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Isolation, Purification and Estimation of IAA from *Pseudomonas* sp. using High-performance Liquid Chromatography

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Plant growth promoting rhizobacteria (PGPR) are group of bacteria that can actively colonize plant roots and can enhance plant growth directly or indirectly. Fluorescent *Pseudomonas* sp. are emerging as largest and potentially most promising group of PGPR bacteria that are involved in plant growth enhancement and plant disease control. Production of auxins is one of the main reason to promote yield because of inoculation with this bacteria. The phytohormone auxin play a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence. In this research eight isolates of Fluorescent *Pseudomonas* sp. were isolated from apple rhizosphere and were investigated for auxin production. All the strains tested produced auxin in concentration of 5.1-16.9µg/ml. Two isolates viz., An-1-kul and An-13-kul were selected on the basis of higher production of auxin. The auxin produced by best isolates (An-1-kul and An-13-kul) were estimated via high performance liquid chromatography (HPLC) in liquid culture. The range of IAA production from fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul was 11.59µg/ml and 13.76µg/ml culture with a retention time of 2.620 and 2.624 respectively.

Key words: Auxin, *Pseudomonas* sp., HPLC.

Plant growth-promoting rhizobacteria (PGPR) are group of bacteria that can actively colonize plant roots and can enhance plant growth using different mechanisms like production of plant growth regulators viz., indole acetic acid, gibberellic acid, cytokinins and ethylene[1] , providing the host plant with fixed nitrogen, solubilization of soil phosphorus, enhance Fe uptake (siderophore production), biocontrol and reducing the concentration of heavy metals. Phytohormones, mainly including auxins, cytokinins, abscisic acid, gibberellins, and ethylene, induce some important physiological responses at different stages of plant development at low concentrations[2].

Different micro-organisms have been recognized to exude different plant growth promoters[3-6]. Among the plant growth promoting micro-organisms which are capable to produce plant hormones we can point to *Azotobacter*, *Pseudomonas*, *Azosprillium*, *Rhizobium*, *Bacillus*, *Enthrobacter* and Mycorrhiza fungus[7-9]. The fluorescent *Pseudomonas* sp. is the most abundant auxin producing micro-organism. Growth regulators especially IAA (Indole-3-Acetic Acid), often affects the root systematic features such as root primary growth, side-root formation and root hairs[10]. Production of plant growth regulators

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especially auxins induces additional root hair and lateral root formation hence thereby enhancing the plants ability to take up nutrients from soil and increase yield[11].

IAA is produced through L-TRP metabolism by plants and many of soil micro-organisms such as bacteria, fungus and algae[12]. Indole-3-Pyruvic acid, Indole-3-Acetaldehid and Indole-3-Acetonirile are pre-substances of Indole-3-Acetic Acid which have a feeble virtue of auxins. Indole-3-butyric acid is very often introduced as a synthesized auxin, although this kind of auxin is usually observed in a natural manner in some plants[13]. Frankberger and Brunner recognized the main indoles in soil as Indole-3-acetamide (IAM), Indole-3-pyrovic acid (IPYA), Indol-3-acetic acid (IAA) by HPLC (High performance liquid chromatography) device[14]. The aim of this research was to evaluating the auxin productivity potential in studied *Pseudomonas* strains through High-performance liquid chromatography **(**HPLC).

MATERIALS AND METHODS

Isolation of fluorescent *Pseudomonas* sp

Isolation of fluorescent *Pseudomonas* sp. was made from apple rhizosphere in Kullu district of Himachal Pradesh (India). The rhizosphere soil particles loosely adhering to the roots were gently teased out with small root pieces and mixed well. The soil thus obtained was crushed in a sterile mortar and pestle and shaken with 100 ml of sterile distilled water for 10-20 min. to obtain standard soil suspension. Isolation of fluorescent *Pseudomonas* sp was made by following the serial dilutions and pour plate method using the specific King's B medium[15].

Pseudomonas sp. isolates were identified on the basis of morphological, biochemical and physiological tests viz., Gram's staining, pigment production, catalase test , oxidase test, growth at optimum temperature i.e. 4°C and 41°C, gelatin liquification, denitrification test and IAA production.

Molecular characterization of fluorescent *Pseudomonas* sp. by 16S rRNA technique

Genomic Deoxyribonucleic acid (DNA) was extracted with DNA isolation kit (Bangalore GeNei), and the 16S rDNA gene was amplified by PCR using the set of primers FP-1(GGTCTGAGAGGATGATCAGT) and RP-1(TTAGCTCCACCTCGCGGC) in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100). The PCR amplification was carried out in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100) with a total of 35 cycles. Amplification was as follows: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 2min. For DNA sequencing, eluted amplified DNA products of two selected *Pseudomonas* sp. (An-1-kul, An-13-kul) was first purified followed by sequencing in Bioserve Private limited (Hyderabad, India). Similarity searches of the Genbank database were performed with BLAST.

Estimation of auxins

Quantitative estimation of auxins was done by colorimetric method[16] with slight modifications i.e. 2 to 3 drops of orthophosphoric acid was added to 2 ml supernatant and 4 ml of salper reagent (1 ml of 0.5M $FeCI_3$ in 50 ml of 30% $HCIO_4$). This mixture was incubated for 60 minutes in dark. Absorbance was measured at 535 nm. Concentration of auxins was estimated by preparing calibration curve using IAA as standard (10-100 µg/ml).

IAA Extraction

Auxins were extracted and separated from supernatant with diethyl ether and detected for homogeneity by thin layer chromatography (TLC)[17]. The isolates viz., An-1-kul and An-13-kul were grown in nutrient broth for 72 h at 28 ± 2°C under shake conditions (90rpm) and supernatants were harvested by centrifugation at 10,000 rpm for 20 minutes.

Thin layer Chromatography

TLC of auxins extracts was carried out on silica gel G pre-coated aluminium plates of Merk brand. 100 ml of the extracted auxins dissolved in methanol were spotted on silica gel G along with IAA standard. Plates were kept in solvent isopropanol-water (30:20 v/v) for 12-14 hours and sprayed with Salper reagent. Plates were observed for presence of pink color spot and R_f value was also calculated using following equation:

R_f = Distance covered by solute / Distance run by the solvent

Avena (oat) coleoptile straight growth bioassay of auxins

Coleoptiles (0.1 cm) length of 3 days old seedlings was floated in Petri dish containing 1ml

solution of test extracted auxins, 1ml standard (IAA:10µg or 100µg) and 1ml water (blank) and was incubated at 25^0 C for 48 h. in dark. Length of section will be measured before and after the experiment[18].

Partial purification of extracted auxins by column Chromatography

Diethyl ether extracted sample from fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul were used for Sephadex G25 column chromatography. 10g of Sephadex G-25 was soaked in distilled water for overnight and was boiled for 4h for complete swelling of the gel. Gel was washed and equilibrated with (0.2M, pH 7.2) tris-HCl buffer and column (50X1.5cm) was packed and equilibrated with tris-HCl buffer. 5ml sample was applied to column and eluted with tris-HCl buffer with flow rate 3ml/12minute. 3ml fractions were collected and observance was detected at different wavelengths (220nm, 240nm, 260nm, 280nm, 300nm, 320nm) (Figure 3). Estimation of auxins were done in each fraction and fraction showing auxin production were pooled, lyophilized and stored at -20^0 C for further use.

HPLC analysis

Analytical method for estimation of auxin was standardized on binary Waters HPLC Unit using Waters HPLC pump 515 with Waters spherisorb 5µm ODS 2 analytical column (4.6 x 250mm) and dual lambda absorbance detector 2487. HPLC system was initially run for overnight with properly cleansed HPLC grade methanol:water (80:20 v/v). HPLC chromatograms were produced by injecting 10µl of filtered extracts of auxins onto Water made (C18 silica column 25 X 4.6mm) in a chromatograph equipped with a differential UV detector absorbing at 270nm. Mobile phase was methanol and water (80:20 v/v) flow rate (1ml/min) for 15 minutes. RT for peaks was compared to those of authentic standards of auxins (indole acetic acid CDH) and quantification was done by comparison of peak heights.

RESULTS AND DISCUSSION

In the present study, eight isolates of Fluorescent *Pseudomonas* sp**.** were isolated from the rhizosphere soil of apple. The results of the biochemical tests performed for the identification of the effective native isolates of Fluorescent *Pseudomonas* sp. showed that all the isolates produced similar results with regard to gram staining (negative), gelatin liquefaction (positive), catalase test (positive), oxidase test (positive) and fluorescent pigmentation (positive). According to Todar (2004), more than half of the *Pseudomonas* bacteria produce pyocyanin which is a blue-green pigment[19]. In this study, all the eight identified gram-negative *Pseudomonas* isolates were found to be green fluorescent on King's B medium under ultraviolet light. The biochemical tests i.e gelatin liquefaction, denitrification, catalase test, oxidase test, IAA production, further confirmed that these isolates belong to genus *Pseudomonas* (Table 1). All the isolates showed positive results in IAA production. Among the isolates An-13-kul produced more quantity (16.92µg/ml) of IAA followed by An-1-kul (14.72µg/ml) and therefore

Table 1. Biochemical characterizations of different isolates of fluorescent *Pseudomonas* sp.

S. No	Parameters	Fluorescent *Pseudomonas* isolates							
		An-1-kul	An-2-kul	An-3-kul	An-4-kul	An-5-kul	An-6-kul	An-13-kul	An-14-kul
1.	Gram staining	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
2.	Gelatin liquefaction	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
3.	Catalase test	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
4.	Oxidase test	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
5.	Fluorescent pigment	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
6.	Growthat 4°C	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7.	Growthat 41°C	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
8.	Denitrification	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
9.	Estimation of IAA (µg/ml)	14.72	7.27	6.94	6.00	5.83	5.11	16.92	10.22

Table 2. Effect of extracted auxins of *Pseudomonas aeruginosa* An-1-kul and An-13-kul on the length of Avena coleoptiles.

Extracted auxins	Length of coleoptiles (cm)			
	Initial length	Final length	Increased length	Auxins (µg/ml)
Control (water)	0.10	0.20	0.10	9.0
An-1-kul	0.1	0.45	0.35	22.0
An-13-kul	0.1	0.50	0.40	25.0
*Standard (IAA)	0.1	65	0.55	32.0

Table 3. Auxins measured by spectrophotemetric and HPLC device

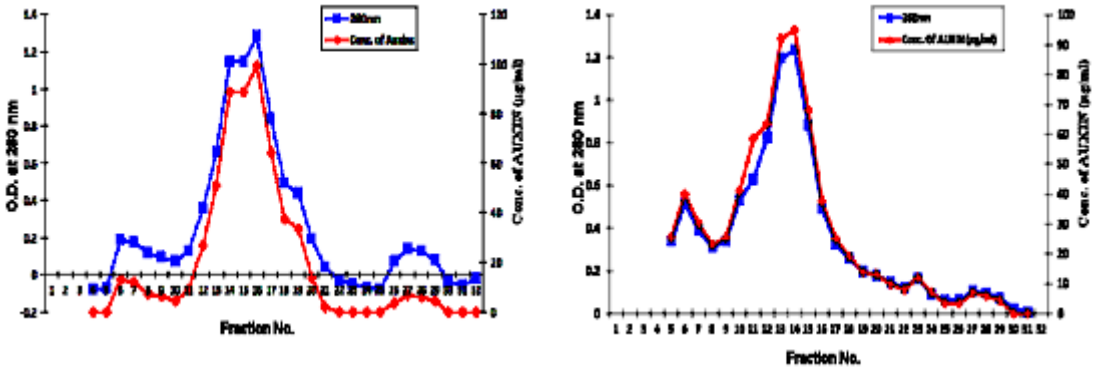
S. No.	Fluorescent *Pseudomonas* isolates	IAA (µg/ml)	
		Spectrophotemetric device	HPLC device
1.	An-1-kul	14.72	11.59
2.	An-13-kul	16.92	13.76

selected for further study. Barea *et al*., reported in an experiment that in 150 bacterial isolation collected from different plant rhizosphere, 58, 86 and 90 percent auxin, gibberellins and semi-auxin compound were exuded respectively[20].

Using the universal primer set, DNA fragment of the 16S rDNA gene was amplified by PCR. The PCR amplified 16S rDNA region was sequenced and sequence from strain An-1-kul and An-13-kul was shown to have a 98% similarity with *Pseudomonas aeruginosa* DK2 (Accession number NC_018080.1) and 99 % similarity with *Pseudomonas aeruginosa* PA7 (Accession number NC_009656.1) respectively via BLAST analysis.
IAA was extracted from the culture of fluorescent *Pseudomonas* using di-ethyl ether. TLC analysis of auxin compounds obtained from both the culture (An-1-kul and An-13-kul) confirmed the presence of IAA with Rf value 0.80 and 0.81 respectively identical to the Rf of the standard (0.85 Partially purified extracts of supernatants of *Pseudomonas aeruginosa* An-1-kul and An-13-kul concentration of IAA is about upto 22-25µg /ml respectively and it has increased the length of Avena coleoptiles (Table 2). This indicates that auxins are present in the partially purified samples.

In the present study we measured the IAA amount of two fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul through HPLC and spectrophotometer. Extracted auxins from strains An-1-kul and An-13-kul showed optimum peak value at 240, 260 and 280nm and showed presence of auxins in fraction number 12 to 18, 12 to 19 and 12-19 respectively in the main peak (Figure 1). Khalifah *et al.*[21] maximum excitation wavelength for auxins (IAA) was 290nm.

IAA extracted from the culture of fluorescent *Pseudomonas* isolates showed a similar peak to that of the standard IAA (Hi-media) in HPLC analysis. The retention time of the extracted auxin (IAA) from both the isolates was 2.620 and 2.624, which matched the retention time of authentic IAA of 2.619min (Figure 2). The range of IAA production from fluorescent *Pseudomonas* isolates An-1-kul and An-13-kul was 11.59µg/ml and 13.76µg/ml culture respectively (Table 3). Our results were the same as results done by Crozier *et al.*[22]. He measured the IAA amount of twenty *Azospirillum lipoferum* and *A. brasilense* strains through both HPLC and Salkowsky methods. He reported the IAA amount in *A. lipoferum* through Salkowsky method from 0 to 14.9µg/ml; and in *A. brasilense* from 0 to 26.0µg/ml. He also reported the IAA amount in *A. lipoferum* through HPLC method from 0.05 to 14.9µg/ml; and in *A. brasilense* from 0 to 4.5µg/ml. Benizri *et al.*[23], reported the auxin excretion in their research on *P. fluorescens* M.3.1 as 2.5µg/ml. In two separate studied carried out on *P. fluorescens* by Leinhos and Vacek [24] and Prikryl *et al.* [25]; the amount of produced auxin



a) Column chromatography of An-1-kul

b) Column chromatography of An-13-kul

Fig. 1. Sephadex G-25 column chromatographic profile of di-ethyl ether extracted auxins from *P. aeruginosa* An-1-Kul (a) and An-13-kul (b) at wavelength 280 nm

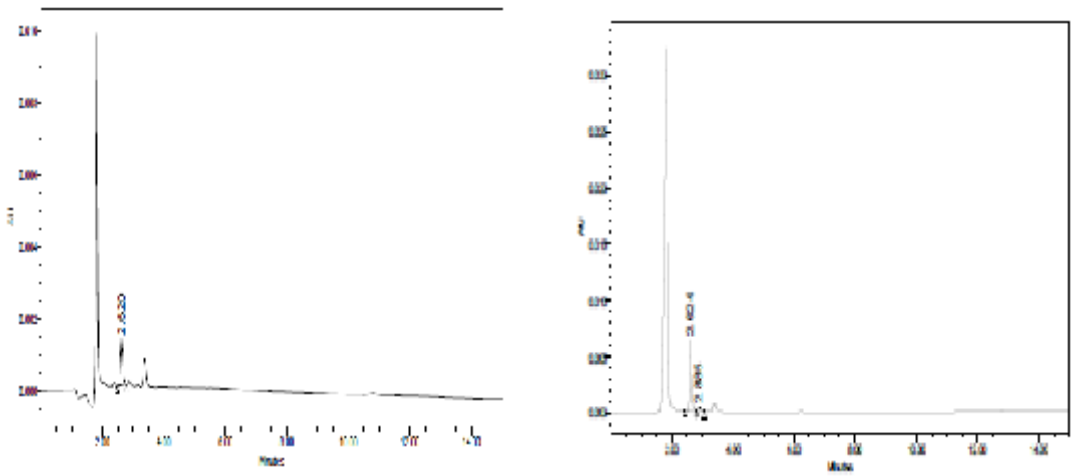


Fig. 2. HPLC chromatograph of auxins (a) An-1-kul (b) An-13-kul (c) standard IAA

reported as 1.6-3.3 mg/l and 0.01-3.93mg/l. Crozier and Reeve [26] reported that HPLC is a powerful method for simplifying the auxins' identification in comparison with mass spectrophotometer method. In HPLC method the goal is to promote the measurable IAA and reduce the amount of unexpected substances in samples.

REFERENCES

1. Zahir, Z.A., Arshad, M., Frankenberger, W.T.J. Plant growth promoting rhizobacteria: applications and perspectives in agriculture. *Advances in Agronomy*., 2003; **81**: 97-168.
2. Ma, Z., Ge, L., Lee. Simultaneous analysis of different classes of phytohormones in coconut (*Cocos nucifera* L.) water using high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry after solid-phase extraction. *Anal Chim Acta*., 2008; **610**: 274-281.
3. Patten, C.L., Glick, B.R. Bacterial biosynthesis of IAA. *Can. J. Microbiol*., 1996; 42: 207-220.
4. Arshad, M., Frankenberger, W.T. Plant growth regulating substances in the rhizosphere: Microbial Production and Functions. *Adv. Agron*., 1998; **62**: 46-151.
5. Frankenberger, W.T., Arshad, M. Phytohormones in soil, New York: Marcel Dekker, 1995; pp 1-135.
6. Epstein, E., Ludwig-Muller, J. Indole-3-butyric acid in plants: Occurrence, Synthesis, metabolism and transport. *Physiol. Plant*., 1993;

88: 382-389.

7. Arshad, M., Frankenberger, W.T. Microbial production of plant hormones. *Plant Soil.*, 1991; 133: 1-8.
8. Fett, W., Osman, S.F., Dunn, M.F. Auxin production by plant-pathogenic Pseudomonads and Xanthomonads. *Appl. Environ. Microbiol.*, 1987; **53**: 1839-1845.
9. Hirsch, A.M., Fang, Y., Asad, S., Kapulnik, Y. The role of phytohormones in plant-microbe symbioses. *Plant Soil.*, 1997; **194**: 171-184.
10. Glick, B.R., Karaturovic, D.M., Newell, P.C. A novel procedure for rapid isolation of pap Pseudomonads. *Can. J. Microbiol.*, 1995; **41**: 533-536.
11. Tien, T.M., Gaskins., Gubble, D.H. Plant growth substance by *Azospirillum brazilance* and their effect on growth of pearl millet. *Applied Environmental Microbiology*., 1979; **37**:1016-1024.
12. Sarwar, M., Kremer, R.J. Determination of bacterially derived auxins using a micro plate method. *Lett. Appl. Microbiol*., 1995; **147**: 282-285.
13. Epstein, E., Ludwig-Muller, J. Indole-3-butyric acid in plants: Occurrence, Synthesis, metabolism and transport. *Physiol. Plant.*, 1993; **88**: 382-389.
14. Frankenberger, W.T., Brunner, W. Method of detection of auxin-IAA in soils by HPLC. *Soil Sci. Soc. Am.*, 1983; **47**: 237-241.
15. King, E.O., Wood, M.K., Raney, D.E. Two simple media for the demonstration of pyocyanin and luorescein. *Journal of Laboratory Clinical Medicine*., 1954; **44**(2): 301-307.
16. Gordon, S.A., Weber, R.P. Colorimetric estimation of indole acetic acid. *Plant Physiology*., 1951; **26**: 192-195.
17. Mahadvan, A., Sridhar, R.K. Method in Physiology. 3rd edn. *Plant Physiology*.1986.
18. Noggle, R., Firtz, J. Introductory Plant Physiology. Niall India Pvt. Ltd. Jay Print Press. New Delhi. 1976.
19. Todar, K. *Pseudomonas* and related bacteria. Todar's online text book of bacteriology.2004 http://textbookofbacteriology.net/*Pseudomonas*. etc.html.
20. Barea, J.M., Navarro, E., Montoya, E. Production of plant growth regulators by rhizosphere phosphate-solubilizing bacteria. *J. Appl. Bacteriol*., 1976; **40**: 129-134.
21. Khalifah, R.A., Lowell, N., Lewis, Coogins, C.W. Differetiation between indole acetic acid the Citrus auxin by column chromatography. *Plant Physiology*., 1966; **41**: 208-210.
22. Crozier, A., Arruda, P., Jasmin, J.M., Monterio, A.M., Sandberg, G. Analysis of IAA and related indoles in culture medium from *Azospirillum lipoferum* and *A. brasilense*. *Appl. Environ. Microbiol*., 1988; **54**: 2833-2837.
23. Benizri, E., Courtade., Picard, A.C., Guckert, A. Role of maize root exudates in the production of auxins *Pseudomonas fluorescens* M. 3.1. *Soil Biol. Biochem*., 1988; **30**: 1481-1484.
24. Leinhos, V., Vacek, O. Biosynthesis of auxins by phosphate-solubilizing rhizobacteria from wheat and rye. *Microbiol. Res.*, 1994; **149**: 31-35.
25. Prikryl, Z., Vancura, V., Wrust, M. Auxin formation by rhizosphere bacteria as a factor of root growth. *Biol. Plantarum*., 1985; **27**: 159-163.
26. Crozier, A., Reeve, D.R. The application of high performance liquid chromatography to the analysis of plant hormones. In (ed.) Plant growth regulation. *Proc. 4 Int. Conf. Plant Growth Substances*. New York: P.E. Pilet, 1977; pp 67-76.