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# Isolation and Characterization of Indole Acetic Acid Producing Bacteria from Rhizosphere Soil and their Effect on Seed Germination

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**A B S T R A C T**

Indole acetic acid (IAA) is one of the most important phytohormones enhances the root structure and plant growth. The present study provides the isolation and characterization of IAA producing bacteria from rhizosphere soil and their effect on seed germination. Out of 30 isolates, eight (*viz.* Aux 4, Aux 9, Aux14, Aux16, Aux 19, Aux 20, Aux 21 and Aux 25) were able to produce IAA which was confirmed by Salkowski reagent test. Isolates were characterized on the basis of visual observations, gram staining and biochemical tests such as oxidase, catalase, indole, TSI, methyl red, Voges-Proskauer, citrate utilization, urease and starch hydrolysis test. The IAA producing bacteria are rod and round shaped gram positive and gram negative bacteria. In biochemical tests, both positive and negative responses were observed. Spectrometric analysis of IAA was done after 24, 48 and 72 hours of culture which showed that the isolated bacteria produced maximum concentration of IAA after 72 hours of incubation period at 37°C. The concentration was measured using a standard IAA curve and the maximum concentration was obtained by Aux 25. The *in vitro* treatment of maize and rice seeds with Aux 25 for germination showed better result than control. Further study is necessary for molecular characterization of isolated bacteria.

## Introduction

Plant growth promoting bacteria (PGPB) are free living soil bacteria found in the rhizosphere soil. They help in the fixation of atmospheric nitrogen (Zehr *et al.,* 2003), production of siderophores (Machua and Milagres, 2003), solubilization of soil minerals (Tilak *et al.,* 2005) and synthesis of phytohormones (Huddedar *et al.,* 2002; Chopade *et al.,* 2008). The synthesis of plant hormones such as auxins, gibberellins, cytokinines and polyamines by rhizosphere microbes are considered to be specific to the microbe-host pheric bacteria synthesize auxins in order to perturb host physiological process for their own benefit (Ahmed *et al.,* 2008; Cassan *et al.,* 2009; Tien *et al.,* 1979; Yang *et al.,* 2009; Rhizos-Yung, 2010). Indole -3-Acetic Acid (IAA) is the principle and first auxin sequestered from plants (Levean and Lindow, 2005; Aziz *et al.,* 2015). The

rhizosphere microflora of various crops have the ability to produce IAA as a secondary metabolite which in turn distracts the host's physiological process that ultimately provide the microflora with necessary amenities. Side by side, IAA has been implicated in virtually all aspects of plant growth and development including the production of longer roots with increased number of root hairs and root laterals which are involved in the nutrient uptake (Datta and Basu, 2000). Furthermore, it enhances embibal activity, inhibit or delay abscission of leaves and promote flowering and fruiting in plant (Zhao, 2010). Increasing global population is driving the agronomic practices where boosting higher yield is a prime objective. Increasing agricultural productivity is currently associated with the extensive application of chemical fertilizers. However, problems like higher cost, pollution of natural resources and health implications have compelled researches to explore alternatives for safe and increased crop productivity. One such alternative is the use of PGPB, particularly IAA producing bacteria. Beneficial microbial allelopathies in rhizosphere are a key agent of change in soil ecosystem and affect crop health and yield (Sturz and Christie, 2003). Due to such valuable attribute, isolation of indigenous bacterial strains with plant growth promoting potential from various environments remains a popular concept. In this regard, the present study was conducted to isolate potential IAA producing bacteria from the South-west Khulna region of Bangladesh followed by their characterization and analysis.

## Materials and Methods

### Collection of sample

Samples were collected from rhizosphere soil of bean plant of breeding plot of Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. Rhizosphere soil samples were taken and kept in a sterile zip lock polyethylene bag. Samples were taken to the laboratory maintaining the aseptic conditions. Ten gram of rhizosphere soil was transferred in a 250 ml sterile Erlenmeyer flask and 90ml sterile distilled water was added. Tenfold serial dilution was carried out with the soil mixture.

### Media preparation

Nutrient agar media were prepared containing (peptone 5.0g/L, beef extract 3.0g/L, sodium chloride 8.0g/L and agar 12.0g/L). The pH of the media was adjusted 7.3. The culture media were sterilized at 121°C for 15 minutes. These media were then plated on sterile petri dish and allowed to solidify. After solidification, the plates were preserved in refrigerator at 8°C.

### Isolation of bacteria from soil sample

Serially diluted soil samples (0.5ml) were placed on the solidified nutrient agar media and both spread plate and pour plate techniques were used for culturing the organisms from the samples. After absorption of all the fluid in the media, the petri dishes were sealed and they were incubated at 37°C for 24 hours under dark. Colonies of different shapes were selected and transferred in nutrient agar media. After several sub-culturing, finally pure isolates were obtained. Isolates tested in the present study were preserved in nutrient broth (NB) medium containing 15% glycerol at -80ºC.

### Screening of bacteria and spectrophotometric analysis

Isolates were screened for auxin production by using Salkowski reagent. Broth cultures were centrifuged and supernatants were mixed with Salkowski reagent in a ratio of 1:2.Salkowski reagent was prepared by mixing 150 ml of concentrated $H_2SO_4$, 250ml of distilled water and 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$ as described

previously (Patten and Glick, 2002). The mixture was allowed to stand for 30 minutes at room temperature in dark for color production. Isolates showing pink to red color were selected as IAA producers and were used in further experiments. The amount of IAA was measured by spectrophotometric method at 535 nm. Then concentration was calculated using standard curve of IAA. The concentration of bacteria was measured by using standard curve at a range of 0.5-10 µg IAA (Sigma-Aldrich).

## Characterization of bacterial isolates

The bacterial isolates were subjected to Gram staining according to the method of Vincent and Humphrey (1970). Smear of bacteria were prepared on slide and fixed with air and heat. Samples were then stained with crystal violet for 30 seconds and rinsed with water. After that samples were covered with gram iodine and allowed to act for 30 seconds and rinsed further with distilled water. A drop of 95% alcohol was added and kept for 10-20 seconds for discoloration. The slides were further rinsed with sterile water. Counter staining was done with safranin for 20-30 seconds and slides were rinsed with sterile water and then dried (Vincent and Humphrey, 1970). Stained slides were examined under compound microscope. This was examined under 10X, 40X and 100X objective lenses of compound microscope. In this study bacterial isolates screened by Salkowsi reagent were subjected to characterization by the biochemical tests e.g. oxidase test, catalase test, starch hydrolysis test, indole test, urease test, methyl red test, Voges-Proskauer test, TSI test and citrate utilization test (Hofwegenet *et al.*, 2016; Cheesbrough, 2006).

## Data analysis

Data analysis was done by using Graph pad software and presumptive detection of isolates was done by using online bacterial identification website.

## *In vitro* seed germination by the highest IAA producing isolate

Highest auxin producing bacterial isolate (Aux 25) was used in maize and rice seed germination treatment. Seeds of maize and rice were surface-sterilized with 0.02% sodium hypochlorite for 2 min, and rinsed thoroughly in sterile distilled water. Both germination tests were carried out by the paper towel method with minor modification (Gholam *et al.,* 2009; Aziz *et al.,* 2015). In both cases of maize and rice 15 seeds were taken for germination for each treatment with three replications and incubated in growth chamber at 28°C with bacterial supernatant of the highest auxin producing isolate (Aux 25) and the control was treated with water and nutrient broth. After 3 days Root length, shoot length and number of root hair of individual seedling was measured in case of maize and root length, shoot length was measured in case of rice and the mean value was compared with the control (Aziz *et al.,* 2015) starting from 48 hours and continued till 96 hours. Plant growth promotion by selected isolate Aux 25was assessed using Vigor Index (VI) according to Baki and Anderson (1973). Germination percent was measured by using the formula, GP = seeds germinated/total seeds x 100.

Vigour index (VI) = (Mean root length + Mean shoot length) × Germination percentage

## Results and Discussion

## Isolation of organism

All three soil rhizosphere samples showed positive bacterial growth on medium. From the plates 30 independent bacterial isolates were collected according to their cultural and

morphological characteristics i.e. by visual observation of the isolates followed by periodic subculture on nutrient agar plate. These plates were designated as Aux-1 to Aux-30. Eight out of 30 isolates exhibited positive reaction by forming pink solution when reacting with Salkowsi reagent. Thus they are considered as potentially IAA producing isolates. These isolates were Aux-4, Aux-9, Aux-14, Aux-16, Aux-19, Aux-20, Aux-21, and Aux-25.

## Quantitative determination of IAA production from bacteria

All eight isolates were able to produce moderate to high amount of IAA under laboratory condition (Fig. 1). During the course of 72 hrs incubation, IAA production increased with time. Highest IAA concentration was observed with Aux 25 (12 µg/ml) followed by Aux 19and Aux 21 (7g/ml) in a 72hrs incubation scheme.

## Characteristics of bacterial isolates

All 8 isolates were subjected to morphological, physiological and biochemical characterization. Cellular Shape and Gram staining were performed for morphological and physiological characterization and the findings are presented in Table 1. Colony morphology, size, shape, color, and growth pattern were recorded after 24 h of growth on NA plates at 37°C and cell size was observed by light microscopy according to the study of Shaikhul, 2015 where they found all of the isolates gram positive. Murat *et al.,* 2017 found red colored colony but in this study one produced yellow colony, one produced white colony and six of them produced off-white colony. In the present study we found three gram positive and five gram negative bacteria. Biochemical characterization was carried out using oxidase test, catalase test, indole test, citrate utilization test, methyl red test, Voges-Proskauer test, gas production $H_2S$ production test, urease test and finally different sugar utilization test. The biochemical characteristics of bacterial isolates are summarized in Table 2.

In oxidase test the oxidation of a PPD derivative (with or without a-naphthol) which is caused by a cytochrome dependent bacterial terminal oxidase reaction and caused blue color (Peter *et al.,* 1976). Karnwal, 2009 found that all isolated bacteria from rhizosphere soil were oxidase positive. In this study we found five oxidase positive (showed blue color) and three oxidase negative bacteria (no color change). Catalase activity can be be measure by catalase test where decomposition of $H_2O_2$ and liberation of oxygen occurs (Aebi, 1974). Inga *et al.,* 2011 all of the isolates were catalase positive but the present study found five catalase positive produced oxygen bubble and three catalase negative bacteria produced no bubble formation. In citrate utilization test positive bacteria produce an enzyme**,** citrate-permease, capable of converting citrate to pyruvate**.** Pyruvate can then enter the organism's metabolic cycle for the production of energy. When the bacteria metabolize citrate, the ammonium salt**s** are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6.

According to the study of Mohite, 2013 all of the bacterial isolate he studied, were able to utilize citrate. Shaikhul et al, 2015 also found all of the isolates which were able to utilize citrate. In the present study we found all of the isolates which can utilize citrate except one. In indole test tryptophan is an amino acid that can undergo deamination and hydrolysis by bacteria that express tryptophanase enzyme. Indole was generated by reductive deamination from tryptophan via the intermediate molecule indolepyruvic acid.

Some found all of the indole positive isolates. In this study we have found four of the isolates as indole positive and four of them were indole negative. Patel, 2014 found four $H_2S$ gas producer and three did not produce $H_2S$ gas. In this study none of them produced $H_2S$ gas.

Mohite (2013) found five IAA producing isolates. Among these isolates four were methyl red negative and one was methyl red positive. In methyl red test yellow color of MR broth changes to red due to the conversion of acid to alkaline. In this study six isolates were methyl red positive and two were negative. In Voges-Proskauer test positive result is found due to the production of acetyl-methyl-carbinol.

Chaiharn and Lumyong (2010) found all of the bacteria as Voges-Proskauer test positive. In this study two showed positive result in this test and six showed negative result.

In urease test urease positive bacteria produce pink color due to the breakdown of urea in the presence of urease. In the study of Ei *et al.*, 2017 all the bacteria were urease positive but in this study three bacteria were urease positive which were able to break down urea.

In this study 3 bacteria had the capacity to hydrolyze starch and others could not. Aziz *et al.*, 2015 found the entire isolates produced maximum amount of IAA after 72 hours incubation period. In this study we also found the same result.

The isolates produced maximum amount of IAA after 72 hours incubation period. They also found more positive response when they treated seed with auxin producing strains than the control. In this study we also treated the seeds with highly auxin producing strain (Aux 25) and found better result than the control.

## In vitro trial of maize and rice seed germination by highly IAA producing strain

Data obtained from seed germination experiment showed positive responses on seed germination are summarized in Table 3.

Both maize and rice seeds treatment with Aux 25 strain increased root length, shoot length and root number ($P<0.0001$). The germination percent of maize was 100% and rice was 100% and the VI of maize was 3.2 and VI of rice was 1.86.

**Table.1** Morphological and physiological characterization of IAA producing isolates

| Isolates | Gram Staining | Shape |
|---|---|---|
| **Aux-4** | - | Rod |
| **Aux-9** | - | Cocci |
| **Aux-14** | + | Rod |
| **Aux-16** | + | Rod |
| **Aux-19** | - | Cocci |
| **Aux-20** | - | Cocci |
| **Aux-21** | + | Cocci |
| **Aux-25** | - | Rod |

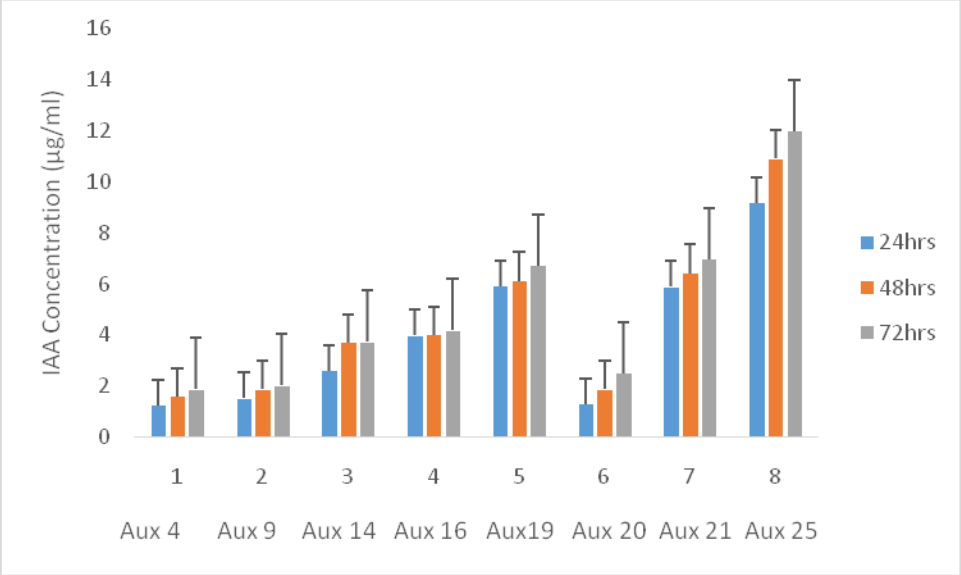
**Table.2** Biochemical characterization of the IAA producing isolates

| Biochemical properties | AUX 4 | AUX 9 | AUX 14 | AUX 16 | AUX 19 | AUX 20 | AUX 21 | AUX 25 |
|---|---|---|---|---|---|---|---|---|
| **Oxidase** | - | + | + | + | - | - | + | + |
| **Catalase** | - | + | + | + | - | - | + | + |
| **Indole** | + | - | + | + | + | - | - | - |
| **Citrate** | + | + | + | + | + | + | + | - |
| **Methyl red** | + | + | + | - | + | + | - | + |
| **Voges-Proskauer** | - | - | - | + | - | - | + | - |
| **Glucose** | - | - | + | + | + | + | - | + |
| **Lactose** | + | + | - | - | - | - | + | - |
| **Sucrose** | + | + | - | - | - | - | + | - |
| **Gas generation** | - | + | + | - | - | - | + | - |
| **$H_2S$ production** | - | - | - | - | - | - | - | - |
| **Urease** | - | + | - | + | - | + | - | - |
| **Starch hydrolysis** | - | - | - | - | + | - | + | + |

**Table.3** Effect of growth stimulatory microbially derived IAA extract on root and shoot length and root number

| Time (hrs) | Maize | | | | | |
|---|---|---|---|---|---|---|
| | Root length(cm) | | Shoot length(cm) | | Root number | |
| | Control | Aux 25 | Control | Aux 25 | Control | Aux 25 |
| | Mean±SD | Mean±SD | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| **48** | - | - | - | - | - | - |
| **72** | 0.45±0.07 | 0.64±0.14 | 0.31±0.12 | 0.52±0.13 | 1.53±0.52 | 3.07±0.70 |
| **96** | 1.00±.08 | 1.57±0.25 | 1.06±0.14 | 1.63±0.12 | 2.53±0.52 | 4.13±0.64 |
| | Rice | | | | | |
| **48** | 0.14±0.02 | 0.34±0.09 | - | - | - | - |
| **72** | 0.44±0.12 | 0.82±0.19 | 0.14±0.02 | 0.34±0.09 | - | - |
| **96** | 0.70±0.17 | 1.12±0.21 | 0.34±0.09 | 0.74±0.099 | - | - |

**Fig.1** Concentration (µg/ml) of IAA from isolates after 24hrs, 48hrs and 72hrs of incubation period




In conclusion, rhizosphere soil is a huge source of nutrient for microbes. There is a complex mechanism between plant root and soil microbes. Bacterial isolates from the rhizosphere soil were more efficient IAA producers than bulk soil. From this study, it can be said that rhizosphere soil is a rich source of IAA producing bacteria. These bacteria produced IAA in media. These eight isolates were characterized by macroscopic observation, microscopic observation and biochemical test. These isolates produced maximum amount of IAA after 72 hours of incubation and have positive response in seed germination. So it can be stated that presence of such growth promoting bacteria are responsible for the beneficial effects on plant growth and they can be used as bio fertilizer instead of industrial chemical.

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