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Production and Optimization of Indole Acetic Acid by Indigenous Micro Flora using Agro Waste as Substrate

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Abstract: Indole Acetic Acid (IAA) producing bacterium was isolated from the Rhizosphere soil and identified as *Rhizobium* sp. and *Bacillus* sp., Optimization of Indole acetic acid production was carried out at different cultural conditions, such as pH, temperature and substrate with *Rhizobium* sp., *Bacillus* sp. and *Rhizobium* sp., produced higher amount of Indole acetic acid (6.1 mg mL^{-1}) than the *Bacillus* sp., (4.4 mg mL^{-1}) at pH 7 and 37°C in the Bengal gram substrate. Partial purification of Indole acetic acid was done by Thin Layer Chromatography (TLC). In conclusion *Rhizobium* sp., appear to be a suitable soil microorganism for high level of IAA production.

Key words: Indole acetic acid, bengal gram, *Rhizobium* sp., *Bacillus* sp., plant hormone, thin layer chromatography

INTRODUCTION

Agriculture is the backbone of India with high yield of legume and cereals. Soil in India needs higher inputs for a stable output; nitrogen and phosphorus are the two most essential nutrients influencing the crop growth and productivity considering the soil health. Soil fertility is essential to increase the productivity of crops to satisfy the demand of food source in over populated India. The plant growth promoting bacteria influence the plant growth in different modes such as the production of plant growth regulators like auxin, cytokines, gibberellins etc. Plant hormones are used extensively in agriculture and horticulture to modify plant growth and development (Fred *et al*., 1932). The microorganism isolated from the rhizosphere region of various crop have an ability to produce Indole acetic acid as a secondary metabolites due to rich supply of substrates. Some of the important morphogenic effects of Indole acetic acid are plant growth elongation of the stem and gall formation representing the reaction of the host in the presence of auxin (Muller *et al*., 1989). Indole acetic acid helps in production of longer roots with increased number of roots hairs and root laterals which involved in the nutrient uptake efficacy. *Rhizobium* sp., produce a maximum amount of Indole acetic acid in glucose containing medium (Datta and Basu, 2000). The microorganisms producing Indole acetic acid include *Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp., *Azospirillum* sp., *Enterobacter* sp. and *Serratia* sp. (Martens and Frankenberger, 1991; Frankenberger and Arshad, 1995). The present study was focused on Indole acetic acid production using substrates like Green gram, Black gram and Bengal gram at different cultural conditions.

MATERIALS AND METHOD

Isolation of indigenous isolates from garden soil: The Rhizosphere garden soil samples were collected from Chinnamanur (Theni District, Tamil Nadu and India) and it was refrigerated at 4°C for using isolation of indigenous isolates. For the isolation of *Rhizobium* sp. and *Bacillus* sp., from the soil on fermentation medium (KH_2PO_4 3 g, K_2HPO_4 0.6 g, $(NH_4)_2Cl_2$ 2 g, $NaCl_2$ 0.5 g, Glucose 0.8 g and $MgSO_4$ 0.1 g), these isolates were showed growth as well as pigment on the selective media and further its sub cultured on fresh nutrient agar medium for 24 h at 37°C.

Screening of bacterial isolates for indole acetic acid (IAA) production: The organisms isolated from rhizosphere region were identified as *Rhizobium* sp. and *Bacillus* sp. and they were screened for their ability to produce IAA. Each inoculated plate was overlaid with nylon 6'6' membrane. Plates are incubated until colonies reached 0.5 to 2 mm in diameter. After an appropriate

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incubation period the membrane was treated with salkowaski reagent prepared as 2% 0.5 M $FeCl_3$ in 35% perchloric acid. Reaction was allowed to proceed until adequate color developed. All reagent incubations were carried out at room temperature. Bacteria producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony. Known concentrations of IAA were also used to check the extent of red halo formed and also for the comparison for their ability to produce IAA. Identification of the indigenous microorganisms was carried out on the basis of different morphological and also biochemical characteristics on the selective media (Holt *et al*., 1994).

Media preparation and Indole Acetic acid production: The fermentation medium contained (per liter of distilled water): KH_2PO_4 3 g, K_2HPO_4 0.6 g, $(NH_4)_2$ Cl_2 2 g, $NaCl_2$ 0.5 g, Glucose 0.8 g and $MgSO_4$ 0.1 g. To the medium the culture was inoculated and incubated for 7 days at 28±2°C for fermentation.

Optimization of Indole acetic acid production

Optimization of substrate: For optimization, different substrate sources were used such as Green gram, Bengal gram and Black gram. Minimal medium was prepared and 2% of different substrates were added in 100 mL fermentation medium in 250 mL Erlenmeyer flasks. The flask were inoculated with isolated cultures and incubated at 28±2°C for 7 days in orbital shaker.

Optimization of pH: The fermentation medium with Bengal gram as substrate was prepared and pH of the medium was adjusted in the range of 3, 7 and 9. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. After inoculation with bacterial cultures the flasks were incubated at 28±2°C for 7 days in orbital shaker.

Optimization of temperature: Optimization of temperature was carried out by incubating the Bengal gram containing at 25, 37 and 45°C for 7 days in orbital shaker.

Extraction of Indole acetic acid: The fermented samples were centrifuged at 10,000 rpm for 30 min and the supernatant was acidified to pH 2.5±3.0 with 1N HCl. It was extracted twice with ethyl acetate at double the volume of supernatant. Ethyl acetate was evaporated at 40°C. Then the extract was dissolved in 300 mL of methanol and kept at 20°C.

Estimation of Indole acetic acid: Fermented samples were centrifuged at 10000 rpm for 15 min. Supernatant was collected and mixed with 2 drops of orthophosphoric acid and 4 mL of Salwosky reagent. Then optical densities were observed at 540 nm.

Partial purification of indole acetic acid: Indole acetic acid was purified by using thin layer chromatography method. Benzene: n-Butanol: Acetic acid solvents were used for the purification of Indole acetic acid (Sridevi *et al*., 2008).

The RF value was calculated by the formula:

$$RF = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Chemical mutation by NTG (n-methyl-n'nitro-n' nitrosoguanidine): The isolated organism was mutagenized with various concentration of NTG like 0.01/-, 0.02% and 0.03% in YEMA medium and incubated at 37°C for 24 h. After incubation the mutated organism was aseptically transferred to YEMA medium and incubated at 37°C for 24 to 72 h.

After incubation isolated colonies were separated and subculture for further analysis.

Physical mutation by UV- rays: The isolated organism was mutated by exposing them to UV radiation for 5, 10 and 15 min in YEMA medium and incubated at 37°C for 24-72 h. After incubation the mutated organism was aseptically transferred to YEMA medium and incubated at 37°C for 24-72 h. After incubation isolated colonies were separated and subculture for further analysis.

Seed germination test: The disease free seed of *Arachis hypogea* were collected. The seeds were surface sterilized with 0.2% mercuric chloride for 2 min to avoid seed borne pathogen adhering to the surface of the seeds followed by the repeated washing with sterile distilled water for 3 times. The seeds were transferred to nitrogen free medium slants. Three drops of both cultures of wild and mutated *Rhizobium* sp., were dispensed into tube containing seed and incubated at 28°C in dark for 7 days. Then it was transferred into conical flask containing nitrogen -free media for the development of leaf under light intensity for 2 days.

RESULTS AND DISCUSSION

Bacteria predominates the rhizosphere and take nutritional substances (amino acids, vitamins and other nutrients) released from plant tissues for growth. The products of microbial metabolism that are released into the soil also influence growth of plants. It has been reported that the bacteria present in the rhizoplane region requires amino acid for its growth (Walker *et al*., 2003). Interaction between plant and microbes is well known for beneficial effect and such free-living soil bacteria isolated from the rhizosphere of plants are known as Plant Growth

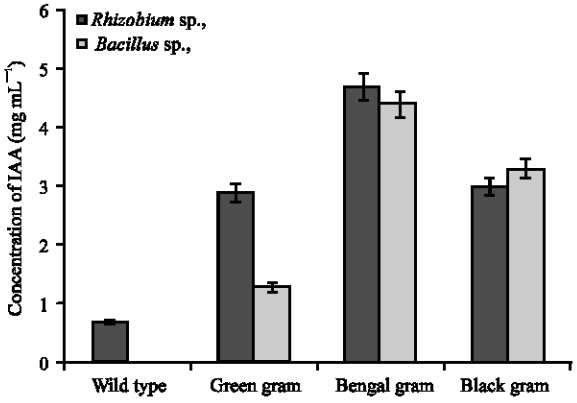

Fig. 1: Estimation of Indole Acetic Acid (IAA) from fermented sample

Table 1: Biochemical characterization of bacterial isolates

Microscopic and Biochemical analysis	*Rhizobium* sp.,	*Bacillus* sp.,
Gram staining	Gram negative	Gram positive
Shape	Rod	Rod
Motility	Motile	Motile
Indole	Positive	Positive
Methyl red	Positive	Negative
Voges proskaver	Positive	Positive
Citrate	Positive	Positive
Oxidase	Positive	Positive
Catalase	Positive	Positive
Starch hydrolysis	Negative	Positive
Case in hydrolysis	Positive	Positive
Lipid hydrolysis	Positive	Positive
Gelatin test	Positive	Positive
Triple sugar iron agar test	Acid production	Acid production
Hoffers alkaline test	No colour change (positive result)	-
Glucose peptone agar test	No growth and no colour change (positive result)	-
Lactose agar test	No growth and no colour change (positive result)	-

Promoting Rhizobacteria (PGPR) (Kloepper *et al*., 1980). Bacteria helps in the nitrogen fixation, production of siderophores 10, solubilization of minerals like phosphorus 11 and synthesis of phytohormones [indole-3-acetic acid (IAA)] (Huddedar *et al*., 2002).

Different bacterial strains were isolated from garden soil at Chinnamanur by serial dilution technique. Of that two cultures, producing Indole acetic acid were used for the further studies. The characteristics of the isolates were tabulated in the Table 1. By morphological and biochemical characterization the isolated organisms were determined as *Rhizobium* sp. and *Bacillus* sp., respectively. The fermentation was carried by *Rhizobium* sp. and *Bacillus* sp., using different substrates like Green gram, Black gram, Bengal Gram and produced Indole acetic acid. Both *Rhizobium* sp. and *Bacillus* sp., shows

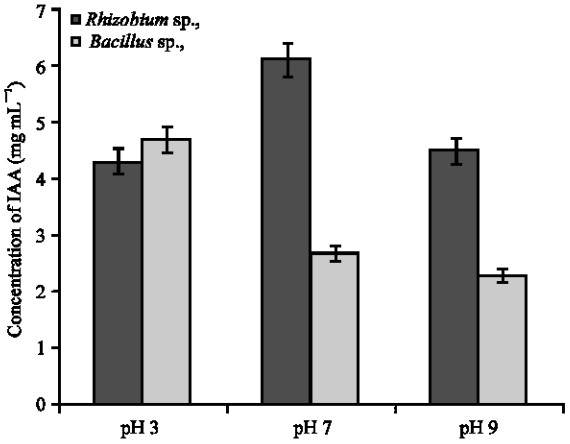

Fig. 2: Optimization of IAA production with various pH (Bengal gram substrate)

the higher production of Indole acetic acid in Bengal gram. The *Rhizobium* sp., produced 4.6 mg mL^{-1} and *Bacillus* sp., produced 4.3 mg mL^{-1} (Fig. 1). Ahemad and Khan (2011) found that the maximum IAA production by *Pseudomonas* sp.

Indole acetic acid produced by *Rhizobium* strain (Nutman, 1977). The effect of pH on Indole acetic acid production was determined at different pH values of 3, 7 and 9. The acidic and alkaline condition was not suitable for IAA production. At pH 7 *Rhizobium* sp., produced 6.1 mg mL^{-1} and *Bacillus* sp., produced 4.7 mg mL^{-1} of Indole acetic acid (Fig. 2). In earlier report (Mandal *et al*., 2007) showed that the IAA synthesis was recorded as higher at the alkaline condition (pH 7.2).

The microorganisms from rhizosphere region of various crops have showed high potential of auxin production (Sarwar and Kremmer, 1995). Optimization was carried out by incubating the fermentation flask at 25, 37 and 45°C. The *Rhizobium* sp., produced 3.2 mg mL^{-1} of Indole acetic acid and *Bacillus* sp., produced 4.4 mg mL^{-1} of Indole acetic acid at 37°C (Fig. 3). Aldesuquy *et al*. (1998) found that temperatures in the range 25-30°C were suitable for growth and Indole Acetic acid production of *Streptomycetes* sp. Fatima *et al*. (2009) and also showed that the germination rate, root, shoot growth of plant were increased by IAA and PGPR .

The Rf value obtained in the Thin layer chromatography is 0.93 and it was equal to the standard value of Indole acetic acid. Chromatograms of culture spots and standard IAA showed almost the same Rf values when it was sprayed with Ehmann's reagent. Our TLC findings are in agreement with reports by other scientists (Xie *et al*., 1996). In addition to IAA, other compounds were also detected on TLC plates which

Table 2: Estimation of Indole Acetic Acid (IAA)

Sample	Various concentration of NTG and time interval of UV mutation	Concentration of IAA (mL) (*Rhizobium* sp.,)	Concentration of IAA (mL) (*Bacillus* sp.,)
Wild type	-	2.70	1.80
Chemically mutated (NTG)	0.01%	3.75	3.15
	0.02%	4.20	3.00
	0.03%	3.55	2.75
Physically mutated (UV)	5 min	1.65	1.15
	10 min	1.48	1.06
	15 min	0.93	0.63

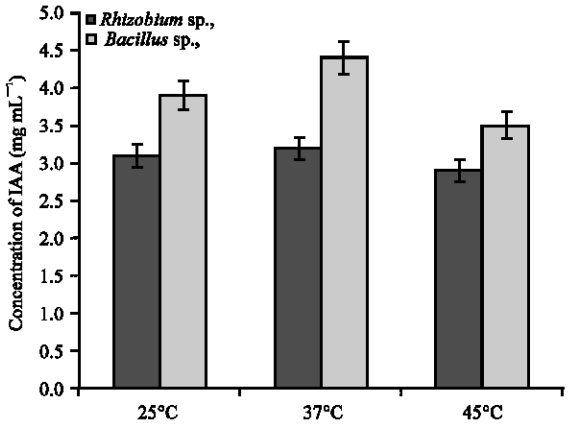

Fig. 3: Optimization of IAA production with various temperatures (Bengal gram substrate)

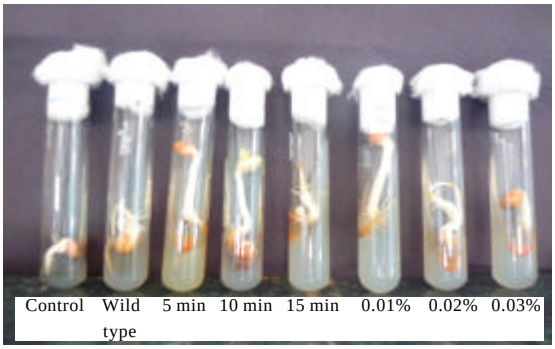

Fig. 4: Effect of IAA production in *Arachis hypogaea* after mutation

remain to be identified. *Rhizobium* sp and *Bacillus* sp., was mutagenized with various concentration of chemical mutagen (NTG) and different time intervals of physical mutation (UV radiation). The efficiency of a variety of common mutagens in producing mutation in *Rhizobium trifoli* P3 was examined. Ethyl methanesulphonate, methyl methane sulphonate, decarbamoyl mitomycinc, nitrous acid and gamma radiation did not mutate *Rhizobium trifoli* P3. N-methyl-N'-nitro-Nitrosoguanidine (NTG) and UV radiation were both mutagenic, the former being the more effective.

The wild type *Rhizobium* and *Bacillus* strain was chemically mutated by exposing them into various concentration of NTG (0.01, 0.02 and 0.03%). Among the mutant strains 0.02% NTG mutant produced higher amount of indole acetic acid (4.20 mg mL^{-1}) than the other two strains (Table 2). The wild type *Rhizobium* sp. and *Bacillus* sp., strain was physically mutated by exposing them into UV radiation for different time intervals (5, 10 and 15 min). Among the mutant strains, which is exposed 5 min, produced higher amount of indole acetic acid (1.65 mg mL^{-1}) than the other two strains. The effect of indole acetic acid production in *Arachis hypogaea* after mutation studies at different schedule which has been observed the growth of root as well as shoot induction in selective media (Fig. 4).

Nutman (1977) has described Indole acetic acid produced by *Rhizobium* strains initiates the plant growth and helps in the formation and development of root nodules . The efficiency of *Rhizobium* was determined by seed germination. In seed germination test when compared to the physical, chemical, wild type and control, the effective range of seed germination was observed in mutant strains. Among the chemically mutated strains, the effective range of seed germination was observed in 0.02% of NTG. Among the physical mutated (UV) strains the effective range of seed germination was observed in the strain which was exposed 5 min.

CONCLUSION

There are numerous efficient microorganisms were able to produce the indole acidic acid for inducing the plant growth level. But some microorganism produce the IAA in the presence of suitable environmental conditions also precursor (Bengal gram substrate). In our study two efficient microorganisms (*Bacillus* sp. and *Rhizbiums* sp.,) were able to produce persistent level IAA, but a significant increase in the production of IAA by *Rhizobium* sp. and it was recorded in the presence of pH, temperature and Bengal gram compared with other microbes. The indole acetic acid was partially purified by using Thin Layer Chromatography (TLC). Thus the

Rhizobium sp., appears to be a suitable soil microorganism for the high level of Indole Acetic Acid (IAA) production.

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