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ORIGINAL RESEARCH ARTICLE

Production, Optimization and Characterization of Phytohormone Indole Acetic Acid by *Pseudomonas fluorescence*

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ABSTRACT

The present study was undertaken for isolation of *Pseudomonas fluorescence* from rhizosphere soil, production, optimization and characterization of indole acetic acid utilizing the isolated bacteria. The isolate was identified as *Pseudomonas fluorescence* by 16S rRNA gene sequencing after following the conventional biochemical tests as per Bergey's manual of systematic Bacteriology. Cultural and nutritional conditions were optimized for indole acetic acid production. The effect of L-tryptophan was studied and the highest yield of 58 µg/ml on 72 hr was obtained using 0.5mg/ml concentration. The effect of various carbon sources such as lactose, galactose, sucrose, cellulose and mannitol was studied. The best yield of 53 µg/ml was obtained with medium supplemented 1% lactose. The effect of nitrogen source was studied by adding soyabean, beefextract, yeast extract, peptone and tryptone. The maximum yield of 48 µg/ml was obtained with supplemented 0.2% peptone as an organic nitrogen source. The IAA produced was subjected to thin layer chromatography for further identification and quantitative estimation was done by high pressure liquid chromatography. The compound was purified and identified as IAA by GC-MS. The result suggests that *Pseudomonas fluorescence* possess plethora of mechanisms to stimulate plant growth and worth applying these bacteria in agriculture.

Key words: Rhizosphere, *Pseudomonas fluorescence*, IAA and HPLC.

1. INTRODUCTION

Plant growth promoting rhizobacteria play an imperative role in agriculture by promoting the exchange of plant nutrients and reducing the application of chemical fertilizers to a large extent. There are several mechanisms by which rhizosphere bacteria may stimulate plant growth. One of the most commonly reported direct growth promotion mechanism by bacteria is the production of plant growth substances. Plant hormones are chemical messengers that affect a plant's ability to respond to its environment.

Hormones are organic compounds that are effective at very low concentration; they are usually synthesized in one part of the plant and are transported to another location [1]. Plant growth promoting rhizobacteria produce phytohormones that are believed to be related to their ability to stimulate plant growth. Indole-3-acetic acid is a phytohormone which is known to be involved in increasing the root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from soil [2]. IAA is a natural auxin with vast physiological effects [3]. IAA is produced through L-TRP metabolism by growth promotion mechanisms such as bacteria, fungus and algae [4]. Root exudates are natural source of L-Trp for rhizospheric microflora, which may enhance IAA biosynthesis in the rhizosphere [5] . Tryptophan is converted to IAA by soil beneficial bacterial activities.

Indole acetic acid is released as secondary metabolites because of rich supplies of substrates exuded from the roots. [6]. IAA (indole-3-acetic acid) is the member of the group of phytohormones and is generally considered the most important native auxin [7]. It functions as an important signal molecule in the regulation of

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plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, and gene regulation [8,9] . Moreover, several recent reports indicate that IAA can also be a signaling molecule in bacteria and therefore, can have a direct effect on bacterial physiology [10]. There are numerous soil microflora involved in the synthesis of auxins in pure culture in soil [11].

Auxin synthesis by cyanobacteria has also been reported [12,13] . The potential for auxin biosynthesis by rhizobacteria can be used as a tool for the screening of effective PGPR strains [14]. *Pseudomonas* bacteria, especially *P. fluorescens* and *P. putida* are the most important kinds of PGPR which produce auxin and promote the yield [15]**.** *Pseudomonas* spp has been reviewed by several workers for the biofertilizer, phytostimulator and phytopathogen biocontrol activities it possess. Hence, the present study was undertaken to isolate *P. fluorescens* from rhizosphere soil, to study Indole acetic acid production and optimization under laboratory condition, evaluating the auxin production quantitatively using HPLC and identification of IAA by GC-MS.

2. MATERIALS AND METHODS

Isolation and identification of *Pseudomonas fluorescence*

Rhizosphere soil samples were collected from different locations of Chennai and bacteria were isolated by serial dilution method using sterile nutrient agar .The isolates were further grown in King's B medium. The strain that showed fluorescence was selected and purified for further study. Identification of the isolates was carried out using colony morphology, Gram reaction and motility. Biochemical characterization was done following the standard method described in Bergey's manual of systematic Bacteriology. The isolate was further confirmed by Molecular method of identification by 16S ribosomal DNA gene sequencing. The sequences were submitted in GenBank, NCBI through 'Sequin' submission tool and accession numbers was obtained. The obtained 16S ribosomal RNA sequences of the strain were aligned with various *Pseudomonas* spp. obtained from GenBank, NCBI using CLUSTAL W aligning tool and Phylogenetic tree was constructed by the Neighbor - joining method and PHYLIP software. Nucleotide sequence determined in this study were submitted to GenBank under the accession number KC913193.

Indole acetic acid production

The isolated *Pseudomonas fluorescence* strain was used for IAA production. The production medium containing (w/v): L - tryptophan - 0.5%, $NaNO_3$ - 0.2%, K_2HPO_4 - 0.1%, $MgSO_4$,$7H_2O$ - 0.01%, $CaCO_3$ - 0.2% and Glucose - 1% was inoculated with the culture and incubated at 28^0C for 72 hrs in a rotary shaker at 120 rpm. Bacterial growth was determined using spectrophotometer taking optical density at 540 nm at every 12 hrs intervals. Bacterial cells were removed by centrifugation at 4,000 rpm for 20 min and the cell free supernatant was used for determining the concentration of Indole acetic acid adopting the method of [16].

Estimation of Indole acetic acid

One ml of the supernatant was mixed with 4 ml of the Salkowski's reagent and the preparation was incubated for 30 minutes at room temperature under darkness. Development of pink colour indicated IAA production. The amount of IAA produced was determined calorimetrically at 540 nm. The extracted IAA was estimated using a standard graph prepared from authentic IAA.

Optimization of cultural and nutritional conditions for Indole acetic acid production

The following parameters were studied to determine their influence on IAA production. The incubation period was studied by inoculating into 100 ml L - tryptophan supplemented production medium and incubated for 72 hrs at 28^0C in a rotary shaker at 120 rpm. Samples were withdrawn every 24 hrs and growth and indole acetic acid concentration were determined. Effect of different levels of tryptophan on the time course of IAA production was determined using production medium supplemented with tryptophan in varying concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg/ml). Impact of different carbon sources (1%) like Galactose, Lactose, Mannitol, Sucrose and Cellulose and different nitrogen sources (0.2%) like Soya bean, beef extract, Yeast extract, peptone and tryptone on IAA production was investigated.

Purification and Detection of Indole acetic acid

Purification of IAA from the sample was done by column chromatography using dry silica gel as absorbent and the solvent used was ethyl acetate and hexane (20:80 v/v). The sample was loaded to the silica gel column and the fractions were collected.

Thin layer chromatography

The fractions (10 - 20 micro litter) and standard IAA were placed on TLC plates (silica gel G f_{254},

thickness 0.25mm). TLC was run by using solvent system benzene: n-butanol: acetic acid in 70:25:5 proportion and spots were detected by spraying the plates using Salkowaski reagent. Rf value of the standard and IAA produced by the isolate was calculated.

Quantitative Estimation of IAA by HPLC

HPLC chromatograms were produced by injecting 10 micro litter of the filtered extracts onto a – (C18, 5 m 25 x 0.46 cm) chromatograph equipped with a differential ultraviolet detector absorbing at 280 nm. Mobile phase was methanol and water (80:20 [v/v]), flow rate was 1.5 ml/min, and retention times for peaks were compared with the standard IAA. Quantification was done by comparison of peak heights.

Identification of IAA by Gas chromatography-Mass spectroscopy

The fraction which gave positive result with Salkowski reagent was collected, concentrated by Lyophilization and analysed by gas chromatography-mass spectroscopy after methylation of the compound.

3. RESULTS AND DISCUSSION

Isolation and Identification of the Strain

Among the total 8 bacterial isolates, the isolate obtained on King's B medium that produced yellowish blue fluorescence, was biochemically identified as *Pseudomonas fluorescens* using Bergey's manual of systematic Bacteriology. BLAST analysis of partial 16S rDNA gene sequences showed that the isolate was closely affiliated with members of the genus *Pseudomonas*. The isolate exhibited 100% similarity to that of *Pseudomonas fluorescens* strain IAM 12022 (Acc # NR043420) and 99% similarity to *Pseudomonas* sp AH2 (Acc # AF451275). Therefore the potential strain was identified as *Pseudomonas fluorescens*. 16S rRNA gene sequence analysis was carried out for identifying fluorescent *Pseudomonas* isolated from sugarcane [17].

Effect of incubation period on IAA production

Indole acetic acid production by *Pseudomonas fluorescens* started after 24 hours and reached maximum at 72 hours when the bacteria reached stationary phase of growth, and then decreased slowly. At maximum growth (72 hours) the indole acetic acid production was found to be 45µg/ml and then the production declined to 19µg/ml at 120 hours. The decrease in indole acetic acid production after 72 hours might be due to release of indole acetic acid degrading enzymes such as indole acetic acid oxidase and peroxidase as has been reported earlier [18]. The maximum IAA production was observed in the stationary phase of growth [19]. Production of IAA is influenced by culture conditions, growth range and availability of substrates and varies greatly among different species [20].

Effect of L-Tryptophan Concentration on IAA production

IAA production was 58 µg/ml when the concentration of tryptophan in the medium was in the range from 0.5 mg/ml and further decreased at the concentration of 0.6 mg/ml. Thus, L-tryptophan at a concentration of 0.5 mg/ml was best. Higher concentration exerted the adverse effects on IAA production in *Acetobacter diazotrophicus* [21]. As the concentration of tryptophan in the medium increases, the amount of IAA produced increased [22], [23]. Maximum IAA production of 170µg/ml was obtained when 0.5% L- tryptophan was used in *Pseudomonas* sp. [24]. *Pseudomonas* isolates varied greatly in their intrinsic ability to produce IAA. Reports of [25] showed production of IAA increased with an increase to 0.2% tryptophan concentration in *Klebsiella*. Presence of tryptophan promoted IAA by *Pseudomonas* sp. isolated from sugarcane [17]. Production of high levels of IAA by *Pseudomonas* is a general characteristic [6]. Several reporters suggested the role of tryptophan in the enhancement of IAA production [14], [26]. Formation and growth of root depends on tryptophan concentration [27].

Effect of Carbon Source on IAA production

For finding out the most favourable carbon source giving maximum IAA production glucose in the production medium is replaced by different sugars. Lactose has been utilized better than other carbon sources compared. *Rhizobium* sp produced a maximum amount of IAA in glucose containing medium as reported by [18], [28]. Effect of carbon sources influenced the growth and IAA production [29]. A wide range of effective carbohydrate utilization is one of the criteria of *Rhizobium* to be considered as PGPR [30].

Effect of Nitrogen Source on IAA production

IAA production at 48 µg/ml was the highest with peptone as nitrogen source followed by yeast extract and beef extract. Both organic and inorganic nitrogen sources had been utilized by *Rhizobium* in IAA production [28] .Yeast extract produced highest amount of IAA in *Pseudomonas* sp [24].

Thin Layer Chromatographic Analysis

Partial purification of indole acetic acid from crude extract was done by using silica gel column chromatography and fractions were collected with solvent system ethyl acetate and hexane (20:80 v/v). Each fraction was tested in thin layer chromatography and then developed with Salkowski reagent. Chromatogram of culture showed a pink spot of purified indole acetic acid at the Rf value (0.62) almost same to standard IAA (0.67). Thin layer chromatography findings are in agreement with reports of [6,19].

High Pressure Liquid Chromatography Analysis

The concentration of IAA in culture broth was analyzed by HPLC. In this study, P*seudomonas fluorescence* produced 40 mg/litre IAA. The amount of exuded IAA by *Pseudomonas fluorescence* strains was varied from 0-31.6 mg/l while it was producing from 0-24.08 mg/l in *Pseudomonas putida* [15]. The only study compared different methods of soil auxin determination is HPLC and considered superior to all of the other methods [31]. HPLC analysis of culture filtrates examined in the comparative spectrophotometric methods confirmed that several L- tryptophan derived auxins were produced by the bacterial isolates [4]. *Azospirillum diazotrophicus* produced 26.28 µg/ml [21] The IAA amount in *Azospirillum lipoferum* and *Azospirillum brasilense* were 0.05 to 14.9 µg/ml and 0 to 4.5 ug/ml respectively as detected by HPLC method [32].

Gas Chromatography-Mass Spectroscopy Analysis

The purified compound was identified as IAA by GC-MS analysis. The retention time of 19.95 and fragmentation pattern of the methylated compound was approximately comparable to standard IAA. IAA from *Vibrio* culture supernatant had an observed retention time of 25.4 min and produced a spectrum identical to that of authentic IAA through GC-MS analysis [33]. Similar study was carried out for *Klebsiella pneumonia* and obtained the retention time of 11.83 and fragmentation patterns of the sample and standard matched [34].

Table 1: Effect of tryptophan concentration on indole acetic acid production

S. No	Tryptophan Concentration (mg/ml)	IAA production (µg/ml)
1	0.1	18
2	0.2	27
3	0.3	35
4	0.4	47
5	0.5	58
6	0.6	50

Table 2: Effect of carbon sources on Indole acetic acid production

S. No	Carbon source	IAA production (µg/ml)
1	Lactose	53
2	Galactose	47
3	Cellulose	26
4	Mannitol	20
5	Sucrose	16

Table 3: Effect of nitrogen sources on Indole acetic acid production

S. No	Nitrogen source	IAA production (µg/ml)
1	Yeast extract	40
2	Beef extract	38
3	Peptone	48
4	Tryptone	20
5.	Soyabean	11

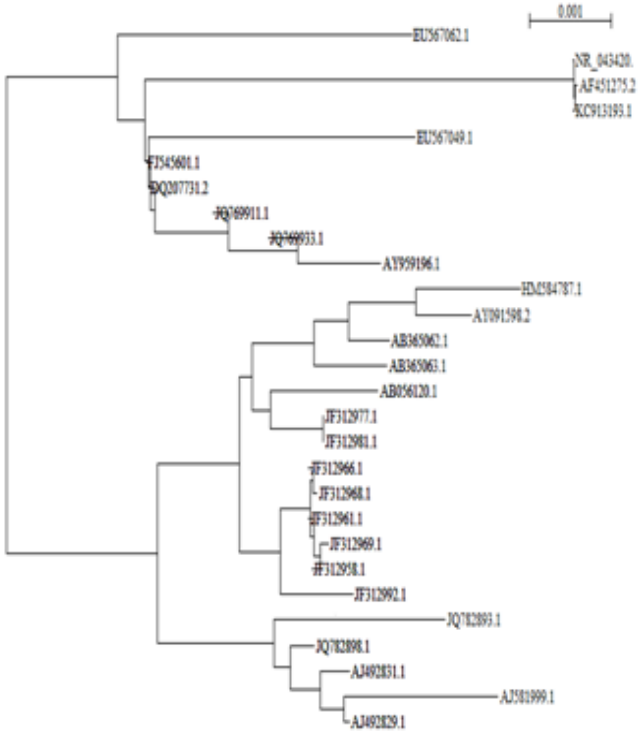

Fig 1: Phylogenetic tree from partial 16S rRNA gene sequences of *Pseudomonas fluorescence*. Sequences of type strains obtained from databases and accession numbers

Pseudomonas fluorescens IAM 12022 **(**NR_043420.1) *Pseudomonas* sp. AH2 (AF451275.2) *Pseudomonas fluorescens* CCM 2115 (DQ207731.2) *Pseudomonas* sp. clone TCCC 11142 (EU567062.1) *Pseudomonas poae* zol-15 (JQ782898.1) *Pseudomonas* sp. PH-03 (AY091598.2)
Pseudomonas sp. PH-03 (AY091598.2)
Pseudomonas sp. SW83 (HM584787.1)
Pseudomonas sp. PH-03 (AY091598.2)
Pseudomonas sp. Pi 3-21(AB365062.1)
Pseudomonas sp. Pi 3-62 (AB365063.1)
Pseudomonas veronii (AB056120.1)
Pseudomonas sp. HC3-7(JF312977.1)
Pseudomonas sp. HC3-11(JF312981.1)
Pseudomonas sp. HC2-27(JF312966.1)
Pseudomonas sp. HC2-29 (JF312968.1)
Pseudomonas sp. HC2-22 (JF312961.1)

Pseudomonas sp. HC2-19 (JF312958.1) *Pseudomonas* sp. HC4-5(JF312992.1) *Pseudomonas poae* zol-15(JQ782898.1) *Pseudomonas trivialis*(AJ492831.1) *Pseudomonas lurida*(AJ581999.1) *P.poae*(AJ492829.1).

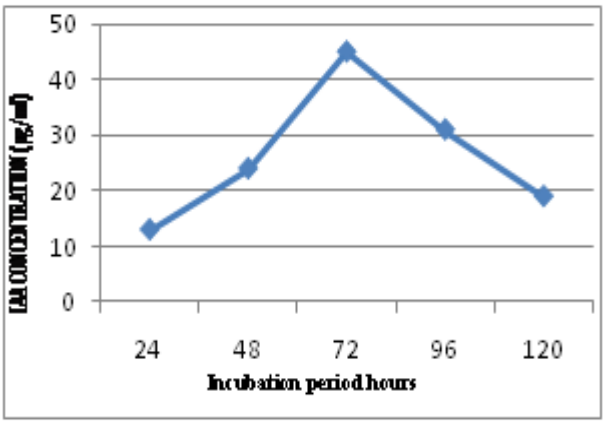

Fig 2: Effect of incubation period on indole acetic acid production

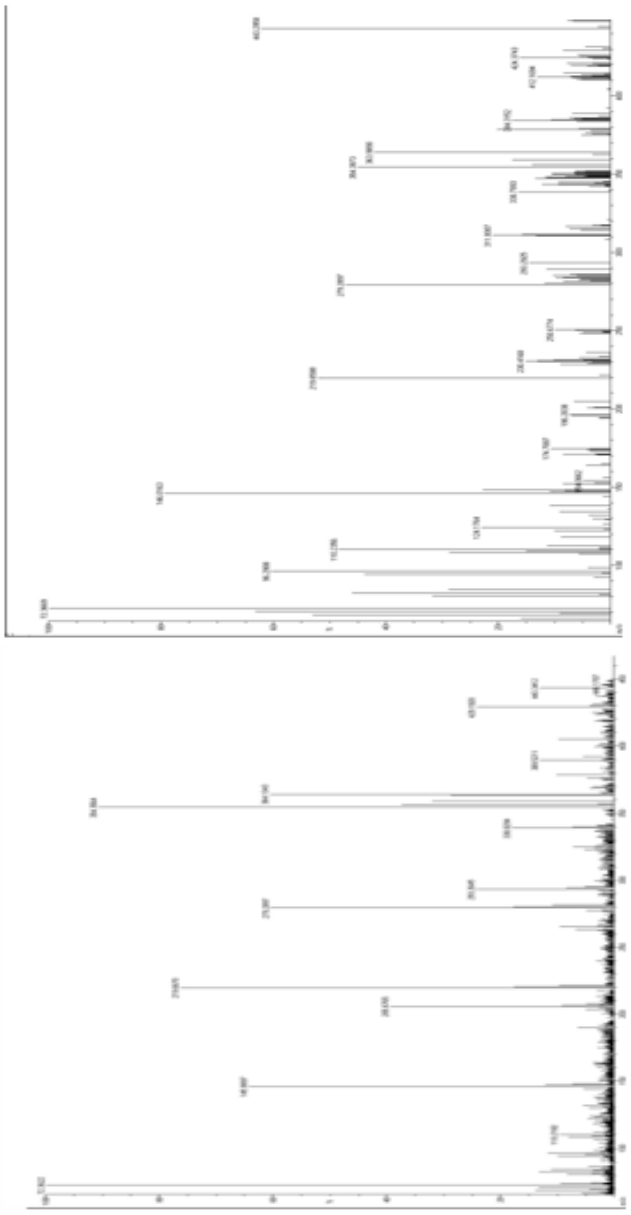

Fig 3: Mass spectra of (a) methylated standard IAA and (b) methylated purified compound from *Pseudomonas fluorescence*

4. CONCLUSION

Pseudomonas fluorescence strains have other traits in addition to IAA production. Hence these strains have the potential of being developed as bioinoculants**.** The effect of IAA on plants is being explored through ongoing plant inoculation studies. Further studies in this strain as formulation of bio fertilizers will help farmers to utilize the bacteria in fields and reduce their dependence on chemical fertilizers.

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