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PRODUCTION OF INDOLE ACETIC ACID AND ALGINATE FROM *AZOTOBACTER VINELANDII* ISOLATED FROM PADDY FIELDS

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ABSTRACT

In the present study, a total of 25 soil sample were taken from the different paddy fields and 15 isolates of *Azotobacter* was obtained. Six isolates of *Azotobacter vinelandii* were screened for the ability to produce Indole acetic acid and a biopolymer alginate. Out of the 6 isolates of 3 isolates showed an increased production of Indole Acetic Acid and alginate.

Azotobacter vinelandii was supplemented with the various concentration of tryptophan (0, 1, 2 and 5mg) and the production of Indole Acetic Acid was estimated by the increasing development of the pink colour. Extraction of Indole Acetic Acid is done by ethyl acetate and thin layer chromatography is done using Ehmann's reagents. Indole-3-acetic acid (IAA) is the major and most abundant auxin in plants. Indole Acetic Acid plays a key role in the regulation of plant growth and development.

A biopolymer Alginate is produced by *Azotobacter vinelandii* and is concentrated by using sodium and Isoproponal. The highest alginate yields occurred in rich medium in nitrogen and phosphate, and glucose as carbon source, by aerating the medium with agitation at 280rpm.

Although, sea weeds are the major source of alginate for commercial use, however, the alginate produced by the bacteria is considered to be of good quality as compared to that obtained from the algae. The bacterial alginate has better qualitative properties. *Azotobacter vinelandi* is more

suitable for the biosynthesis of alginate in view of its latent utilization as a food stabilizer. The alginate is widely used as thickening stabilizing, gelling and emulsifying agent in food, textile, paper and pharmaceutical industries.

Keywords: Indole Acetic Acid, Tryptophan, Alginate, *Azotobacter vinelandii*

INTRODUCTION

Soil is an ecosystem inhabited by various groups of microorganisms that constitute its live component. Active microbiological processes in soil increase the speed of synthesis and mineralization of organic matter leading to better plant nutrition. Chemical fertilizers and bio-fertilizers enhance the sustainability of crop production. However excessive use of chemical fertilizers has generated several environmental problems. Some of these problems can be tackled with the use of natural bio fertilizers, which are beneficial and ecologically friendly. Knowledge about microbiological processes and factors on which they depend allows for the possible use of many microorganisms in agriculture. Symbiotic nitrogen fixators have been commonly used in legume production and there is now a growing interest in the use of free nitrogen fixators in other agricultural production.

Nitrogen fixation can be considered as one of the most interesting microbial activity as it makes the recycling of nitrogen on earth possible and gives a fundamental contribution to nitrogen homeostasis in the biosphere. Among the free-living nitrogen-fixing bacteria those belonging to genus *Azotobacter* play a remarkable role, being broadly dispersed in different environments, such as soil, water and sediments. In fact, field trials have demonstrated that under certain environmental conditions, inoculation with *Azotobacter* has beneficial effects on plant yields **[1]**. Due to the increase of fixed nitrogen content in soil and to the microbial secretion of stimulating hormones.

Experiencing the adverse effects of synthetic input dependent agriculture the concept of organic agriculture is gaining momentum **[2]**. The excessive use of chemical fertilizers has generated several environmental problems including the greenhouse effect, ozone layer depletion and acidification of water. These problems can be tackled by use of biofertilizers .Soil microbes are of great importance in cycling nutrients such as carbon (C), nitrogen (N), phosphorus (P), and sulphur (S). Not only do they control the forms of these elements (e.g. specialized soil bacteria convert ammonium N (NH_4^+) to nitrate (NO_3^-), they can also regulate the quantities of N available to plants **[3]**. Beside their effects on the availability of nutrients the bacterial soil life prevents the

uptake of several harmful ions. The use of living bacteria (biofertilizer) accelerates mineralization of organic residues in soil, therefore makes the nutrients more available. At the same time due to effect of living bacteria from biofertilizer, the uptake of heavy metals decrease. To minimize the uses of chemical fertilizers, to improve the soil fertility status and for enhancement of crop production by their biological activity in the rhizosphere, application of beneficial microbes in agricultural practices started 60 years ago and there is now increasing evidence that these beneficial microbial populations can also enhance plant resistance to adverse environmental stresses, e.g. water and nutrient deficiency and salt stress.

Salinity is a major environmental constraint to crop productivity throughout the arid and semi arid regions of the world. A third of arable land resources in the world are affect by salinity The salt stress negatively affects plant growth and biological stability of ego systems. Seed germination is an important factor limiting plant growth the saline condition can decrease seed germination either by creating osmotic potential that prevents the absorption of water by toxic effects resulting high concentration of Na^+ in the soil thus, the limitation of water absorption can cause various structural, physiological and bio chemical modifications of seeds that can reduce the rate of germination and retard plant development.**[4].**

Plant Growth Promoting Rhizobacteria (PGPB) is considered to promote plant growth directly or indirectly. PGPB can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (auxin, gibberellin, ethylene etc.), siderophores, HCN and antibiotics **[5]**. Indole acetic acid (IAA) is one of the most physiologically active auxins.

Indole-3-acetic acid is a common product of L-tryptophan metabolism by several microorganisms including PGPR **[6]**. Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites, because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils. Plant morphogenic effects may also be a result of different ratios of plant hormones produced by roots as well as by rhizosphere bacteria. Growth regulators are produced by Azotobacter in culture media **[7]**. Indole-3-acetic acid (IAA) is the main auxin in plants, controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity. Bacterial Indole-3-acetic acid producers (BIPs) have the potential to interfere with any of these processes

by input of Indole-3-acetic acid into the plant's auxin pool **[8]**.

Alginates form an important family of biopolymers of both technological and scientific interest. These polymers are linear polysaccharides, which are composed of variable amounts of (1–4)-β-D-mannuronic acid and its epimer, α-L-guluronic acid. Optimal conditions enhance bacterial alginate production **[9]**. Bacterial alginates are produced by a mutant of *Azotobacter vinelandii* **[10]**. Alginates present a wide range of applications, acting for example as stabilizing, thickening, gel or film-forming agents, in various industrial fields.

In vitro mutagenesis is an important technique which can induce stress tolerance and improve the yield and quality of crop plants. Plant improvement based on mutations can change one or more specific traits of a cultivar, which can enhance the quality and quantity of crops. Conventional induced mutations have well-defined limitations, especially in crop-breeding applications but the use of in vitro techniques with the conjunction of conventional mutagenesis has overcome this barrier. Molecular techniques can provide a better understanding about the potential and limitations of mutation breeding. In vitro mutation induction has high potential to enhance the crop yields that can be used for the improvement of life style of the mankind **[11]**.

This present work aims at the production of Indole acetic acid and alginate from *Azotobacter vinelandii* from different paddy field.

MATERIALS AND METHODS

Collection of Soil Sample

Soil samples were collected from rhizosphere region of different paddy field. Samples were collected in polythene bags from the selected sites at a depth of 10-15cm. the samples were then immediately transported to lab for further processing.

Preparation of Medium

Jensen's medium was used to be the selective medium for the isolation of *Azotobacter*.

Isolation of *Azotobacter*

Azotobacter was isolated from each sample by serial dilution and spread plate method. 1g of soil sample was mixed with 9ml of autoclaved distilled water and was thoroughly shaken.1ml of the above solution was again transferred to 9ml of sterile distilled water to obtain 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions for each soil sample0.lml of each dilution was spread on Jensen's medium and incubated at 27-30˚C for 72-80 hours. Large mucoid, dewdrop like colonies were characterized for bio-chemical properties.

Characterization of the Isolate

The isolates were characterized as per the biochemical characterization and the results were recorded.

Production of Bacterial Alginate

Burk nitrogen-free medium fortified with 2% sucrose and 1.5% agar was used as the medium for slant culture. 100ml of Jensen's medium was prepared and inoculated with the culture and the pH was adjusted to 6.5.

Concentration of Bacterial Cells (*Azotobacter*)

Reagents required: 20% EDTA disodium salt: 20g of EDTA disodium salt were dissolved in 100ml of distilled water.

Procedure

100ml of the Jensen's culture broth was diluted with the 6ml of 20% EDTA disodium salt. The mixture was centrifuged at 15,000 rpm for 30minutes.The cell precipitate was washed with the 120ml of distilled water and then centrifuged again for 15,000rpm for 30minutes.Supernatant was discarded and the cell precipitate was tapped. Finally, the cell precipitate was dried.

Concentration of Alginate From the Bacterial Cells

Reagents required: 20% sodium chloride: 2g of sodium chloride was dissolved in 10ml of distilled water. 95% isopropanol: 95ml of isopropanol was diluted in 5ml of water.

Procedure

100ml of culture broth was taken and 1ml of 20% sodium chloride was added. Alginate and the cells of the mixture were precipitated with 40ml of 95% isopropanol. The collected precipitated was then dehydrated with a small amount of 95% isopropanol. The mixture was centrifuged at 10,000rpm for 15minutes.The precipitate was dried to a constant weight at 105˚c.The alginate weight was obtained by subtracting the dried cell weight from the dried precipitate weight. The concentration of cells and alginate was expressed in grams per liter.

Immobilization of Bacterial Cell With Bacterial Alginate *(Azotobacter*)

Reagents: 1% Barium Chloride: 1g of Barium chloride was dissolved in 100ml of distilled water.

Medium

Jensen's medium-100ml., Bacterial Alginate obtained from the above procedure.

Procedure

Overnight culture of *Azotobacter vinelandii* was pelleted out at 10,000rpm for 10 minutes. The harvested cell were treated with sterile saline solution and suspended in 25ml of sterile distilled water. Separately 2% sterile bacterial Alginate solution was prepared. It was mixed with a suspension and resulting mixture extruded drop wise through a hypodermic needle into sterile ice cold barium chloride solution. Special beads of size 2 to 3mm were formed and kept in barium chloride for one hour for stabilization. Then they were washed thrice with sterile distilled water. The activity of

immobilized cells was assayed by incubating with 5mg/ml of tryptophan for 15days.

Production of Indole Acetic Acid

Reagents Required

Tryptophan – (1,2 and 5mg/ml), 35% perchloric acid: 35ml of perchloric acid was dissolved in 100ml of distilled water., 0.5g of Ferric chloride: 0.5g of ferric chloride was dissolved in 1ml of distilled water., and Solawaski's reagent: 98ml of 35% perchloric acid was mixed with 2ml of ferric chloride.

Procedure

100ml of sterilized Jensen's medium was taken in a conical flask. Test bacterial culture was inoculated in the medium. To the 100ml of Jensen's medium, tryptophan was added in a various concentration (1, 2 and 5mg/ml) and one set up was kept without tryptophan. Culture medium were incubated at 28-30˚c for 15 days. Cultures were centrifuged at 3000rpm for 30 minutes. Supernatant was removed and 2ml of supernatant was mixed with 4ml of solawaski's reagent. OD value read at 530nm using spectrophotometer. The level of Indole acetic acid production was estimated.

Extraction of Partially Purified Indole Acetic Acid

Reagents

Tryptophan (1mg and 5mg/ml).,1N of Hydrochloric acid**:** 0.1ml of hydrochloric acid was dissolved in 50ml of distilled water**,** Ethyl acetate-50ml and Methanol -300ml

Procedure

Isolated *Azotobacter* colonies were inoculated in 200ml of Jensen's broth.1mg/ml and 5mg/ml of tryptophan were added to the medium. The culture broth were incubated at 28-30˚c for one week on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 10,000rpm for 30minutes. The supernatant was acidified with 1N HCL and pH was adjusted to 3. Ethyl acetate was added at the double the volume of supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotatory evaporator at 40˚c.The extract was dissolved in 300ml of methanol and stored at -20˚c

Thin Layer chromatography

Reagents

Silica gel (2:1 ratio): 50g of silica gel was dissolved in 100ml of water (stationary phase).
Solvent (70:25:5): 70ml of benzene was added to 25ml of n-butanol and dissolved in 5ml of acetic acid (mobile phase)**.**

Ehmann's Reagent

Reagent A (Van Urk37 Reagent)

1 g p-dimethylaminobenzaldehyde decolorized with activated carbon and recrystallized from ethanol and water was dissolved in 50 ml of concentrated Hydrochloric acid and 50 ml

absolute ethanol was added and stored in a brown glass bottle.

Reagent B (Salkowski Reagent)

2.03g ferric chloride were dissolved in 500 ml water and 300 ml concentrated sulphuric acid.

Procedure

Silica gel was prepared and poured on the TLC plate. The silica gel was equally spread on the plates with the spreader. The thickness of the gel was 0.25mm.The plates were dried in hot air oven for 30 minutes. The plates were spotted by 5µl of ethyl acetate fraction. The plates were placed against the solvent in the tank. Ehmann's reagent was sprayed on the plate. The spots were identified under UV lightspots were measured and the Rf values were calculated.

Calculation

$$(Rf) = \frac{\text{Distance moved by the Indole acetic acid}}{\text{Distance moved by the solvent}}$$

Rf= Resolving front

RESULTS AND DISCUSSION

Biofertilizers are used extensively as an eco-friendly approach to minimize the use of chemical fertilizer, improve soil fertility status and for enhancement of crop production by their biological activity in the rhizosphere. Plant Growth Promoting Rhizobacteria (PGPR) enhance the supply of other nutrients (P mobilization, S oxidization, Fe chelating), phytochrome production leading to increases in root surface (IAA, cytokinin, gibberllin).

Microbial isolates from the rhizosphere of different crops appear to have a greater potential to synthesize and release Indole acetic acid as secondary metabolites because of the relatively rich supply of substrates. Indole-3-acetic acid (IAA) is the main auxin in plants, controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity. *Azotobacter vinelandii* is more suitable for the biosynthesis of alginate in view of its latent utilization as a food stabilizer.

In the present study, **Table 1** shows a total of 25 soil samples were taken from different paddy fields and 15 isolates of *Azotobacter* was obtained, 4 isolates were obtained (AzR1, AzR2, AzR3, AzR4) from Redhills paddy fields, 6 isolates (AzN1, AzN2, AzN3, AzN4, AzN5, AzN6) from Nellore, 3 isolates (AzM1, AzM2, AzM3) from Madharpakam and 2 isolates (AzP1, AzP2) from Ponari paddy field. They were found to be a fast grower and dew drop colonies were observed **(Figure 1)** in 72 hours incubation period. Among the 15 isolates, the 6 isolates of *Azotobacter vinelandii* were isolated based on biochemicals test and sugar utilization. They were selected for further study.

In the present study, *Azotobacter vinelandii* was supplemented with the various concentration of tryptophan (0, 1, 2 and 5mg/ml), and the production of Indole acetic acid was estimated

by the increasing development of the pink color **(Figure 2)**. OD value was taken for the 6 *Azotobacter vinelandii* isolates. **Table 2** shows that among the 6 isolates of *Azotobacter vinelandii*, the 3 isolates namely AzR2, AzN6 and AzM2 showed an increasing absorbance value.

The crude extraction of Indole acetic acid was done for these three isolates. These 3 isolates were screened for their ability to produce plant growth regulator Indole acetic acid. Varying levels of Indole acetic acid production were recorded with different concentrations of tryptophan (0, 1 and 5 mg/ml). The range of Indole acetic acid production in *Azotobacter vinelandii* isolates without tryptophan was 4.40- 9.44mg/ml. A significant increase in the production of Indole acetic acid was recorded in the presence of 1 and 5mg/ml of tryptophan, 7.25-12.69 mg/ml and 27.0-30.80mg/ml respectively was shown in **Figure 3**. Our findings of Indole acetic acid production in *Azotobacter vinelandii* isolates are in agreement with those of other researchers.

Thin Layer Chromatography was performed for these 3 isolates of *Azotobacter vinelandii*, the Indole acetic acid migration was measured. There was a considerable increase in the R*f* values of AzR2 (6.0-7.0), AzN6 (7.2-8.0) and AzM2 (6.7-7.9) shown in **Table 3** and **4**. The R*f*values concurrent with that observed by **[12]** on his work on the production of Indole acetic acid. The highest Indole acetic acid producing *Azotobacter vinelandii s*was found to be AzN6 isolate **(Figure 3)**.

In the present study, **Table 5** shows that the production of alginate by the six isolates of *Azotobacter vinelandii*. Cell concentration was high for the isolates (AzN2, AzN6 and AzM2). These isolates were further tested for alginate production. The highest Indole acetic acid and alginate producing *Azotobacter vinelandii* was found to be AzN6 isolate **(Figure 4)**.

Table 1: Sample for Study

S.NO	PADDY FIELD	NO OF SOIL SAMPLE	NO OF ISOLATES
1	Redhills	7	4 (AzR1, AzR2, AzR3, AzR4)
2	Nellore	8	6 (AzN1,AzN2,AzN3,AzN4,AzN5,AzN6)
3	Madharpakam	6	3 (AzM1,AzM2,AzM3)
4	Ponari	4	2 (AzP1,AzP2)

Table 2: Absorbance Values

TYRPTOPHAN CONCENTRATION (mg/ml)	AzR2	AzN6	AzM2
0	**0.021**	**0.528**	**0.271**
1	**0.534**	**1.725**	**0.933**
2	**1.642**	**2.067**	**1.037**
5	**2.449**	**4.392**	**3.001**

Table 3: R*f* Values

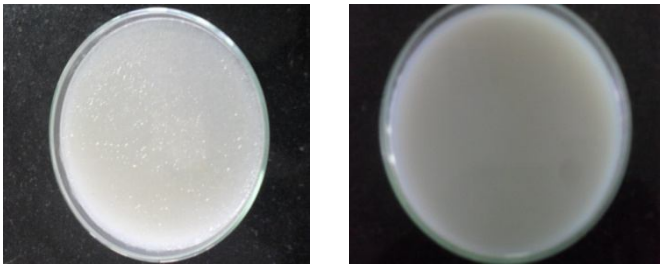
TYRPTOPHAN CONCENTRATION (mg/ml)	AzR2	AzN6	AzM2
0	6.0	7.2	6.7
1	6.4	7.6	7.0
5	7.0	8.0	7.9

Table 4: R*f* Values

TYRPTOPHAN CONCENTRATION (mg/ml)	R*f* VALUES		
	AzR2	AzN6	AzM2
0	0.6	0.72	0.67
1	0.64	0.76	0.7
5	0.7	0.8	0.79

Table 5: Alginate Production

ISOLATES	ALGINATE CONCENTRATION mg/ml
AzN2	**5.24**
AzN6	**6.0**
AzM2	**5.10**



Azotobacter **Control**

Figure 1: Azotobacter and Controll Plates

Figure 2: Estimation of IAA Production

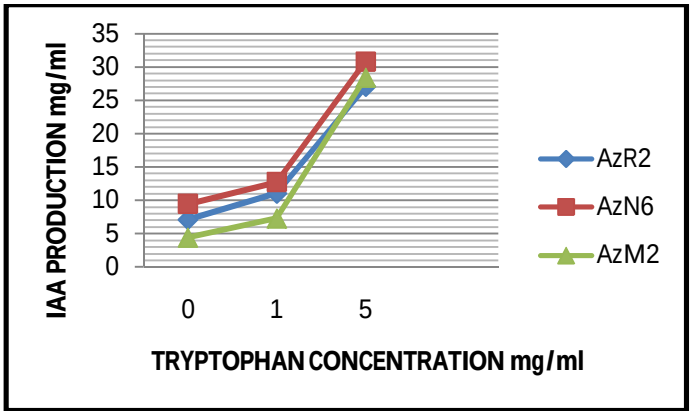


Figure 3:

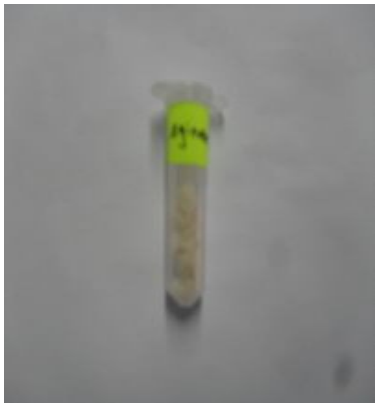


Figure 4: Alginate Production

CONCLUSION

The present study investigated the influence of tryptophan in inducing indole acid production, the precursor for an important plant hormone auxin. Optimum conditions for production of alginate were characterized and thus the cost of alginate to various industries could be reduced dramatically if bacterial alginate were chosen. Literatures have revealed that bacterial alginates were superior

to those of other chemical alginates and strain improvement will further augment its industrial application

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