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# First Report of Antibacterial Properties of a New Strain of *Streptomyces plicatus* (Strain 101) Against *Erwinia carotovora* subsp. *Carotovora* from Iran

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**Abstract:** This is the first report of antibacterial activity of Iranian actinomycetes isolates against *Erwinia carotovora* subsp. *carotovora* (Jones) Dye. *E. carotovora* subsp. *carotovora* is a soil born destructive plant pathogen with worldwide economical importance. Chemical measures have lost their attractiveness because of development of resistant strains of pathogens and due to undesirable effects on environment. Therefore, the studies on biological control of plant pathogens in soil have gained great importance worldwide. Actinomycetes, by virtue of their wide distribution, filamentous growth in soil, their ability to colonize the root surface and the rhizosphere, inhibitory effect on microorganisms and their ability to produce copious amounts of secondary metabolites especially antibiotics, role as a influential biological control agents have received special attentions. In order to obtain antibacterial antagonists from soil actinomycetes, a survey performed as follows. Soil samples taken from agricultural soils of Kerman. Serial dilutions provided and cultured in Casein Glycerin Agar (CGA). Over 110 isolates were screened among which one isolate showed high level of activity in Agar disk and Well diffusion methods against *E. carotovora* subsp. *carotovora* and identified as a new strain of *Streptomyces plicatus* (strain 101). This strain was propagated in submerged cultures and active crude was prepared upon which some biological characterization performed. High concentration of antibacterial agent was detected in 10th to 11th day in shake cultures. The active substance was water-soluble and insoluble in chloroform, hexan and dichloromethane. Minimal Inhibitory Concentration (MIC), by solving the crude extract in the dimethylsulfoxide plus methanol (v/v, 1:1) was determined as 5 mg $mL^{-1}$. Longevity *in vitro* (LIV) of active crude of *S. plicatus* strain 101 against *E. carotovora* subsp. *carotovora* in soluble state determined about 30 days at room temperature. In thermal inactivation point studies, active crude retained activity up to 135°C. Antibacterial genes from this strain may be proper candidate for genetic engineering of plants for increased tolerance against the tested cosmopolitan bacterial pathogen.

**Key words:** Antibacterial, *Streptomyces plicatus*, *Erwinia carotovora* subsp. *carotovora,* biocontrol, biological control, actinomycetes

## INTRODUCTION

Saprophytic Gram-positive bacteria in the genus *Streptomyces* are the most widely studied and well known genus of the Actinomycetals. Streptomycetes usually inhabit soil and are important decomposers. They are able to metabolize many different compounds including sugars, alcohols, amino acids and aromatic compounds by producing extracellular hydrolytic enzymes. Their metabolic diversity is due to their extremely large genome which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs[1]. Streptomycetes are also of medical and industrial importance because they synthesize antibiotics. There are several theories which may explain antibiotic production; the most widely accepted one being that antimicrobial compounds help the organism compete with other organisms in the relatively nutrient-depleted environment of the soil by reducing competition. Over 50 main antibiotics have been isolated from Streptomycetes species, including Streptomycin, Neomycin, Chloramphenicol and Tetracyclines[2].

In modern agriculture, pesticide application is still an invaluable and effective method to control plant diseases. However, since use of agrochemicals is falling into disfavor because of environmental pollution and detrimental effects on a variety of nontarget organisms, potential use of microbes based biocontrol agents as

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replacement or supplements for agrochemicals has been addressed in many recent reports[3]. With the increased concern about conserving natural resources as air, soil and water, natural or biological control of plant diseases has received increased emphasis. Biological control of plant diseases is slow, gives few quick profits, but can be long lasting, inexpensive and harmless to life. Biocontrol systems do not eliminate neither pathogen nor disease but bring them into natural balance[4]. In search for biocontrol agents, several bacteria as *Pseudomonas flourescens, Bacillus subtilis* and *Erwinia herbicola* Eh252 have shown activity against *Erwinia carotovora* subsp. *carotovora*[5-7].

*Streptomyces* spp. have been shown to have characteristics which make them useful as biocontrol agents against soil-borne bacterial plant pathogens. These characteristics include the production of different kinds of secondary metabolites and biologically active substances of high commercial value such as enzymes and antibiotics and they are of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conductive to crop production[8-11]. Several workers have reported that *in vitro* studies have documented satisfactory results in use of *Streptomyces* against some root pathogens. For example, *Streptomyces* sp. strain 5406 has been used in China for the last 35 years to protect cotton crops against soil-borne pathogens. The results even show that use of *Streptomyces* enhances growth of the crops and vegetables[12,13].

With extended environmental diversity, however, the actinomycetes microflora of the Iranian soils has not been well explored with the goal of exploring new means of biocontrols. With the respect to their role in biological control of soil-borne bacterial-pathogens, at the present research 110 isolates of actinomycetes were isolated from agricultural soils of Kerman province, Iran and screened against worldwide destructive soil born plant pathogen, *E. carotovora* subsp. *carotovora*. However, there is no published reports on *Streptomyces* use as biocontrol agent against *E. carotovora* subsp. *carotovora* and so this research is being the first report on the issue.

## MATERIALS AND METHODS

**Culture media:** A synthetic medium, Casein Glycerin (or starch) Agar (CGA) was used for screening and isolating of actinomycetes which composed of: glycerol or soluble starch, 10 g; casein, 0.3 g; $KNO_3$, 2 g; NaCl, 2 g; $K_2HPO_4$, 2 g; $MgSO_4.7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g and agar, 18 g in 1 L of distilled $H_2O$ (pH 7.2)[4]. In submerged cultures, agar was excluded (CG medium). Actinomycetes colonies with different morphologies were selected and transferred to CGA slants under refrigeration for further studies[14,15].

**Preparation of bacterium:** *E. carotovora* subsp. *carotovora* was kind gift of Dr. Rahimian, Department of Plant Pathology, College of Agricultural Sciences, University of Sari, Iran. This bacterium was rejuvenated on nutrient agar (NA) (Difco) at 27-29°C. Stock cultures stored at 4°C and subcultured as needed.

**Isolation of actinomycetes from soil:** Soil samples were collected from grasslands, orchards and vegetable fields in different localities of Kerman province, Iran. Several samples were selected randomly from mentioned localities using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang[16]. Soil samples were taken from a depth of 10-20 cm below the soil surface. The soil of the top region (10 cm from the surface) was excluded. Samples were air-dried at room temperature for 7-10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature before use. Ten grams samples of air-dried soil were mixed with 100 mL sterile distilled water. The mixtures were shaken vigorously for 1 h and then allowed to settle for 1 h. One milliliter portions of soil suspensions (diluted $10^{-1}$) were transferred to 9 mL of sterile distilled water and subsequently diluted to $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$. Inocula consisted of adding aliquots of $10^{-3}$ to $10^{-6}$ soil dilutions to autoclaved CGA (1 $mL^{-25}$ mL CGA) at 50°C before pouring the plates and solidification. Three replicates were considered for each dilution. Plates were incubated at 30°C for up to 20 days. From day 7 on, actinomycetes colonies were isolated on CGA, incubated at 28°C for one week and stored refrigerated as pure cultures before use. For screening studies 110 pure actinomycetes isolates were collected.

**Screening procedures and *in vitro* antibacterial bioassays**

**Agar disk-method:** Each actinomycetes isolate was smeared on CGA medium as a single streak and after incubation at 28°C for 4-6 days, from well-grown streaks 6 mm agar disks of actinomycetes colony mass was prepared by using sterile cork borers. Disks were then aseptically transferred to NA plates having fresh lawn cultures of bacterial isolates. Controls included using plain disks from CGA medium. Plates were incubated at 29°C for 24 h and bioactivity was evaluated by measuring the diameter of inhibition zones (DIZ, mm)[4,17].

**Well diffusion-method:** For evaluation of antibacterial activity of aqueous samples, by use of sterile cork borer, wells (6x4 mm, 2 cm apart) were punctured in fresh bacterial lawn-cultures. Respective concentrations in dimethylsulfoxide: methanol (1/1: v/v) solvent (DM solvent) were then administered to fullness in each well. Plates were incubated at 29°C for 24 h. Bioactivity was determined by measuring inhibitory zones (mm). Each experiment was repeated three times and the mean of inhibitory zones recorded. Controls included use of blank wells and use of DM solvent without test compounds[4].

**Preparation of crude extract from submerged cultures:** Active strain was grown in submerged cultures of CG medium on rotary shakers under 130 rpm at 30°C. To monitor the activity, aseptically small aliquots of culture media were taken every 24 h for 37 days and the activity was evaluated by well diffusion-method[4,17]. To prepare crude extracts, 10th or 11th day of post inoculation which the activity reached maximum, the cultures were harvested; spores and mycelia were excluded by filtration through two layers of cheese cloth. The clarified sap was then dried to dark crude under reduced air at 50°C and kept refrigerated before use.

**Classification of *Streptomyces* strain 101:** Streptomyces colonies were characterized morphologically and physiologically following the direction mentioned in the methods manual of international cooperative project for description and deposition of cultures of *Streptomyces* (ISP)[18].

**Morphological characterization:** Streptomyces colonies on glycerol-nitrate-casein agar were transferred onto oatmeal agar and streaked across the plate and incubated in the dark at 27°C for 21 days.

**Color determination:** This made for: a) Mass color or mature, sporulating aerial surface growth, b) The color of substrate mycelium as viewed from the reverse side and c) Diffusible soluble pigments other than melanin. Observation was made after 21 days and was limited to mature cultures with heavy spore mass surface using code for determining the color of aerial mycelium of Streptomycetes composed by Prauser[19] for color tabs of Baumann Farbtonkarte Atlas I.

**Determination of morphological characteristics of the sporebearing hyphae:** The spore-bearing hyphae characteristics were determined by direct microscopic examination of the culture surface (21 days old) on opened dishes of the crosshatched cultures using 100 x magnification. The species involved in the genus *Streptomyces* divide into sections: Rectus (R) or straight, flexible (F) or flexeous, Retinaculum-Apertum (RA) and spiral (S)[21].

**Spore morphology surface:** The spore surface of the isolate was examined under scanning electron microscope at a magnification of 8000 to 100000 x. Preparation for the scanning electron microscope consisted of scratching the culture of 14-21 day old growing on oatmeal agar by a sterile needle and depositing the specimen onto specimen aluminum stubs which held by a piece of double stick scotch tape. The stubs were placed in a sputter coater (Polaron Equipment Ltd. E 5000) for 2-3 min. (approximately 150 A° of gold deposited). The gold sputterer was set at 1-4 kv, 20 mA and 0.1 torr. After coating, the specimens were viewed with a Lutz 100 A scanning electron microscope with an accelerating voltage of 20 kv. Secondary electron images were recorded with black and white film.

**Melanin production:** Peptone iron agar was used for the detection of deep brown to black diffusible pigment (+). Absence of the color was recorded as negative (-).

**Carbon utilization:** The following sugars were tested, L-arabinose, D-xylose, *meso*-inositol, D-mannitol, D-fructose, rhamnose, raffinose and sucrose. Preparation was done as described in the ISP[18].

Characterization of *Streptomyces* strain 101 to species level was based on morphological, cultural and physiological characteristics following the directions given for the International *Streptomyces* Project (ISP)[20]. General morphology was determined on oatmeal agar plates, incubated in the dark at 27°C for 21 days, by direct light microscopy examination of the surface of crosshatched cultures. Colors were determined according to the scale adopted by Prauser[21] and melanin reactions were detected by growing the isolate on at least one of the ISP media (Nos. 6 and 7)[18,21]. Strain 101 was identified as a new strain of *Streptomyces plicatus*.

**Solubility studies of active crude in organic solvents:** To evaluate the relative polarity of the active principle (s) present in the crude, 2 mL of each of $H_2O$, methanol, DMSO: Methanol (1:1, v/v), chloroform, dichloromethane and hexane were added to 20 mg pulverized-crude samples separately and vortexed for 20 min. Each sample was then centrifuged at 3000 rpm for 15 min using a bench low speed centrifuge. Supernatants and pellets were separated, dried under reduced air at 50°C and assayed at concentration of 10 mg $mL^{-1}$ by agar diffusion-method[22].

**Determination of Minimum Inhibitory Concentrations (MIC):** To measure the MIC values, two-fold serial dilutions of 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 mg $mL^{-1}$ of the crude extract were prepared in DM solvent and assayed by well diffusion-method as described by Shahidi Bonjar[23,24]. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. All data represent average of three replicated experiments.

**Determination of shelf life or stability of active crude:** To measure the stability of the active crude in soluble state, 5 mg $mL^{-1}$ samples were prepared in distilled water and placed in small vials. These samples were kept at room temperature and tested using agar diffusion-method for anti *E. carotovora* subsp. *carotovora* activity at 14 days intervals as long as the activity persisted.

## RESULTS

**Screening and bioassays:** In screening for actinomycetes having antibacterial activity, over 110 isolates were screened from which one isolate showed activity against *E. carotovora* subsp. *carotovora*. Colony morphology of *Streptomyces plicatus* strain 101 at 40 x magnification is shown in Fig. 1. Spore chain of this strain is shown in Fig. 2. The bioassay results against *E. carotovora* subsp. *carotovora* are indicated in Fig. 3.

Table 1: Morphological and physiological characterization of *Streptomyces plicatus* strain 101

| Morphological criteria | | Physiological criteria | |
|---|---|---|---|
| Spore chain | S | Reverse side pigment | 0 |
| Sporulation of aerial mycelia | LC | Soluble pigment | 0 |
| Aerial mass color | Gy | Arabinose | - |
| Fragmentation of substrate mycelia | 0 | Xylose | + |
| Spore surface | Sm | Inositol | + |
| Sclerotia formation | 0 | Mannitol | + |
| Melanoid pigment | 0 | Fructose | + |
| | | Rhamnose | + |
| | | Sucrose | - |
| | | Raffinose | - |

S: Spiral, LC: Long chain, Gy: Grey, 0: Negative, Sm: Smooth, -: No utilization, +: Utilization

Table 2: Bioassay results of solubility tests of the antibacterial principle (s) of *Streptomyces plicatus* strain 101 against *Erwinia carotovora* subsp. *carotovora* in fractions of different solvents indicated by well diffusion-method at 10 mg $mL^{-1}$ of dry crude

| Solvent | Fraction | Activity |
|---|---|---|
| $H_2O$ | S* | + |
| | P* | - |
| Methanol | S | + |
| | P | - |
| DMSO: Methanol (1:1, v/v) | S | + |
| | P | - |
| Chloroform | S | - |
| | P | + |
| Dichloromethane | S | - |
| | P | + |
| Hexane | S | - |
| | P | + |

S*: supernatant, P*: pellet

**Taxonomy of actinomycetes:** The active strain was identified as *Streptomyces plicatus* (strain 101). Based on the literature reviews; it is concluded that it is a new record from Iran. Table 1 shows the complete characterization criteria for this isolate based on morphological and biochemical properties. Scanning electron micrograph of spore chains of strain 101 is indicated in Fig. 4.

**Determination of MIC:** In well diffusion-method, MIC of the crude was determined as 5 mg $mL^{-1}$ against *E. carotovora* subsp. *carotovora*.

**Solubility of active crude in organic solvents:** Solubility results are indicated in Table 2. As the results show, apparently the active principle (s) has a polar nature since activity is recoverable only in $H_2O$, methanol supernatants and pellets of chloroform, dichloromethane and hexane treatments.

**Shelf life or stability of active crude:** Stability of the active crude in distilled water at room temperature (12-30°C) was about 30 days, assayed by using agar diffusion-method against *E. carotovora* subsp. *carotovora*.

## DISCUSSION

Actinomycetes produce more than half of the world's antimicrobials and are consequently becoming valuable tools in the field of biological control. Antibacterial activity of the isolate found in this study highlights its importance as candidate for further investigation in biological control of tested pathogen.

In ideal biological control measures, proper microorganisms are those having well adaptation in soil and rhizosphere exerting effective antagonistic activity against soil pathogens persistently. In this regard soil-driven actinomycetes do not have adverse effect or alter the biological buffering of soils as chemical measures do. They should receive higher attention in research for biological controls worldwide. In this regard, soil-driven *Streptomyces* do not have adverse effect or alter the biological buffering of soils; so one possible approach to biological control of *E. carotovora* subsp. *carotovora* is to inoculate soil with selected antagonists[25].

*E. carotovora* subsp. *carotovora* is an important problem in world agriculture. Genetic engineering provides an opportunity to protect plants from bacterial

**Fig. 1: Colony morphology of *Streptomyces plicatus* strain 101 under binocular microscope at 40 x magnification**

**Fig. 2: Spore chain morphology of *Streptomyces plicatus* strain 101 under light microscope at 400 x magnification**

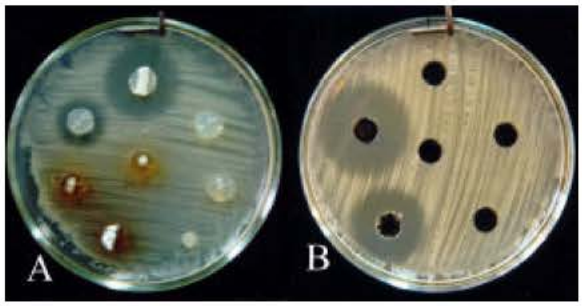


**Fig. 3: Bioassay results of *Streptomyces plicatus* strain 101 against *Erwinia carotovora* subsp. *carotovora* measured in two methods; A) Agar disk-method, Clockwise from top: *S. plicatus* strain 101 and 7 other tested Actinomycetes isolates. B) Well Diffusion-Method, Clockwise from top: Blank 1 (control), DMSO: Methanol solvent (control), Growth medium (control), *S. plicatus* strain 101 crude extract at 10 mg $mL^{-1}$, *S. plicatus* strain 101 crude extract at 20 mg $mL^{-1}$ and middle: Blank 2 (control)**

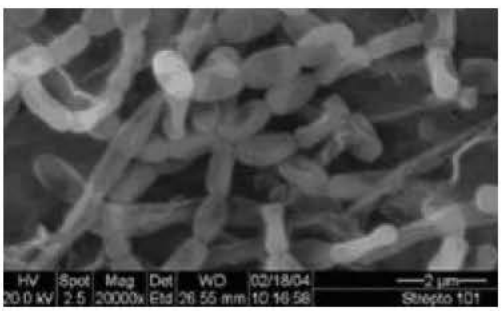


**Fig. 4: Scanning electron micrograph of spore chains of *Streptomyces plicatus* strain 101**

diseases and to reduce the use of synthetic bactericides. The genes for antibacterial metabolites can be engineered into plants to increase the resistance of crop plants to bacterial attack, decreasing the use of environmentally unfriendly chemicals. The major factor limiting the application of this technology is the identification and isolation of useful genes that code for antibacterial metabolites.

*Streptomyces plicatus* strain 101 is a proper candidate for genetic engineering of agriculturally important crop plants for increased tolerance against *E. carotovora* subsp. *carotovora*. Having special environmental characteristics and being rich in actinomycetes population, the microbiology of the Iranian soils has to be further explored for new active isolates of actinomycetes.

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