4. Antimicrobial-producing isolates recovered from two different Soils and the relative activity of cell-free culture filtrates against a panel of microorganisms. MIC values for organic extracts of some of the isolates are provided in mg/ml.

Identification	Colony Morphology	*S. aureus*	*M. smegmatis*	*S. cerevisiae*	*E. coli*	*S. acnes*
Actinobacter sp.	Yellow	+	-	+	+	+
Actinobacter sp.	Brown	+	-	+	+	ND
Actinobacter sp.	Green	-	-	-	+	ND

a Antimicrobial activity expressed as clearing (+) or no clearing (-) on lawn of target microorganism spotted with 10 µl cell-free culture filtrate and as minimal inhibitory concentration (MIC) of organic extracts in mg/ml prepared as described in Experimental Procedures. b ND = Not Determined. C Isolated on copper agar. d Isolated from a zone of inhibition.

This is likely due to the proliferation of a spectrum of microorganisms producing a suite of antimicrobial compounds, their ability to kill would be expected to be magnified by the production of extracellular as in (Table 4).

The goal of this study was to ascertain if antimicrobial-producing bacteria have the ability to increase diversity in their microbial community, but the limited artificial community does not appear to have indicated that this is the case. A larger microbial community in the microcosm may help to resolve this issue but would also complicate the DNA based comparison due to the increase in band numbers For example, the absorbance (as a reflection of metabolism) of N-acetyl-D-glucosamine (wells E4 and E6) varied over a range in duplicate assays of the same suspension (Table 2) and (wells E2 and E5) as in Table 3.

Further work with larger microcosm communities as well as varied antimicrobial producers would need to be done in order to determine if these antimicrobial producers are helping to drive microbial community diversity through either a predatory or allelopathic interactions (Matsumoto, A., 2012) .

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الملخص العربى

انتاج المضادات الميكروبية بواسطة سلالة الاكتينوبكتر الطبيعية والمشعة .

ماجد سيد احمد و مدحت عبد الفتاح عبد المحسن و مصطفى محمد سلامه و حسين محمد القبانى.

قسم النبات - كلية العلوم- جامعة بنى سويف - و قسم البحوث الاشعاعية ، المركز القومى لبحوث وتكنولوجيا الاشعاع ، هيئة الطاقة الذرية .

الإنتاج من عدّة مواد متطايرة مثل جوسمين مسؤولة ومميّزة عن رائحة الارض المبلّلة و توزّيع أرضتها فى نظام بيئيّ مائيّ وخصوصا في التربة .

هذه المجموعة يمكنها إنتاج عقارات مفيدة مضادّة للأورام مثل (أنثراسيكلين) و مضادات ، (أريسرومايسين) ، (جانتاميسين) و (فانكوميسين) .

دنا تفلج بى توب ايزومريز إي أو إيي ذ، ميتوكندريا بوليميريزيشن و يمنع تيومر أنجوجبنسيس و تنتج الاكتينوباكتر من المضادات الحيوية الفعالة وهى تستغل كمنتج اساسى للمضادات الحيوية فى صناعة الادوية .

عمليّة عزل و تطهير أكتينوباكتر من مصادر مختلفة وتعيين أنشطة أحيائيّة أنتيميكروبيل / أنتي تيومر

و أخيرا , تعيين النشاط أنتي - ثيومر من ال كمبوند (س) بيوأكتيف أكتينوبكتر ضدّ أورام مختلفة .