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Isolation, Purification and Estimation of Zeatin from *Corynebacterium aurimucosum*

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ABSTRACT

Plant pathogenic organisms are known to affect plants by secreting enzymes, toxic compounds and phytohormones (IAA, KIN, GA, Zeatin and ABA). In the present study, bacteria were isolated from infected Prunus salicina. It was identified by biochemical tests and rDNA sequencing. It was studied for production of zeatin, a phytohormone known to influence plant growth and development. Conditions for zeatin production were optimized and after 10 days of incubation zeatin was extracted from the broth. The extract was subjected to isolation of zeatin using TLC. The further purification of zeatin was performed using HPLC and tested by immunoassay.

Key words: Bacteria, Zeatin, HPLC, Immunoassay.

INTRODUCTION

Microorganisms interact with plants because plants offer a wide diversity of habitats including the phyllosphere (aerial plant part), the rhizosphere (zone of influence of the root system), and the endosphere (internal transport system) [1]. Several bacteria also secrete the small molecules such as toxins, plant hormones, and exopolysaccharides (EPS) and antibiotics [2] during the pathogenesis [3].

Several microorganisms are able to promote the plant growth [4]. Some bacteria provide beneficial effects on host plants by producing plant growth regulators [5, 6]. Plant growth hormones, which are synthesized in minute amounts, affect many activities in plant growth and development [7]. These hormones are not only synthesized by higher plants [8, 9] they have also been synthesized by lichens [10, 11, 12], mosses [12], fungi [13, 14] and bacteria [15, 16, 9, 17, 18, 19].

It is becoming increasingly apparent that many of the pathogenic and non pathogenic microbes have the ability to synthesize cytokinins and/or auxins [20, 21]. Cytokinins are adenine derivative phytohormones that control cell division, cell cycle and stimulate developmental processes in plants [22]. Stimulatory or inhibitory function of cytokinins in different developmental processes such as regulation of root and shoot growth as well as branching, control of apical dominance in the shoot, chloroplast development, and leaf senescence [23, 24]. Cytokinin influence cell division activity in embryonic as well as mature plants by altering the size and activity of meristems [23]. Yang et al. [25] showed that the rate of endosperm cell division is closely associated with cytokinin level in endosperm.

Cytokinin biosynthesis in microorganisms has been known for many years that tRNA degradation is a source of cytokinins in bacteria e.g. *Escherichia coli* [26, 27]. However, microorganisms are also able to synthesize cytokinins with the first isopentenyl ATP enzyme identified in the slime mould *Dictyostelium discoideum* [28]. Since then, *ipt*

genes have being identified in a number of bacteria e.g. *ptz* of *Pseudomonas savastanoi*, *ipt* of *Rhodococcus fascians* and *ipt of Erwinia harbicola* [29].

Cytokinins were detected in the culture medium of several bacteria including *Halomonas desiderata*, *Proteus mirabilis*, *P. vulgaris*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *B. cereus*, *B. subtilis* and *Escherichia coli* [30, 31, 32]. Different cytokinins are detected not only in the biomass of microorganisms (in free state or bound to certain tRNAs) but also in the culture medium in the form of either adenine derivatives, isoprenylated at N^6 position or their ribosides, such as 6-benzyladenine, N6-isopentenyladenosine, zeatin riboside [33]. Trans zeatin has also been found in the culture of *Agrobacterium tumefaciens* [34]. Considering to this, in the present study Bacteria isolate from the infected *Prunus salicina* plant material was studied for zeatin production and estimation by Immunoassay.

MATERIALS AND METHODS

Isolation and Identification of bacteria

Bacteria isolated from the infected *Prunus salicina* fruit .It was cultured on Nutrient Agar (N-agar) media and identified on basis of biochemical tests and 16s rDNA sequencing.

DNA Extraction

Bacterial cells were pelleted by centrifugation, resuspended in 500 µl of 10mM Tris-EDTA buffer, and treated with 30 µl SDS (10% W/V) , 2 µl Proteinase K (10mg/ml) mix well and incubated for 1 h at 37ºC of. Then after 20 µl of cetyltrimethylammonium bromide (10%, W/V) and 100 µl of NaCl (5 M) were added, the solutions were incubated for 10 min at 65°C. DNA was purified by two 1:1extractions in which we used (i) Chloroform: Isoamyl alcohol (24:1) (ii) Phenol: Chloroform: Isoamyl alcohol (25:24:1) and then was precipitated with isopropanol, washed with ethanol (70%), and dissolved in Tris-EDTA buffer. The quality and concentration of the DNA were checked by measuring optical density (260/280 ratio).

16S rDNA gene amplification

The 16S rRNA gene was amplified using universal primer pair .DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl 10X buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Trion X100), 1.5mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 10 µM primer and 1U of *Taq* DNA polymerase , 200 ng bacterial DNA. Profile of PCR was: initial Denaturation 95º C – 5min: followed by 35 cycles: Denaturation 95ºC – 30 s , Annealing 52 ºC- 45s, Extension 72ºC – 2min and Final extension 72ºC -12 min. Amplified DNA fragments were electrophoresed through a 1.5% Low melting agarose gel.

Sequencing of 16s rDNA gene

The eluted PCR products were sequenced by using a Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Bio system) on a model ABI 3130 genetic analyzer

Zeatin extraction from Bacteria

Strain PKV001 (HQ454508) was grown in 200 ml of N-broth medium, under constant agitation at 30 ± 2 ºC for 10 days. After incubation period the broth was centrifuged at 10,000 g for 10 min and supernatant was collected. The pH of the solution was adjusted to 7.0 using 7M HCl and 7M NaoH. This solution was then transferred to a funnel and the extraction proceeded by the use of 15 ml of ethyl acetate, thrice. The ethyl acetate solution was dried in air and the final residue was resuspended in 1 ml of methanol.

Thin layer chromatography

Ethyl acetate fraction was plated on TLC plates (Silica gel 60 F- f254, thickness 0.25 mm) and developed in isopropanol/ammonia/distilled water (10:1:1v/v/v) Spots with Rf values identical to standard zeatin was identified under UV light (354 nm).Bands detected were scraped from the TLC plate and dissolved in methanol and analyzed by HPLC.

HPLC analysis

Zeatin was separated on a 250 × 4.6mm, reverse phase Luna 5U C-18 column on a HPLC-Shimadzu. Sample was analyzed under isocratic condition with 70% methanol. The UV detector was set at 254nm. The total run time for separation was approximately 30 min at a flow rate of 1ml/min.

Raising of antibodies against Zeatin

Zeatin-BSA conjugation was prepared by modified protocol of Weiler [35]. For preparation of zeatin-BSA conjugate, 10.3 mg zeatin was suspended in 1ml of methanol and 0.01m Na_2SO_4 was added over a period of 7min.

The solution was stirred for another 13min and 0.3ml of 0.1M ethylene glycol (30µmol) was added. After 5 min, the reaction mixture was added drop wise to a stirred solution of 110 mg BSA in 5 ml distilled water. During addition of the oxidized riboside the pH was kept constant between 9.2 to 9.4 by addition of 5% K_2Co_3. After 60 min solid $NaBH_4$ (5mg) was added and its repeated addition was done after 40 min. Then, pH was adjusted to 6.5 by the addition of 1M acetic acid and the solution was stirred for another 2 hr. The conjugate was purified by dialysis against water for 3 days at 4°C and stored at 0°C.

Immunization

Two rabbits for each zeatin were immunized by intramuscular rout according to Chudasama and Thaker [36]. Antigen was prepared by mixing zeatin-BSA conjugate thoroughly with equal volume of adjuvant and injected into rabbits. Booster injections were given periodically to raise the titer. Rabbits were bled periodically, serum was separated and antibodies were purified by ion exchange chromatography using DEAE cellulose cake. Purified antibodies of each hormone were stored in freeze before used.

Extraction of Hormones

For the extraction of zeatin strain PKV001 was grown in 200 ml of N-broth medium with different concentration of adenine Sulphate, respectably 100, 150, 200, 250, 300 and 350 mg under constant agitation at 30 ± 2ºC for 10 days. After incubation period the broth was centrifuged at 10,000 g for 10 min and supernatant was collected. The pH of the solution was adjusted to 7.0 using 7M HCl and 7M NaoH. This solution was then transferred to a funnel and the extraction proceeded by the use of 15 ml of ethyl acetate, thrice. The ethyl acetate solution was dried in air and the final residue was resuspended in 5 ml of phosphate buffer saline pH 7.0.

Estimation of endogenous hormones

Endogenous level of hormones zeatin was estimated by a comparatively more sensitive and specific technique i.e. indirect ELISA [36]. Zeatin-casein conjugate (300 µl) was coated on ELISA plate and incubated for overnight at 4°C, followed by washing with PBS-T. The next step involved was blocking of free protein binding sites of well with egg albumin and incubated for 1 h at 37°C. Antibodies against zeatin mixed with samples, were coated and incubated for 3 h at 37°C. Finally, the plate was coated with anti Rabbit IgG, tagged with peroxidase and the color was developed using O-phenylene diamine as a substrate. The reaction was terminated by addition of 1 M sulfuric acid (50 µl). After each coating, the ELISA plate was washed thoroughly with PBS containing 0.05 % tweeen-20. The color developed was measured at 490 nm by ELISA Reader (µ Quant, Biotek, USA). Assay for each hormone was performed thrice and mean value was calculated.

RESULTS AND DISCUSSION

In the present study, bacterium isolated from the *Prunus salicina* was gram positive oval shaped cells, having small round, smooth textured and opaque colonies. The results of biochemical tested revealed that this bacterium belongs to genus *Coryneforms* (Table 1). Using the universal primer set, 1517bp DNA fragment of the 16s rDNA gene was amplified by PCR (Figure 1). PCR amplified 16S rDNA region was sequenced and sequence (Figure 2) from strain PKV001 was shown to have a 99 % similarity with *Corynebacterium aurimucosum* (Accession number HQ454508) via BLAST analysis.

Production of Zeatin from the *Corynebacterium aurimucosum*

In this study, zeatin extracted from the culture of *Cornybacteroium aurimucosum* using ethyl acetate, TLC analysis of Zeatin compounds obtained from the culture confirmed the presence of zeatin with Rf value 0.53 identical to the Rf of the standard (Figure 3). Zeatin extracted from the culture of *Corynebacterium aurimucosum* showed a similar peak to that of the standard zeatin (Himedia) in HPLC analysis. The retention time of the extracted zeatin was 3.2, which matched the retention time of authentic zeatin of 3.2 min (Figure 4).

Zeatin production was optimized by using the adenine sulphate a promoter for zeatin synthesis. Adenine sulphate is frequently used in plant tissue culture for induction of more numbers of multiple shoots from the explant [37] and its probable role in kinetin synthesis is presumed. In this experiment, varying levels of zeatin production were recorded with different concentrations of adenine sulphate i.e 100 – 350 mg /100 ml. The range of zeatin production from the *Corynebacterium aurimucosum without* adenine sulphate was 23.52µg zeatin/100 ml culture. A gradually increased in the production of zeatin in the presence of adenine sulphate was recorded in the concentrations of 100-300 then after decreased. The value of zeatin in concentration of adenine sulphate 100, 150, 200, 250, 300 and 350 was 41.13µg, 42.96µg, 39.46µg, 69.72µg, 69.93µg and 1812µg zeatin per 100ml culture , respectively (Figure 5). Previously Taller and Wong [38] determined cytokinins as equivalent to 0.75 µg per litre in *A. vinelandii* culture medium. Similarity Barea and Brown [39] determined 20 µg/l of cytokinin equivalent per liter for *Azotobacter paspali* and 50 µg/l for *Azotobacter vinelandii*. In addition, it was determined that the *A. vinelandii* culture medium

contained 1 mg of cytokinin per liter [31]. In earlier Norimoto et al. [40] isolate the zeatin from *Corynebacterium fascians* causes fasciation disease or witches' broom in dicotyledonous plants. Nearly 70 µg/l zeatin productions by this organism support the view that adenine sulphate enhance the zeatin production and can be exploit commercially.

Table 1: Result of Biochemical test

No.	Biochemical Test	*Corynebacterium aurimucosum*
1	Sugar fermentation	
	Glucose	Acid & Gas
	Lactose	-Ve
	Fructose	Acid
	Mannitol	-Ve
	Sorbitol	-Ve
	Sucrose	Acid & gas
	Galactose	Acid
2	Methyl Red	+Ve
3	Voges Proskauer	-Ve
4	Indole Production	-Ve
5	H2S Production	+ve
6	Ammonia Production	-Ve
7	Nitrate Reduction	+Ve
8	Starch hydrolysis test	-Ve
9	Gelatin hydrolysis	-Ve
10	Catalase test	+Ve
11	Lactose utilization test	+Ve
12	Citrate utilization test	+Ve
13	Reduction test for TSI	Slant Acidic
14	Urea hydrolysis	-Ve

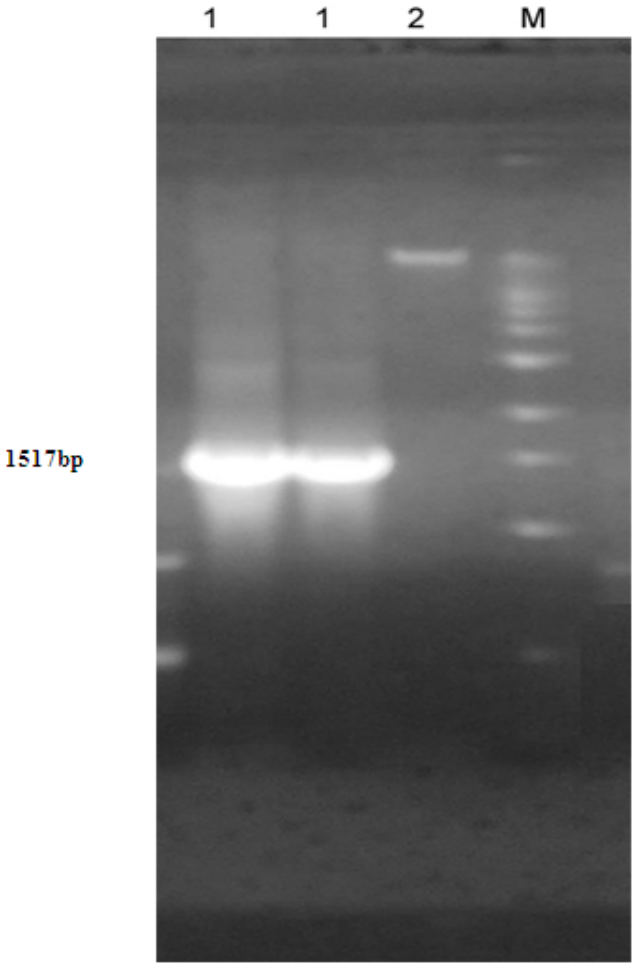

Figure 1: Gel electrophoresis of DNA products amplified using universal primer (lane 1, 2, M) respectively contain 1517bp, Genomic DNA and marker

ccaatccaccgttcgaagctccctacagtttaggccactggcttcgggtgttaccaactttcatgacgtgacgggcggtgtgtacaaggcccgggaacgtattcaccgca
gcgttgctgatctgcgattactagcgactccgacttcatggggtcgagttgcagaccccaatccgaactaaggccggctttcagcgattcgctccacctcacagtgtcgct
gcgcgttgtaccgaccattgtagcatgtgtgaagccctggacataaggggcatgatgatttgacgtcatccccaccttcctccgagttaaccccggcagtctctcatgagtc
cccaaccaaatgctggcaacataagacaagggttgcgctcgttgcgggacttaacccaacatctcacgacacgagctgacgacaaccatgcaccacctgtacaccagc
cacaagggaaactacatctctgcaccaatccgga

Figure 2: Sequence of *Corynebacterium aurimucosum* (Accession no. HQ454508).

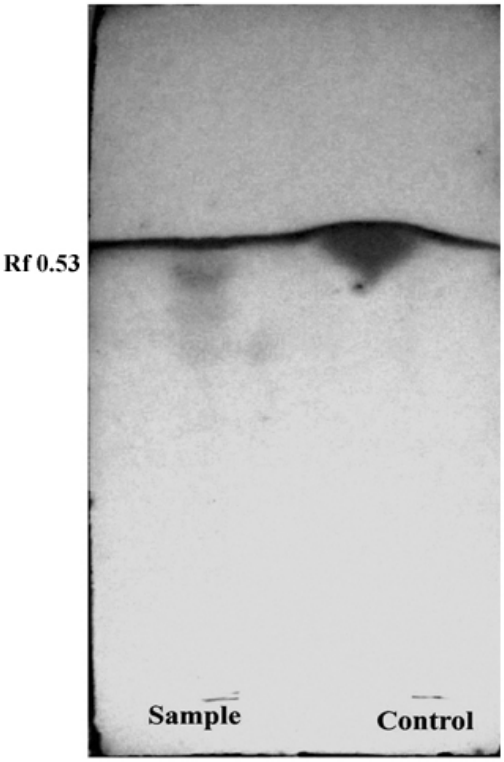

Figure 3: TLC of Zeatin produced by *Corynebacterium aurimucosum*

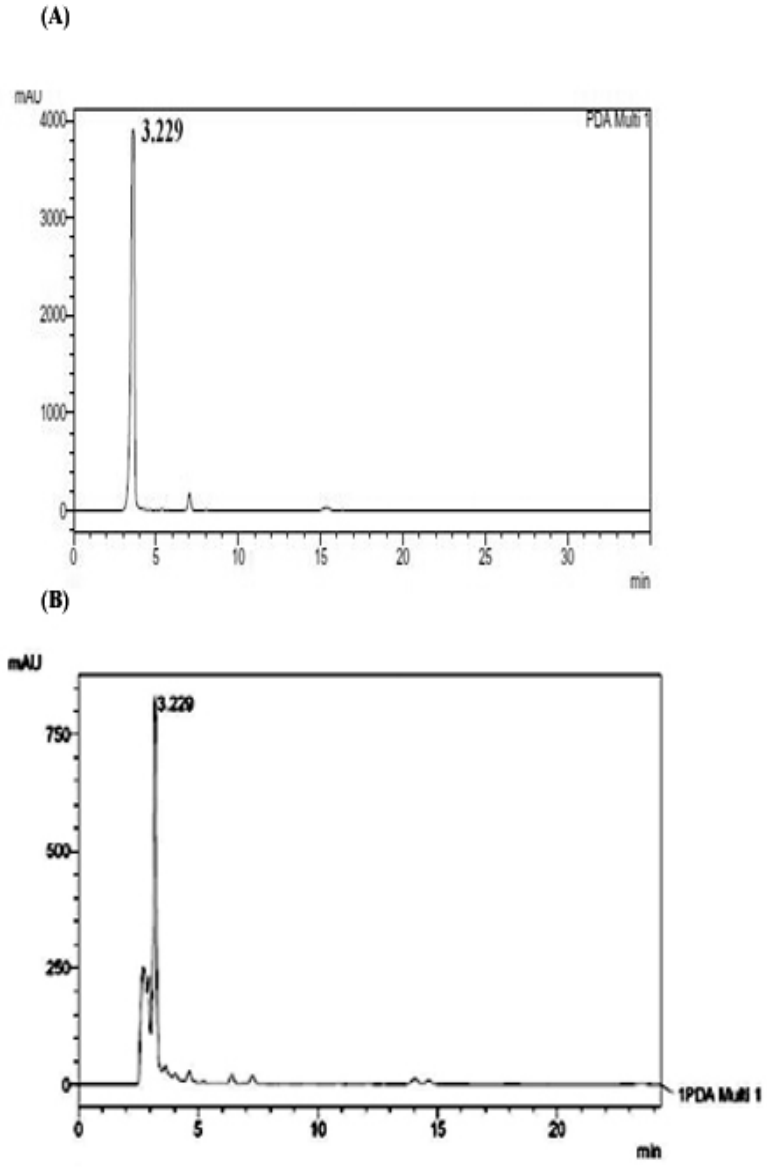

Figure 4: HPLC chromatogram for the production of (A) Standard Zeatin (B) Zeatin from *Corynebacterium aurimucosum*

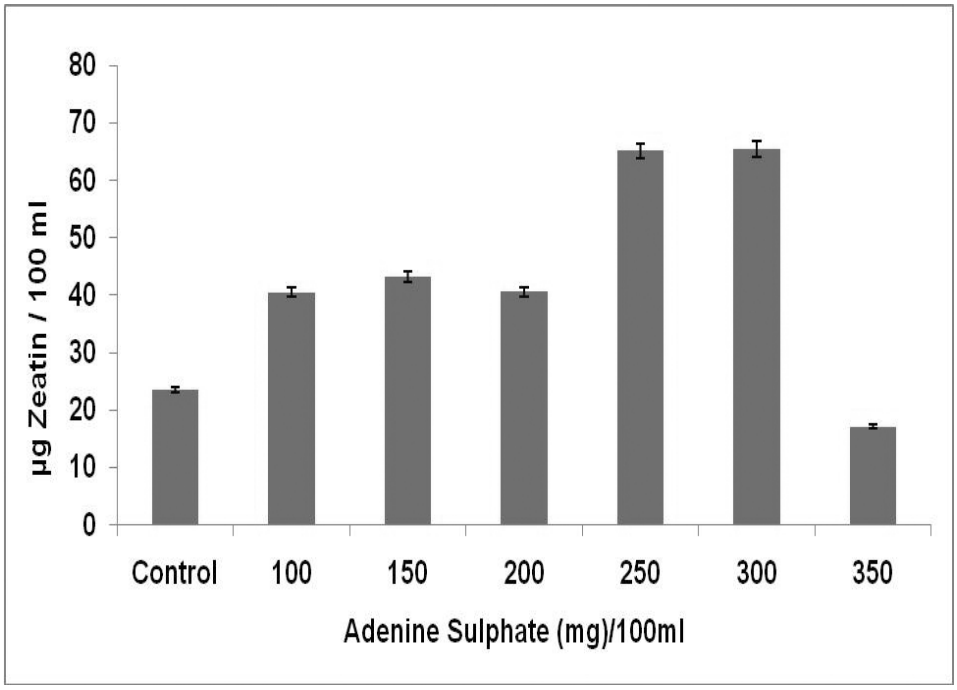

Figure 5: Zeatin productions from the *Corynebacterium aurimucosum* by Immunoassay

CONCLUSION

Present study revealed that studied plant pathogenic organism is corynebacterium aurimucosum, confirmed using analysis of 16s rDNA sequences and characteristics of the organism on biochemical basis (Bergey's manual).This organism produces plant growth hormone Zeatin as primary and secondary metabolite in the culture growth medium, which is identified & confirmed using TLC, HPLC and immunological techniques. Production of Zeatin is increased remarkably in the media with adenine sulphate (250-300 mg/lit).This organism may commercially be exploited for zeatin production.

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