Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth

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Abstract

Indole acetic acid (IAA) production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. The present work deals with isolation, characterization and identification of indole acetic acid producing bacteria from the rhizospheric soil. Out of ten Indole acetic acid producing isolates, five were selected as efficient producers. Optimization of indole acetic acid production was carried out at different cultural conditions of pH and temperature with varying media components such as carbon and nitrogen source, tryptophan concentration. Partial purification of IAA was done and purity was confirmed with Thin layer chromatography. Subsequently, effect on plant growth was tested by pot assay. In conclusion the study suggests the IAA producing bacteria as efficient biofertilizer inoculants to promote plant growth.

Keywords: Tryptophan, rhizobacteria, purification, optimization, plant nutrition

1. Introduction

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism produced by several microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR) (Lynch, 1985). Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as PGPR. PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (like auxin, gibberellin, and ethylene), siderophores, HCN and antibiotics (Arshad et al., 1992). Bacteria synthesize auxins in order to perturb host physiological processes for their own benefit (Shih-Yung, 2010). The microorganisms isolated from rhizosphere region of various crop have an ability to produce Indole acetic acid as secondary metabolites due to rich supply of substrates. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake (Datta and Basu, 2000). IAA stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis and inducing specific RXA and
protein synthesis. It promotes embial activity, inhibit or delay abscission of leaves, induce flowering and fruiting. (Zhao, 2010)

IAA is a metabolite derived from Trp by many Trp-dependent and Trp-independent pathways in plants and bacteria. More than one pathway could be present in a bacterium (Pattern and Glick, 1996). Physiological evidence for different Trp-dependent pathways for synthesis in *Azospirillum brasilense* has been reported (Carreno-Lopez *et al.*, 2000). In Trp dependent pathway, tryptophan is converted to indole-3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is metabolized to IAA by IAM-hydrolase (Matsukawa *et al.*, 2007). Horemans and Vlassak (1985) demonstrated that *A. brasilense* could produce IAA in the absence of tryptophan when grown aerobically showed that the highest levels of auxin were produced in the presence of NH₄. It appears to be of particular importance during embryogenesis, when fine control over low levels of IAA is critical to polar development. Trp-independent pathway might contribute significantly to the newly synthesized IAA; however, extensive Trp-to-IAA conversion also occurs in such preparations. The first objective of this study was to isolate and screen indigenous Indole acetic acid producing bacteria from different rhizospheric soil. The second was to purify the IAA and screen their abilities of plant growth promoting rhizobacteria attributes. Besides, optimization study intended for high IAA production was carried out with physicochemical parameters such as carbon and nitrogen source, with and without supplement of tryptophan, pH and temperature.

2. Materials and Methods

2.1. Isolation of IAA producing bacteria from rhizospheric soil

Ten soil samples each from banana, cotton, maize and wheat rhizosphere were collected. Physicochemical analysis of samples based on soil texture, pH and temperature was done. The soil texture was slit, pH in the range of 7.5 to 8.5 and temperature was 30 to 32 °C. The isolation of the microorganisms was done as follows. 10g of rhizosphere soil in 250mL flask was taken and 90 mL sterile distilled water was added. It was incubated on rotary shaker at 120 rpm for 10 min. 1ml sample was serially diluted upto 10⁻⁷. 0.1 mL of diluted sample was plated on sterile Luria Bertani (LB) agar medium (Himedia, India) and incubated for 3 days at 28 °C. Single colonies were picked up and streaked on sterile LB agar plates to get pure culture. Well isolated colonies were observed for morphological characterization.

Total 10 isolates were obtained from different rhizospheric soil. The isolates were further checked for IAA production.

2.2. Identification of isolates

The isolates based on micromorphological observation and biochemical characterization were identified. The tests involved, were Gram staining, amylase and gelatinase, catalase like enzyme production, citrate utilization, indole test, Vogus Proskaur test, methyl red test, H₂S production, sugar fermentation etc. (Aneja, 2001).

2.3. Characterization of IAA production

To determine the amounts of IAA produced by each isolate, a colorimetric technique was performed with Van Urk Salkowski reagent using the Salkowski’s method (Ehmann, 1977). The isolates were grown in yeast malt dextrose broth (YMD broth) (Himedia, India) and incubated at 28 °C for 4 days. The broth was centrifuged after incubation. Supernatant was reserved and 1ml was mixed with 2ml of Salkowski’s reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution) and kept in the dark. The optical density (OD) was recorded at 530 nm after 30 min and 120 min.
IAA production was compared in YMD and LB media. YMD medium was compared with and without tryptophan.

2.4. Extraction and purification of IAA

Isolates were cultivated in YMD broth and it was centrifuged by Beckman centrifuge SW 40 Ti rotor with 17738 × g for 15 min. The supernatant was collected and mixed with ethyl acetate (1: 2). After vigorous shaking it was allowed to stand for 10 min. IAA was extracted within solvent layer. The procedure was repeated 3 to 4 times.

Thin layer chromatography

TLC slide was prepared with silica gel G and calcium carbonate. Propanol: Water (8:2) was used as Solvent system. The extracted sample and standard IAA (10mg/100ml) were spotted on TLC plate. Chromatogram was developed with the Salkowski’s reagent (Kuang-Ren et al., 2003).

2.5 Optimization of media and physical factors for IAA production

Optimization of carbon and nitrogen source, tryptophan concentration and process parameters such as pH, temperature were made for improved yield of IAA by one factor at a time analysis. The effect of pH was tested in the range of 5 to 9 using buffered broth prepared in phosphate buffer. YMD medium with tryptophan supplement was used as basis for optimization of IAA production.

2.6. Effect of IAA producing isolates on plant growth by pot assay

To study the effect of IAA producing rhizospheric isolates on plant growth, pot assay was performed. Local wheat (var. Lokvan) seeds were used for seed coating. The wheat seeds were surface sterilized by immersing in 95% ethanol for 30 s and mercury chloride (0.2%) for 3 min. Then further to remove traces of mercury chloride, the disinfected seeds were washed 5 times by sterile distilled water. 0.1ml overnight grown culture (0.5 OD) was applied on seed surface for seed coating. Seeds were dried and sowed into sterile soil as carrier. Six seeds were sown in each pot used per pot at equal distance and experiment was performed in triplicates for each isolates. The uncoated seeds were used as control. After appearing seedlings of soil 0.1 g of Trp per kg soil after being solving in water was added to every pot. Pots were irrigated with sterile distilled water every day and kept in sunlight. At the interval of every 5th day, plant was uprooted and seedlings were measured for shoot and root length and chlorophyll content upto 15th day.

3. Results

3.1. Isolation and Identification of rhizospheric isolates

10 bacterial isolates were successfully isolated as IAA producer from rhizosphere soil among which 5 were selected based on IAA production ability. The isolates were coded as br1, br2, br3 (from banana rhizosphere), wr2 (from wheat rhizosphere) and mr2 (from maize rhizosphere).

The isolates were identified based on morphological observation and biochemical characterization (Table 1). Bergey’s manual of determinative of bacteriology was used as a reference to identify the isolates (MacFaddin, 2000). The isolates were identified as B. megaterium, Lactobacillus casei, B. subtilis, B. cereus and Lactobacillus acidophilus, respectively for isolates coded as br1, br2, br3, wr2 and mr2 based on Bergey’s manual.
Table 1. Morphological and Biochemical characterization of IAA producing rhizospheric isolates

| Characteristics       | br1     | br2     | br3     | mr2     | wr2     |
|-----------------------|---------|---------|---------|---------|---------|
| Gram Staining         | Gram positive | Gram positive | Gram positive | Gram positive | Gram positive |
| Shape                 | Rod     | Rod     | Rod     | Rod     | Rod     |
| Motility              | Positive| Positive| Positive| Positive| Positive|
| Capsule staining      | Positive| Positive| Positive| Positive| Positive|
| Acid Fast nature      | Negative| Negative| Negative| Negative| Negative|
| Endospore             | Positive| Negative| Positive| Negative| Positive|
| Oxygen Requirement    | Aerobic | Aerobic | Aerobic | Aerobic | Aerobic |
| Colony                | Off white| White   | White   | Pale yellow| Off white |
| Catalase              | Positive| Negative| Positive| Negative| Positive|
| Urease                | Positive| Positive| Positive| Positive| Positive|
| Amylase               | Positive| Positive| Positive| Positive| Positive|
| Protease              | Positive| Positive| Positive| Positive| Positive|
| Pectinase             | Positive| Positive| Positive| Positive| Positive|
| H₂S production        | Negative| Negative| Negative| Negative| Negative|
| Indole Production     | Negative| Negative| Negative| Negative| Positive|
| Methyl red test       | Negative| Negative| Negative| Negative| Positive|
| Vogus Proskau Test    | Negative| Negative| Positive| Negative| Negative|
| Citrate Utilization   | Positive| Positive| Positive| Positive| Positive|
| Glucose               | Positive| Positive| Positive| Negative| Negative|
| Mannitol              | Positive| Positive| Positive| Negative| Negative|
| Sucrose               | Positive| Positive| Positive| Positive| Positive|
| Xylose                | Negative| Negative| Negative| Negative| Negative|
IAA producing plant growth promoting rhizobacteria

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Figure 1. Comparison of IAA production by the bacterial isolates in LB medium with tryptophan (n= 3*)

![Graph showing IAA production in LB medium with and without tryptophan](image)

Figure 2. Comparison of IAA production by the bacterial isolates in YMD medium a) without tryptophan and b) with tryptophan (n= 3*)

![Graph showing IAA production in YMD medium with and without tryptophan](image)

3.2. Characterization of IAA production potential

IAA production was checked with use of Salkowski reagent. Color development was first visible at the highest IAA concentration within minutes and continued to increase in intensity for a period of 30 min. Hence optical density was measured after 30 and 120 min. If colour development was not observed after 30 min, it was not kept for further incubation up to 120 min.

IAA production when compared between YMD and LB media, the production of IAA was more in YMD media (Figure 1 and 2a). YMD media with tryptophan was more suitable for IAA production compared with YMD without tryptophan as reported earlier (Figure 2a and 2b). The results were in support to previous study (Ghosh and Basu, 2002).
3.3. Detection of IAA by thin layer chromatography

Purified IAA sample was compared with standard IAA on TLC chromatograms. TLC of ethyl acetate extract showed pink colour spot at the Rf corresponding to the authentic IAA (0.57) as shown in Figure 3. It confirmed IAA producing potential of rhizospheric isolates.

![Figure 3](image)

Figure 3. Thin layer chromatogram of bacterially sized IAA detected by Salkowiski’s reagent compared with standard

3.4. Effect of Different Carbon and Nitrogen Sources on IAA production

The mannitol (as carbon source) and ammonium nitrate (as nitrogen source) in addition to tryptophan was tested as carbon and nitrogen sources for IAA production (Figure 4a). The most suitable carbon source for IAA production was glucose for br1, Mannitol for br2 and br3, Glucose and Sucrose for mr2 while Glucose and Mannitol for wr2. The suitable nitrogen source for IAA production was different with the isolate type as NaNO₃ for br1, KNO₃ and peptone for br2. KNO₃ and peptone for br3 and mr2 while NaNO₃ and peptone for wr2 (Figure 4b). There was a significant difference between the concentrations of IAA which indicates the effect of nitrogen. Basu and Ghosh (2001) have reported that Glucose and KNO₃ as the best carbon and nitrogen sources of IAA production by Rhizobium spp. Shilts et al. (2005) have reported high IAA production in medium containing mannitol and galactose.

3.5. Effect of L-Tryptophan concentration on IAA

L-Tryptophan is generally considered as an IAA precursor, because of its addition to IAA producing bacterial culture enhances IAA biosynthesis (Costacurta and Venderleyden, 1995). All 5 isolates preferred Tryptophan for IAA production. Maximum IAA production was found in the medium amended with 0.1% tryptophan for br1, br2 and br3, 1.5% for mr2 and 0.05% wr2 (Figure 5a).

IAA was not produced or produced in negligible quantity in the L-Tryptophan free medium. There was a significant different level of L-tryptophan for varying microorganisms. For many bacteria, the conversion of tryptophan into IAA is most important. Manulis et al. (1994) have reported various Stryptomyces spp. that secrete Indole 3-acetic acid (IAA) when fed with tryptophan while Swain et al. (2007) have reported IAA producing Bacillus subtilis spp. Tryptophan dependant IAA synthesis had been also determined in several other bacteria (Patten and Glick, 2002). In Enterobacter Cloacae, IAA was synthesized via indole-3 pyruvic acid (Koga et al., 1991). In Pseudomonas syringae, IAA biosynthesis occurs mostly from tryptophan via indole-3-acitamide (Kosuge and Sanger, 1987) and in Pseudomonas fluorescens, tryptophan bypassing the indole 3-actaldehyde, which is further converted into IAA (Oberhansli et al., 1991). IAA synthesis has also been found to occur via tryptamine in a Agrobacterium tumefaciens and via indole 3-acetonitreile in Alcaligenes faecalis and A. tumefaciens (Costacurta and Vanderleyden, 1995).
Figure 4. Effect of a) carbon and b) nitrogen sources on biosynthesis of IAA by the bacterial isolates grown in YMD medium supplemented with tryptophan (n= 3*)

Figure 5. Effect of a) tryptophan concentration b) pH and c) temperature on biosynthesis of IAA by the bacterial isolates grown in YMD medium supplemented with tryptophan (n= 3*)
Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells. The application of organic fertilizer can increase the levels of tryptophan in soil and tryptophan found in organic wastes and fertilizers may be produced by aerobic or anaerobic microbial transformation (Arkhipchenko et al., 2006).

3.6. Effect of pH and temperature on IAA production

The low pH limits plant growth, because the concentrations of metals (Al\(^{3+}\) and Mn\(^{2+}\)) in the soil solution can reach toxic levels. It is known that soil pH and metal cations may affect many processes occurring in the rhizosphere. The impact of different levels of pH (5 – 9) was determined. The maximum amount of IAA was produced when pH of the culture medium was 8 for isolates br1, br2, br3 while pH 7 for mr2 and pH 9 for wr2 (Figure 5b). Acidic pH (below 6) was found to be unfavorable for IAA production. The effect of different ranges of temperature (20 – 40 °C) was studied. The optimum temperature for IAA production was 30 °C (Figure 5c). According to Sudha et al. (2012) 37 °C temperature was optimum for Rhizobium and Bacillus spp. for IAA production. Mandal et al. (2007) have reported the Rhizobium strain VMA 301 for elaborated high levels of IAA production in a medium having pH 7.2. Khamna et al. (2010) have reported temperature 30 °C and pH 7.0 was suitable for maximum IAA production by Streptomyces sp.

3.7. Biological feasibility of Rhizobacteria for plant growth

The rhizosphere soil isolates were significantly augment the plant height and root length of wheat seedlings along with increase in chlorophyll content when compared with control (Figure 6, Table 2). In earlier report, root elongation was found to occur in Sesbania aculeata by inoculation with Azotobacter spp. and Pseudomonas spp., in Brassica campestris by Bacillus spp (Ghosh et al., 2003), in Vigna radiata by Pseudomonas putida (Patten and Glick, 2002) and in Pennisetum americanum by Azospirillum brasilense (Tien et al., 1979). This indirectly confirms the involvement of bacterial isolates in enhancing the plant growth by synthesizing IAA.

Figure 6. Growth stimulatory effect of bacterially synthesized IAA on wheat root and shoot length elongation compared with untreated control (n= 3')
4. Discussion

IAA, a member of the group of phytoharmones, is generally considered to be the most important native auxin. All ten isolates are positive for IAA production but among those five isolates br1, br2, br3, mr2 and wr2 were selected as potential IAA producers. Most of studies from the earlier work showed that IAA producing organisms are Gram negative (Lindow et al., 1998; Datta and Basu, 2000). Few Gram positive strains belong to Bacillus strain known to produce IAA (Wahyudi et al., 2011). Present study showed that five IAA positive strains were Gram positive.

It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mutluru and Konada, 2007). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Sarwar and Kremer, 1992).

The use of the technique for the detection of IAA using the Van Urk Salkowski reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. The amount of IAA produced by the bacteria was within the detection limits of Salkowski reagent (Ehmann, 1977). The reagent gives reaction with IAA and does not interact with L-tryptophan and Na-acetyl-L-tryptophan and used by and large (Vaghasiat et al., 2011). Among the isolates br3 and wr2 were found to be the best producer of IAA. On the other hand br1, br2 and mr2 were found to be a medium producer of IAA as shown in Figure 2. Hence for further characterization these isolates were selected.

Auxin production by all isolates increased when culture medium supplemented with an IAA precursor; tryptophan which confirm the results of other scholar (Mutluru and Konada, 2007). Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. The tryptophan increases the production of IAA in Bacillus amyloliquefaciens FZB42. Tien et al. (1979) showed that Azospirillum is able to produce auxins when exposed to tryptophan. Plants inoculated with the rhizobia together with Ag+ ion and L-tryptophan (Trp), give the highest root dry weight, and significantly increase the uptake of N, P and K compared to non-inoculated control plants. Karnwal (2009) tested Fluorescent Pseudomonas isolates for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan and found that for both strains, indole production enhanced with increases in tryptophan concentration.
Effect of carbon sources (1.0%) in the basal YMD revealed that the bacterial isolates vary in their utilization and production of IAA in different carbon sources. Most of the isolates gave maximum IAA production in mannitol containing media. Effect of different nitrogen sources (0.1%) was studied by replacing yeast extract in the original YMD medium supplemented with L-tryptophan. The isolates show differential nitrogen utilization pattern.

The effect of pH on IAA production differed between the Bacillus strains. The effect of pH on IAA is in agree with studies on other production is ement with studies on other Bacillus spp. IAA production by Bacillus spp. MQH−19 was highest at pH 6.0 and decreased by 62% at pH 5.0. For Paenibacillus spp. SPT−03, IAA production was highest at pH 5.0 and decreased by 42% at pH 7.0 (Acuna et al., 2011).

The property of synthesizing IAA is considered as effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria have profound effect on plant growth (Wahyudi et al., 2011). Inoculation with IAA producing bacteria induces the proliferation of lateral roots and root hairs. Fatima et al. (2009) also showed that germination rate, roots, shoot growth of plant were increased by IAA and PGPR. Therefore these isolates were studied for their effect on plant growth under controlled conditions. There was a significant increase in root and shoot elongation and chlorophyll contents. Among the isolates wr2 and mr2 were found much effective to show potential increase. Data obtained from pot experiment and seed germination demonstrated positive effect on plant growth and thus can be considered as plant growth promoter.

5. Conclusion

From this study, it is clear that rhizospheric soil can provide a rich source of IAA producing bacteria and has the ability to produce a significant amount of IAA in a tryptophan-supplemented medium. Overall ten isolates were identified as IAA producing strains among which five efficient IAA producing bacteria were characterized and media components, physical parameters were optimized for IAA production. Among the isolates wheat and maize isolates (wr2 and mr2) show best growth promoting activity. It is concluded that presence of such growth promoting rhizoflora accountable for the beneficial effects on crop growth and yield. The significance of the study could be stated as the potential of these IAA producing isolates and optimization study for IAA production will flourish the growth and ultimately IAA production in the field and prevent environmental pollution by avoiding excessive applications of industrially produced fertilizers to cultivated fields.

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