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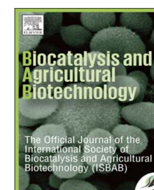

Production, characterization and optimization of actinomycin D from Streptomyces hydrogenans IB310, a(n antagonistic bacterium against phytopathogens

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ABSTRACT

Disease in agriculture field is a major issue from food security point of view. In each year different types of diseases (mainly bacterial, fungal and viral) results a huge loss in agricultural production. A wide range of chemical agents are available in the market to reduce the degree of infection in crop plants. However, these are not safe for health and as well as for environment, thus, the demand of bio-control agents are increasing rapidly. In this present investigation, we have demonstrated the antagonictic activity of an agricultural soil bacterium *Streptomyces hydrogenans* IB310 against both bacterial and fungal phytopathogens (*Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Xanthomonas campestris*, *Botrytis allii, Fusarium oxysporum* and *Ustilago maydis*). The active antimicrobial compound produced by the bacterium *S. hydrogenans* IB310 was purified, characterized and identified as actinomycin D. Furthermore, the parameters (glycerol, oat meal and tween 80) responsible for the production of actinomycin D were optimized through Response Surface Methodology (RSM). Highest yield (18.912 mg/L of actinomycin D) was obtained at glycerol 1.949 g/L, oat meal 2.676 g/L and tween 80 0.524 g/L. Due to antagonistic nature against the bacterial and fungal phytopathogens, the strain *S. hydrogenans* IB310 might be useful as a bio-controlling agent in agricultural field. To the author's best knowledge, it is the first report of actinomycin D production by *S. hydrogenans*.

1. Introduction

The status of global food security, i.e., the balance between the growing food demand of the world population and global agricultural output, is alarming (United Nations, Department of Economic and Social Affairs, 2011; Savary et al., 2012). In a report, Zadoks (2008) has stated that crop losses caused by plant disease not only directly affect the quantity, but also affect the quality directly or indirectly through the fabrics of trade, policies and societies. The scientific management or the protection of plant against diseases, has an obvious role to play in meeting the growing demand for food quality and quantity (Strange and Scott, 2005). Furthermore, Savary et al. (2012) reported that disease in agricultural field cause a huge losses (20% and 40%) and directly affect the consumers, public health, societies, environments and country economy. In last few years, a wide range of bacterial and fungal candidates have been identified as potential pathogens in several crop plants (Mansfield et al., 2012; Göhre et al., 2013). Hence, it is very important to address the issue related to plant diseases infestation by harmful bacterial (*Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Xanthomonas campestris*) and fungal (*Botrytis allii*, *Fusarium oxysporum*, *Ustilago maydis*) phytopathogens (Dean et al., 2012; Mansfield et al., 2012).

The use of chemicals in agricultural field is very popular to control the bacterial and fungal phytopathogens, and to maximize the yield (Yuliar et al., 2015). However, from toxicity point of view, these chemicals are not good for health and environment. Thus, the use of such hazardous chemicals should be restricted in agricultural fields. Biological control offers better alternative to synthetic chemicals, as they are target specific, easily biodegradable, having less shelf life and user friendly (Sayyed and Patel, 2011; Kumar and Singh, 2015). The disease management in agricultural field using bio-control agents is an important part of integrated crop management (ICM) system (Sayyed and Patel, 2011; Chattopadhyay et al., 2014). Among the 20 genera of bacteria, *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. are widely used as bio-control agents (Islam et al., 2012). Among them, *Streptomyces* spp. are well known for producing divers array of

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secondary metabolite, and is therefore selected for the present investigation (Palazzini et al., 2017). Until now, several biocontrol agents have been reported against diverse group of bacterial and fungal phytopathogens (Wattana-Amorn et al., 2016; Palazzini et al., 2017). However, the information regarding a single biocontrol candidate against both bacterial and fungal phytopathogens are still lacking. Therefore, the aim of our present investigation was (1) isolation and characterization of *Streptomyces* strains, (2) evaluation of their antagonistic activity against bacterial and fungal phytopathogens, (3) purification and characterization of active compound from one of the potent selected isolate, and (4) statistical optimization of culture parameters for the enhanced production of active compound.

2. Materials and methods

2.1. Sample collection

Soil samples were collected from agricultural field of anjeer orchard of Purandar area of Pune District, Maharastra State of India (adjacent agricultural land of Purandar fort 19° 12' N /73° 51' E). The collected samples were stored into a sterile glass screw cap bottle and sealed tightly.

2.2. Bacterial and fungal phytopathogens

Three bacterial (*Agrobacterium tumefaciens* MTCC No. 609, *Pseudomonas syringae* MTCC No. 1604 and *Xanthomonas campestris* NCIM No. 2956) and three fungal (*Botrytis allii* MTCC No. 154, *Fusarium oxysporum* MTCC No. 284 and *Ustilago maydis* MTCC No. 1474) phytopathogens have obtained from Microbial Type Culture Collection, Chandigrah, India.

2.3. Isolation and pure culture of Actinobacteria

In order to isolate the *Actinobacteria*, 1 g of soil sample was suspended in 10 mL of distilled water, mixed thoroughly and heated at 55 °C for 5 min. Serial dilution was done by the 10- fold dilution method. An aliquot of 0.1 mL from each dilution was poured on the starch casein agar medium [containing Cycloheximide (5 g/mL) and Rifampicin (g/ mL)] and incubated at 27 °C for 2–3 weeks. The colonies obtained were picked up and pure culture was done on International *Streptomyces* Project (ISP2) media (Shirling and Gottlieb, 1966) by repeated streaking method. The cultural characteristics of the pure cultured isolates were done on ISP 2 media. The colors of substrate, aerial mycelium and soluble pigments were determined using the NBS/IBCC color chart (Mundie, 1995).

2.4. Screening for antimicrobial activity

In order to check the antimicrobial activity, each isolate was grown (200 rpm, 28 °C) in 10 mL of 301 seed medium (2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% CaCO3, pH 7.0). The 5-day-cultured broth was extracted with 10 mL of ethyl acetate in the ratio of 3:1 by shaking (200 rpm) for 30 min. In order to separate the organic phase from the aqueous phase, the organic phase was collected and concentrated in rotary vacuum evaporator. The crude extract was then dried in desiccators for few days. The dried crude extract was then examined for antimicrobial activity against bacterial phytopathogens (viz., *A. tumefaciens* MTCC No. 609, *P. syringae* MTCC No. 1604 and *X. campestris* NCIM No. 2956), and fungal phytopathogens (viz., *B. allii* MTCC No. 154, *F. oxysporum* MTCC No. 284 and *U. maydis* MTCC No. 1474) by cup plate method (Shetty et al., 2014). Nutrient agar (NA) medium and potato dextrose agar (PDA) were used for bacterial phytopathogens and fungal phytopathogens, respectively. The zone of inhibition was measured by using calibrated scale. The isolate exhibited highest activity against these phytopathogens was selected for further studies.

2.5. Identification of the most potent strain

Genomic DNA of the selected isolate was prepared following the method of Kutchma et al. (1998). The 16 S rDNA gene was amplified using the primers described by Takahashi et al. (2002). The quality of the PCR product was checked by agarose gel electrophoresis. The PCR product was sequenced on a DNA sequencer (Applied Biosystems 3130 Genetic Analyzer) using a BigDye Terminator v3.1 cycle Sequencing kit (Applied Biosystems), according to the manufacturer's instructions. The obtained sequence was then submitted to GenBank and compared with all sequences available in GenBank using the BLAST program. The clustalw program was used for multiple alignments (Kimura, 1980). The phylogenetic tree was constructed using the neighbor-joining method using the Mega 6.0 programme (Tamura et al., 2007). Bootstrap values (10,000 replicates) were calculated to validate the reproducibility of the branching pattern (Felsenstein, 1985).

2.6. Purification and identification of antimicrobial compound

The broth media was extracted with 10 mL of ethyl acetate in the ratio of 3:1 by shaking (200 rpm) for 30 min. The ethyl acetate fraction was collected and concentrated in rotary vacuum evaporator at 45 °C until a reddish-yellow solid mass was obtained. The dried crude extract was dissolved in DMSO and subjected to silica gel column chromatography as described by Shetty et al. (2014). The crude extract is adsorbed on to stationary phase of 100: 200 silica gel mesh size. Mobile phase was a mixture of dichloromethane and ethyl acetate (20:80). The fraction showing highest zone of inhibition was taken and further subjected to high performance liquid chromatography (HPLC, Shimadzu, Japan). The major single pick was selected and identified using UV visible spectroscopy (UV- 1700, Shimadzu, Japan) and Reverse phase HPLC (Shimadzu, Japan) equipped with C18 column. Retention time was noted and compared with pure reference compound (Sigma-Aldrich, USA). The molecular weight was determined by SDS gel electrophoresis and compared to the pure compound obtained from Sigma-Aldrich, USA.

2.7. Optimization of actinomycin D production by response surface methodology

The medium for maximum actinomycin D production has been first optimized by an ‘one-variable-at-a-time’ approach (Vohra and Satyanarayana, 2001). The medium composition that resulted in the highest actinomycin D titre was considered as the basal medium. The optimization was done by response surface methodology (RSM) using Central Composite Design (CCD). The levels of three independent variables, viz. glycerol (A), oat meal (B) and tween 80 (C) chosen for this study were optimized by the experimental plan. The statistical software package ‘DESIGN-EXPERT® 10.0.3′, StatEase, Inc., Minneapolis, USA was used to analyze the experimental design. Each factor in the design was studied at five different levels (−α, −1, 0, +1 and +α) (Table 1). A set of 20 experiments were carried out. All variables were taken at a central coded value considered as zero. The

Table 1
Coding and assigned concentration of variables (medium components) of different levels of the central composite design.

Variable Code	Variable name (Medium components)	Unit	Variable range	Coded level				
				-α	−1	0	+1	+α
A	Glycerol	g/ L	0.5–2.5	0.5	1.0	1.5	2.0	2.5
B	Oat meal	g/ L	1.0–5.0	1.0	2.0	3.0	4.0	5.0
C	Tween 80	g/ L	0.2–1.8	0.2	0.6	1.0	1.4	1.8

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