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Screening of earthworm cast actinobacteria for plant growth promoting properties and production of indole acetic acid (IAA) from selected actinobacterial isolates

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ABSTRACT

Actinobacteria are the diverse group of bacteria which are widely distributed in various natural and man-made environments. This present study is attempted for the isolation of actinobacteria from earth worm cast and the production of indole acetic acid (IAA) from selected isolates. Totally 10 actinobacterial colonies were isolated using starch casein agar and cast extract agar from earthworm cast material collected from Agricultural area, Kanchipuram. All the isolated were confirmed as actinobacteria based on their colonial and microscopic appearance. All the isolates were screened for the production of ammonia, acetoin, phosphate solubilisation and indole acetic acid production. Out of 10 isolates, 5 showed acetoin production, 9 showed phosphate solubilisation and six isolates showed indole acetic acid production. Further study was concentrated on IAA production from two actinobacterial isolates namely CA4 and CA6. Production, extraction, separation and assay of IAA were performed by adopting standard procedures. The quantity of IAA produced by the strain CA4 and CA6 was estimated as 15.4µg/ml and 19.8 µg/ml, respectively. Microscopic, cultural and physiological characteristics of actinobacterial isolates were studied by adopting standard procedures. Based on the phenotypic characteristics both the actinobacterial isolates (CA4 and CA6) are suspected as *Streptomyces* species. Finding of the present work concludes that cast soil is a potential source for plant growth promoting actinobacteria (PGPA). Optimization studies are needed to prove the potential of cast soil actinobacteria such as CA4 and CA6 as producer of Indole Acetic Acid (IAA).

KEYWORDS

Earthworm cast;
Actinobacteria;
Plant growth promotion;
IAA production;
Streptomyces.

INTRODUCTION

Actinobacteria are the group of Gram positive filamentous bacteria which are recognized as a major source for bioactive metabolites. These microorganisms are abundant in soils and act in the degradation of

complex molecules as well as recalcitrant substances, especially cellulose, lignocellulose, xylan and lignin that play an important role in soil organic matter decomposition processes[1]. Besides acting as organic matter decomposers, these microorganisms have great potential as agents for control of plant pathogens and/or for plant growth promotion[2]. This is due to their capacity to produce antibiotics, siderophores, enzymes that have antimicrobial activity, substances that promote plant growth, solubilization of phosphates and competition with plant pathogens for substratum and nutrients.

Earth worms contribute to cycling and accumulation of nutrients by casting at the soil surface. Earth worm cast consists of mixed inorganic and organic materials from the soil that are voided is an essential function within earth worm communities which maintain their living space[3]. Fertility of world soil is greatly determined by the quantity of earthworm cast material present in soil, Worm castings contain a high percentage of humus which helps soil particles form into clusters. They, in turn, create channels for the passage of air and improve its capacity to hold the water. The castings are in the form of tiny pellets which are coated with a gel. This crumb like structure helps improve drainage and aeration. Castings are high in soluble nitrogen, potassium, calcium, magnesium and many other trace elements. Worm castings allow plants to quickly and easily absorb all essential nutrients and trace elements because the earth worms grinds and uniformly mixes the nutrients and trace elements into simple forms. Plants need minimal effort to absorb these nutrients[4].

Though the distribution of actinobacteria in earthworm cast material is well documented, their utilization for plant growth promoting activities is very less. With this view an attempt was made to study the actinobacteria from earth worm cast with special reference to plant growth promoting properties.

MATERIALS AND METHODS

Collection of sample and pretreatment

The soil samples were collected from two different places at Kanchipuram, Tamil Nadu. The samples were transported in a sterile polythene bag into the laboratory and dried at room temperature for 3 days. One gram cast sample was weighed and heat treated at 55ºC for 10 minutes.

Isolation and selection of actinobacteria

One gram soil was suspended in 9ml of sterile distilled water. The samples were serially diluted upto 10^2 dilution using 9ml sterile distilled water blanks. Hundred microlitre of aliquot from 10^3, 10^4 and 10^5 dilutions was transferred to starch casein agar pH 7 supplemented with nystatin and nalidixic acid and cast extract agar medium plates and spreaded using sterile L-rod. The plates were incubated at room temperature for 1 month[5].

The actinobacterial colonies selected from isolation agar medium was streaked on ISP2 agar plates (yeast extract 4.0%; malt extract 10.0 %; dextrose 4.0%; pH 7.0; agar 1.8%; distilled water 100 ml)[6] and incubated at 28ºC for 5 to 7 days. After incubation cultural characteristics such as aerial mass color, reverse side pigment, soluble pigment and microscopic appearance such as the presence of substrate and aerial mycelium of all the actinobacterial isolates were detected by observing under bright field microscope with 40x magnification.

Screening for plant growth promoting properties

The selected actinobacterial isolates were screened for certain plant growth promoting properties such as ammonia production, acetoin production, growth on nitrogen free medium, phosphate solubilisation, indole acetic acid production by adopting the methods described by Gayathri *et al.*,[7] with certain modifications.

For ammonia production, the actinobacterial cultures were inoculated into each 5 ml of peptone broth and incubated at 28^0C for 7 days in rotary shaker. Development of pink colour after the addition of 0.5 ml of Nessler's reagent is the indication for ammonia production.

The actinobacterial cultures were inoculated into each 5 ml of MR-VP broth and incubated at 28^0C for 7 days in rotary shaker for acetoin production. The development of red color after the addition of 1ml of KOH and 3ml of 5% α-napthol is the indication for acetoin production.

To confirm nitrogen fixation, all the actinobacterial cultures were inoculated into nitrogen free medium namely Jensens medium and incubated at 28^0C for 10 days for the growth of actinobacteria.

For phosphate solubilisation testing, actinobacterial cultures were inoculated into Pikovaskaya's agar plates and incubated at 28^0C for 7 days. The halo formation

around the growth is the indication for phosphate solubilisation.

For indole acetic acid (IAA) production, the well grown actinobacterial cultures were inoculated into the nutrient broth supplemented with tryptophan (2mg/ml) and incubated in rotary shaker for 7 days at 28^0C. After incubation, the cell free supernatant was prepared by centrifugation at 3000 rpm for 10 minutes. Two ml of the supernatant was mixed with 1ml of solawaski's reagent. The development of pink color is the indication for IAA production.

Production and extraction of IAA from selected isolates

Further studies were concentration on the production and assay of IAA from selected actinobacterial isolates. About 10% of actinobacterial inoculum from ISP2 broth was transferred into 50 ml of nutrient broth supplemented with 2mg/ml, 4 mg/ml and 6 mg/ml concentration of tryptophan and incubated in rotary shaker with 95 rpm at 28^0C for 7 days. After incubation the cell free supernatant was collected by centrifugation at 10000 rpm for 10 minutes. The resulting supernatant was acidified to pH 2.5-3.0 using 0.1N HCl and extracted twice with two volume of ethyl acetate. The extracted ethyl acetate fraction was evaporated to dryness and stored in refrigerated conditions[8].

Estimation of IAA

To estimate the IAA concentration, about 1ml of cell free supernatant obtained from nutrient broth supplemented with different concentration of tryptophan was taken in a test tube and mixed with 2 ml of Solawaski's reagent. After 30 minutes of incubation, the color absorbance was read using spectrophotometer at 535nm. Standard IAA was prepared at the concentration of 0, 5, 10, 15, 20, 25, 30, 35, 40µg/ml[9].

Characterization of potential actinobacteria

Micromorphological characteristics of actinobacterial strain was studied by slide culture technique and observed under bright field microscope at 40X magnification. Recorded microscopic characteristics include aerial mycelium, substrate mycelium and spore chain morphology[10]. Growth pattern of actinobacterial isolates was studied using different ISP media described by Shirling and Gottileb[6]. Physiological characteristics which are studied include carbon utilization, pH tolerance and temperature tolerance[10].

RESULTS AND DISCUSSION

The fertility of world soil is determined by the amount of earth worm cast and its microbial flora. There are many microorganisms and their plant growth promoting activities are well documented. Total cast production by earthworm is an indicator of burrowing and soil turnover, because 99.9% of ingested material is egested as casts[3]. Microbial diversity studies on earthworm casts were well documented in various countries even before five decades[11]. But the prospecting microbes from cast material for commercial purpose are less documented. With this view an attempt was made to isolate actinobacteria bacteria for studying their plant growth promoting activities.

In the present study, cast material from the agricultural soil around Kanchipuram city was collected and actinobacteria were isolated using starch casein agar and cast extract agar. After incubation only few actinobacterial colonies were developed on both the isolation agar plates. Though there are good reports are available on isolation of actinobacteria from soil and aquatic sediments using starch casein agar[5,12], eight morphologically different colonies were isolated in this study using starch casein agar. Only two colonies recovered from agar medium supplemented with cast extract. Hamaki *et al.*,[13] isolated novel bacteria and actinomycetes using soil extract agar. More interestingly such bacteria are not able to grow on other conventional media. But in the present study only two actinobacteria colonies (CEA1 and CEA2) are recovered from cast extract agar but both the isolates showed good growth on yeast extract malt extract agar medium. The above observation suggests that knowing the chemistry and physical property of the cast material is mandatory and useful before the isolation of actinobacteria since the chemical and microbial flora of cast vary with its age, size and associated earthworm.

The microbial flora present in cast material play an important role in the degradation of complex and partially digested material present thereby it determine the nutritional quality of the cast material. Ultimately the cast material associated bacteria support the growth of plants. In the present study actinobacteria isolated from cast soil was screened for plant growth promoting activities. Ryu *et al.*,[14] reported that the volatile substances such as 2,3 butanediol and acetoin produced by bacteria seem to be a newly discovered mechanism responsible for plant

growth promotion. In the present study, five out of ten isolates showed acetoin production (TABLE 1).

Phosphorus (P) is one of the major essential macronutrients for plants and is applied to soil in the form of phosphatic fertilizers. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizers is immobilized rapidly and becomes unavailable to plants. Microorganisms are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle[15]. Vinotha *et al.*,[16] reported that the enhanced phosphatase activity in earth worm casts is more of microbial origin. In the present study nine out of ten actinobacterial isolates showed phosphate solubilisation activity (TABLE 1). But none of the actinobacterial isolates showed ammonia production (TABLE 1).

TABLE 1 : Plant growth promoting properties of cast soil actinobacteria

S. No	Strain No	Plant growth promoting properties				
		Ammonia production	Acetoin production	Phosphate solubilisation	Nitrogen fixation	Indole Acetic Acid Production
1	CEA1	-	-	+	-	+
2	CEA2	-	+	+	-	+
3	CA2	-	+	+	-	+
4	CA4	-	-	+	-	-
5	CA6	-	+	-	+	+
6	CA8	-	+	+	+	+
7	CA11	-	-	+	-	-
8	CA17	-	-	+	-	-
9	CA18	-	-	+	-	+
10	CA22	-	+	+	-	-

One of the major activity by which the microbes supporting the plant growth is the production of plant growth hormones like auxins and giberllins. Indole acetic acid (IAA) is a common natural auxin and is a product of L-tryptophan metabolism in microorganisms. Approximately 80% of the rhizosphere bacteria can secrete IAA. *Streptomyces* species inhabiting the rhizosphere of various plants can also serves as good source of IAA[17]. In the present study out of 10 actinobacterial isolates, two isolates namely CA4 and CA6 are showed good IAA producing activity (TABLE 1). Hence further study was concentrated on these two isolates with special reference to IAA production. Production, extraction, separation and assay of IAA were performed by adopting standard procedures. The quantity of IAA produced by the strain CA4 and CA6 was estimated as 15.4µg/ml and 19.8 µg/ml, respectively (TABLE 2).

TABLE 2 : Estimation of IAA produced by cast soil actinobacteria

S. No	Strains	IAA production (µg/ml) with Tryptophan Concentration (mg/ml)			
		0	2	4	6
1	CA 4	8.32	10.11	13.31	15.45
2	CA 6	9.50	12.15	17.40	19.85

In characterization studies, both the isolates showed good growth on all the media tested. But no growth was observed on carbon utilization studies. This finding insisted that the characterization method designed for normal terrestrial actinobacteria is giving some difficulties when it used for cast soil actinobacteria. Further optimization and designing of media is needed to overcome this problem. Based on the phenotypic characteristics both the actinobacterial isolates (CA4 and CA6) are suspected as *Streptomyces* species (TABLE 3). Chemotaxonomic and molecular characterization is needed for its further confirmation.

Finding of the present work concludes that cast soil is a potential source for plant growth promoting actinobacteria (PGPA). But the unsuitability of the isolation agar used in this study results in recovery of less number of actinobacteria. Designing of suitable media and protocol for the recovery of diverse culturable actinobacteria from the earthworm cast – complex but fertile habitat - is needed to be strengthened. Optimization studies are needed to prove the potential of cast soil actinobacteria such as CA4 and CA6 as producer of Indole Acetic Acid (IAA).

TABLE 3 : Characteristics of cast soil actinobacteria

Characters	Actinobacterial strains	
	CA4	CA6
Micromorphology		
Aerial mycelium	+	+
Substrate mycelium	+	+
Spore chain morphology	Rectus Flexible [RF]	Rectus Flexible [RF]
Cultural characteristics		
Colony consistency	Powdery	Powdery
Aerial mass color	Brown	Brown
Reverse side pigment	+	+
Soluble pigment	-	+
Growth on different ISP medium		
ISP1	No growth	No growth
ISP2	Good	Good
ISP3	No growth	Moderate
ISP4	Moderate	Good
ISP5	No growth	Good
ISP6	Moderate	No growth
ISP7	No growth	No growth
Carbon compounds		
Glucose	-	-
Sucrose	-	-
Xylose	-	-
Inositol	-	-
Mannitol	-	-
Fructose	-	-
Rhamnose	-	-
Raffinose	-	-
Arabinose	-	-
Cellulose	-	-
Enzyme activities		
Amylase	+	+
Lipase	-	-
Protease	+	+
Temperature tolerance (ºC)		
20	Moderate	Moderate
30	Good	Good
40	Good	Good
45	Poor	Moderate
pH tolerance		
5	-	-
7	Good	Good
9	Good	Good
11	Poor	-
Anaerobic condition	No growth	No growth

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