

## Production of Auxin Hormone by Fluorescent Pseudomonads

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**Abstract:** Plant growth promoting rhizobacteria (PGPR) are considered to promote plant growth directly or indirectly. *Pseudomonas* bacteria, specially *P. fluorescens* and *P. putida* are the most important kinds of PGPR. Production of auxin is one of the main reasons to promote yield because of inoculation with this bacteria. In this research fifty strains of Fluorescent pseudomonads belong to microbial bank of Soil and Water Research Institute, isolated from Iran soils, selected and evaluated about secretion of auxin compounds. In HPLC device, 72% of the strains exuded at least one type of indolic auxin composites. The amount of exuded IAA by *P. fluorescens* strains was varied from zero to 31.6 mg/l while it was producing from zero to 24.08 mg/l in *P. putida*. The amount of exuded IAM by *P. fluorescens* and *P. putida* was between 0-16.2 and 0-17.2 mg/l, respectively. Also these strains exuded 0-7.2 mg/l ILA for *P. fluorescens* and 0-10 mg/l for *P. putida*. Neither of experimented strains exuded the IBA. The results showed that 65% of the studied *P. fluorescens* used IAM pathway to synthesize IAA and 35% used the IAM and IPyA path, while 48% of the *P. putida* through IAM, 41% through both paths and 7% used the only IPyA path towards IAA synthesize. But 78% of the strains studied in spectrophotometry exuded auxins which their amounts were producing between 0-7.09 mg/l for *P. fluorescens* and 0-4.40 mg/l for *P. putida* strains.

**Key words:** Auxin • Pseudomonads • HPLC • Colorimetric Method

### INTRODUCTION

Growth regulators are those of carbonic composites which are able to control physiological processes in low densities. These composites are found in natural and artificial forms and are categorized into five main groups based on their chemical structure and physiological effects. These groups are auxins Gibberellins, Cytokinins, Absciscic acids and Ethylene [1].

Plant growth excretion is one of the chief plant growth promotion mechanisms [2]. Abundant studies have emphasized the instigation of plant growth and the seed positive reaction or inoculated root with promoters. Soil microbiota, especially rhizospheral microflora are the sources of these materials instigation [3, 4]. Different micro-organisms have been recognized to exude different plant growth promoters [3- 6]. Among the plant growth micro-organisms which are capable to produce herbal hormones we can point to *Azotobacter*, *Pseudomonas*, *Azospirillum*, *Rhizobium*, *Bacillus*, *Enthrobacter* and Mycorrhiza fungus [3, 7-9].

Misco and Germida 2002, reported through an experiment that the *Pseudomonas* is the most abundant

auxin producer 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]. Auxins are a group of herbal hormones which IAA is the most important of them [11].

IAA is a natural auxin with vast physiological effects [12] which plays an important role in growth, increasing and distinction [11].

IAA is produced through L-TRP metabolism by plants and many of soil micro-organisms such as bacteria, fungus and algae [13]. Indole-3-Pyruvic acid, Indole-3-Acetaldehyd and Indole-3-Acetonitrile 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 [6]. Frankberger and Brunner recognized the main indoles in soil as Indole-3-acetamide (IAM), Indole-3-pyruvic acid (IPYA), Indole-3-acetic acid (IAA) by HPLC (High performance liquid chromatography) device. There is a report that in ILA anaerobic conditions, the IPYA reduced form is framing, Tryptamin is also a natural

composition and the median process of changing Tryptophan to IAA [13].

There are five observed paths to produce IAA from Tryptophan but the bacteria use only three ways of IAA metabolic synthesise [12, 14]. In IAM path, two enzymes are engaged in IAA production Tyrptophan-2-mono oxygen is the first enzyme which catalyzes the changing L-TRP to Indole-Acetamides. The second enzyme is Indole-Acetamide hydrolyser which change IAM to IAA [15]. In IPYA path, Tryptophan is changed to IPYA by the use of D-Amination and following is changed to IAAId on the effect of carbonization and eventually is oxidized and changed to IAA [7].

In Tryptophan side-chain oxidizes, IAAId plays a median path role [15] which IAA is formed through its oxidation by Acetaldehyde D-hydrogenised enzyme.

The aim of this research was evaluating the auxin productivity potential in studied *Pseudomonas* strains through chromatography, using HPLC devise; comparing two above mentioned methods and appointing IAA synthesise method by the studied strains in the applied cultivars.

## MATERIALS AND METHODS

At this stage, 50 strains of *P. fluorescens* and *P. Putida* were selected in a random block from the soil and water research institute microbial bank, isolated from the Iranian soils. After ensuring of bacteria's purity, we cultivated them in a Broth containing environment.

Then the samples prepared for read and measuring by HPLC and spectrophotometer in two different stages. To prepare the bacteria's for read by HPLC device; the containing broth transferred to a refrigerator centrifuge and kept in 20000 rpm for 20 min in a 4°C temperature. The above mentioned auxin containing solution's pH fixed on 3 through applying 2<sup>nd</sup> HCL. Then the solution transferred to D-counter, 50 ml Ethyl-Acetate added and shacked well. After the tranquility condition, the upper layer of the solution which contains the auxin, separated and the inferior layer extracted with e-applying of 50ml of ethyl-Acetate. The upper solution of both extraction stages dried in vacuum evaporator and the resulted sediment solved in 1 ml menthol and prepared for injecting to HPLC device.

In this research the Beckman HPLC device model and the 168 type of detector was applied. The specification of column and model are as:

Column=C18, Beckman Ultrasphere, 4.6mm\*25cm  
Mobile phase: 70% H<sub>2</sub>O + 0.1% TFA

Flow rate: 0.8 ml/min

Wave Length: 260nm

To prepare the bacteria to be read by the spectrophotometer device, 2ml of bacteria containing broth transferred to 2ml tips refrigerator centrifuge and centrifuged for 10min in 15000rpm. Then 1ml of above mentioned solution mixed with 4ml of Salkowsky indicator (containing 150ml HCL, 250 ml of purified water and 7.5 ml of 0.5 molar FeCl<sub>2</sub>).

Then it was kept for 20 min in dark room temperature and the light absorption measured in 535 nanometer of wave length by spectrophotometer device. The auxin amount calculated with the comparison of this absorption standard curve in 0, 5, 10, 15 and 20 mg/lit Indole-Acetic density.

To supply the spectrophotometer standard we prepaid 1lit of the primary environment, then 0.05 gram of IAA weighted and added to 50ml of above mentioned solution. The resulted solution is 100mg/kg which 0, 5, 10, 15 and 20 mg/lit standards are obtained from.

## RESULTS AND DISCUSSION

In this study 72 percent of experimented *Pseudomonas fluorescens* and *Pseudomonas Putida* strains, exuded at least one composite of Indole auxins.

In different *P. fluorescens* strains of this research, the abundance of capabale to produce IAA, IAM, ILA and IBA were 10, 15, 8 and 0 respectively. In different *P. fluorescence* strains of this research, the abundance of capable to produce IAA, IAM, ILA and IBA were 12, 13, 11 and 0 respectively. Totally no one of the used *Pseudomonads*' here was able to produce IBA. Accumulation of different auxin kinds in this bacteria metabolite were IAM, IAA and ILA in high to low ranking.

In Tables 2 and 3 the amounts of IAA, IAM, ILA and IBA produced by experimented strains is mentioned.

Table 1: The bacteria composition prepared environment for auxin hormone measuring through HPLC method [6]

Amount	Chemical Substance
K <sub>2</sub> HPO <sub>4</sub>	0.4 gr
KH <sub>2</sub> PO <sub>4</sub>	0.1 gr
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 gr
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.5 gr
Nacl	0.1 gr
CaCl <sub>2</sub>	0.1 gr
Malic acid	2.5 gr
Yeast extract	0.5 gr
L-Trp	10 mg
Distilled water	1000ml

Table 2: The amounts of IAA, IAM, ILA and IBA produced by *P. fluorescens* in HPLC device

Strain	The amount of produced auxin hormone (mg/l)			
	IBA	ILA	IAM	IAA
P4	4.72	6	5.8	-
P10	0.6	-	-	-
P11	24.08	16.4	-	-
P34	0.96	17.2	-	-
P41	2	1.84	0.88	-
P50	-	1.2	1.76	-
P53	1.12	7.68	-	-
P56	2.64	16.4	0.16	-
P74	-	1.52	-	-
P98	0.96	-	-	-
P100	-	-	1.92	-
P101	2	-	5	-
P103	1.24	-	-	-
P139	-	-	1.24	-
P143	7.04	5.52	10	-
P147	-	1.52	1.32	-
P168	-	1.52	-	-
P179	0.16	0.6	2	-
P183	-	6.12	0.88	-

Table 3: The amounts of IAA, IAM, ILA and IBA produced by *P. putida* in HPLC device

Strain	The amount of produced auxin hormone (mg/l)			
	IBA	ILA	IAM	IAA
P4	4.72	6	5.8	-
P10	0.6	-	-	-
P11	24.08	16.4	-	-
P34	0.96	17.2	-	-
P41	2	1.84	0.88	-
P50	-	1.2	1.76	-
P53	1.12	7.68	-	-
P56	2.64	16.4	0.16	-
P74	-	1.52	-	-
P98	0.96	-	-	-
P100	-	-	1.92	-
P101	2	-	5	-
P103	1.24	-	-	-
P139	-	-	1.24	-
P143	7.04	5.52	10	-
P147	-	1.52	1.32	-
P168	-	1.52	-	-
P179	0.16	0.6	2	-
P183	-	6.12	0.88	-

Table 4: Auxins measured by the spectrophotometer device

<i>P. putida</i>		<i>P. fluorescens</i>	
IAA (mg/l)	Isolation No.	IAA (mg/l)	Isolation No.
0.63	P4	0.17	P3
0	P9	1.47	P6
0	P10	0	P31
1.44	P11	3.67	P65
0.28	P34	0.98	P71
3.02	P41	0.91	P79
0	P50	0	P82
1.58	P53	3.91	P87
4.31	P56	0	P88
0	P68	2.15	P99
3	P74	0.11	P111
4.46	P98	2.48	P120
0.52	P100	0.71	P145
0.51	P101	1.04	P153
0	P103	0	P157
0.90	P108	0	P161
0.031	P112	5.95	P162
2.24	P123	7.09	P169
4.01	P130	1.95	P173
3.67	P139	0.17	P174
2.24	P143	0.04	P189
0.78	P147	2.87	P194
3.05	P159	7.08	P196
0.8	P168		
2.03	P179		

The amount of auxin measured by spectrophotometer device, produced by *P. fluorescens* strain was tolerated between 0 and 7.09 mg/l. Six of the 23 experimented strains were disable to produce auxin. The amount of auxin measured by spectrophotometer device, produced by *P. Putida* strain was tolerated between 0 and 4.46 mg/l.

Many studies have reported auxin excretion by *Pseudomonas* strains. In most of these studies, it is measured through spectrophotometer device. This is a color measuring method which is so easy and accessible and based on measuring the darkness, evaluates the existing inodole rings in the environment.

Barea *et al.* [17], reported in an experiment that in 150 bacterial isolation collected from different plant rhizosphers, 58, 86 and 90 percent auxin, gibberellins and semi-auxin compound were exuded respectively. Moreover, in another research the amount of auxin in *P. putida* strain and its mutant measured in L-TRP and reported, the amount of auxin in the absence of L-TRP was 0.5µg/ml and the scope of produced auxin was

32.7µg/ml in the presence of 500µg/ml L-TRP. The mutant of mentioned bacteria in the presence of 500µg/ml L-TRP exuded only 2µg/ml. Both of the strains and their mutants exuded a little auxin (0.5µg/ml) in the absence of L-TRP [18].

Benizri *et al.* [19], reported the auxin excretion in their research on *P. fluorescens* M.3.1 as 2.5µg/ml. In two separate studies carried out on *P. fluorescens* by Leinhos and Vacek [20] and Prikryl *et al.* [21]; the amount of produced auxin reported as 1.6-3.3 mg/l and 0.01-3.93mg/l.

Ataei [22] measured the amount of auxin production in 202 *Pseudomonas* strains isolated from Iran's soils by spectrophotometer device and reported the facts. In the absence of L-TRP the auxin excretion was between 3.316mg/l to 76.697mg/l, meanwhile it was between 3.997mg/l to 67.987mg/l in the presence of 50mg L-TRP.

Abbaszadeh [23] experimented the ability of auxin production in 40 *P. fluorescens* in two DF and TSB environments and with different L-TRP densities. The amount of produced auxin in DF environment with no L-TRP was 0.13-0.22 mg/l and in L-TRP=200 mg environment was 0.91-63.7 mg/l. Either, the amount of produced auxin in TSB environment with no L-TRP was 0.71-23.1 mg/l and in L-TRP=200mg environment was 0.86-21.27mg/l. The IAA excretion in 18 *Acetobacter diazotrophicus* strains measured by spectrophotometer device was between 19 to 56 mg/l auxin [24]. These results were fully in agreement with the reported results for *A. brasilense* [25, 26].

In the present study we got a regression between the amounts of IAA, IAM and ILA; evaluated by HPLC device and spectrophotometer auxin but we had no significant correlation. In another study, Crozier *et al.* [27] measured the IAA amount of 20 *A. 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. All of the studied strains in HPLC produced IAA; meanwhile some of them did not in Salkowsky method. The results showed that we have a low correlation between the extracted results in two HPLC and Salkowsky methods. For instance, the amount of produced IAA by *A. brasilense* 703 Ebc strain in Salkowsky method was 26.1 µg/ml, it was 0.5 µg/ml in HPLC method, though. Considering the results he suggested that the results in Salkowsky method have less accuracy and they must be considered with a bit of obsession. Our results were the same as results done by

Crozier *et al.* [27]. We also could not find any significant correlation between the HPLC and Salkowsky. The only study by Frankenberger and Brunner, [28] compared different methods of soil auxin determination and concluded that HPLC method is superior to all of the other methods including TLC and GC. In Asghar *et al.* [29] report, the capability of rhizospheric bacteria's in auxin production measured by spectrophotometer device and only two strains have been measured by HPLC device in manner of indole rings separation, which only the type of them is mentioned and their amount is not mentioned. In another research carried out by Fuentes-Ramirez *et al.* [30], the amount of IAA, IAAId, ILA and IPYA in 10 strains isolated from different parts of sugarcane plant and rhizosphere, measured in HPLC method. These results were contrary to the results of researches carried out by Tang and Bonner [24], Hartmann *et al.* [25] and Jain and Patriquin [26], which had measured auxin in *A. brasilense* through Salkowsky method. This is a proved fact that measuring IAA through Salkowsky method could not be reliable [27]. In a study by Crozier *et al.* [27], the measured amounts of IAA for rhizospheric *Azospirillum* through HPLC method were the same as results reported by Fuentes-Ramirez *et al.* [30].

Plat and Timan [31], believed that presence of different reducers such as Hydroquinone, Ascorbic Acid, Cistein and phenols such as Katcol, Risorkinol, Fluoroglokinol and brilliant medians which have similar colors with IAA compounds are the most important factors in errors of spectrophotometer method. On the other hand, the spectrophotometer methods are not capable to separate different indole compounds [28].

Through an experiment by Crozier and Reeve [32], they 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. Ethyl acetate is used as a vaporizable un-polar solvent, because the IAA is simply solved in this carbonic solvent and distried from liquid phase. On the other hand, Ethyl acetate is simply vaporized in Vacuum Evaporator device and leaves the solved auxin as sediment without any oxidation or explosion [28].

The samples preparation method is different and more convenient in spectrophotometer rather than HPLC. In this device we can only report the darkness of the samples as a total indole compounds called auxin, in comparison to defined standards. This device can not separate indole compounds and report them independently.

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