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Optimization of Indole-3-Acetic production by phosphate solubilization bacteria isolated from waste mushroom bed of *Agaricus bisporus*

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ABSTRACT – **A total of 35 phosphate solubilizing bacterial strains were isolated from waste mushroom bed of *Agaricus bisporus* in Buyeo-Gun, Chungnam and screened for the production of indole acetic acid (IAA). The best IAA producing strain was identified as *Pantoea rodasii* using 16S rRNA analysis. In addition to the IAA production, this strain could act as an efficient phosphate solubilizer (1100 μg ml^{-1} after 5 days of incubation) also. The selected strain was cultured under different conditions in order to assess the optimum conditions for maximum IAA production. The nutrient broth (NB) medium was recorded as the best medium, where the maximum IAA production (229 μg ml^{-1}) was recorded at the start of stationary phase (12 hours after inoculation) of the bacteria growth. The performance of the strain was found to be maximum at the temperature of 30°C followed by 25°C. IAA production was found to be increased with increasing tryptophan concentration (from 0.1 to 0.6%), however beyond this limit, a slight reduction in IAA production was observed. The strains' ability to produce IAA was further confirmed by extraction of crude IAA and subsequent TLC analysis. A specific spot from the extracted IAA preparation was found corresponding with the standard spot of IAA with same R_f value. The results of HPLC analysis conducted in identifying and quantifying the IAA production more precisely, are in agreement with the results of the assessment done with colorimetric method. As revealed by the results of the pot experiment, the isolated strain could significantly enhance the growth (as measured by shoot and root growth) of mung bean plants compared to that of non-inoculated plants. Therefore it can be concluded that the present strain, *Pantoea rodasii* has great potential to be used as bio-inoculants.**

KEYWORDS – *Pantoea rodasii*, indole acetic acid, bio-inoculants, phosphate solubilizer

Introduction

Microorganisms can act directly or indirectly resulting in beneficial or detrimental effects on the soil environment. Soil microorganisms such as bacteria, fungi and algae, which are capable of producing physiologically active quantities of auxins may exert pronounced effects on plant growth and development. The most common, best characterized and physiologically most active auxin is known to be indole acetic acid (IAA), which is mainly produced by tryptophan dependent pathway (Datta and Basu, 2000; Ahmad *et al*., 2005; Gosh and Basu, 2006; Mandal *et al*., 2007). Some microorganisms are known to produce auxin in the rhizosphere using a substrate of plant exudates which contain amino acid L-tryptophan (Ahmad *et al*., 2005)

As stated by Ahmad *et al*. (2008), both rapid response (e.g. increased cell elongation) as well as long-term response (e.g. cell division and differentiation) is distinguished when IAA stimulates plant growth. Furthermore, IAA enhances lateral root formation which in turn could facilitate high root surface area for better absorption of nutrients (Compant *et al*., 2010). Therefore, microbes through the production of IAA could have definite effect on growth of the host plant. It has been reported that microbial IAA promotes root growth either directly by stimulating plant cell elongation or cell division or indirectly by its influence on the 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Patten and Glick, 2002). It has also reported that IAA

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could effectively contribute towards the defense mechanism in plants against external stress conditions (Bianco and Defez, 2009).

Microbial IAA production has been investigated over the years by several researchers (Ahmad *et al*., 2008; Gulati *et al*., 2009; Jung *et al*., 2011). The phosphate solubilizing bacteria which are capable of producing IAA include *Pseudomonas* sp., *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Klebsiella* sp., *Azospirium* sp., *Pantoea* sp. and *Serratia* sp. (Mirza *et al*., 2001; Vessey, 2003; Patil *et al*., 2011; Kapoor *et al*., 2012; Kumar *et al*., 2001).

In vitro screening of the phosphate solubilizing bacteria for their potential of IAA production could provide a reliable base for selection of effective plant growth promoting bacteria to be used as bio-inoculants. Therefore, the present study was carried out to quantify and optimize the production of IAA by *Pantoea rodasii*, an efficient phosphate solubilizing bacteria isolated from green house soils and to assess the strains' ability to promote the growth of mung bean (*Vigna radiata*).

Materials and Methods

Isolation of phosphate solubilizing bacterial strains

The phosphate solubilizing bacterial (PSB) strains were isolated from tomato growing rhizosphere soil samples collected from green houses at Chungchugnam-do province, Buyeo-Gun area in South Korea. Serially diluted aliquots of soil samples were inoculated on NBRIP medium (National Botanical Research Institute Phosphate medium) containing 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water (Nautiyal, 1999). The pH of the media was adjusted to 7. The petri plates were incubated for 7 days at 30°C and examined for colonies developing clear zone. Colonies with conspicuous clear zones around them were picked up and further purified by repeated sub-culturing. Pure cultures were maintained as a glycerol suspension (30% v/v) at –80°C until use.

Screening of bacterial isolates for IAA production

For qualitative assay of IAA production, nutrient agar plates containing 0.1% tryptophan were inoculated individually with phosphate solubilizing isolates in aseptic conditions. Each inoculated plate was overlaid with an 82 mm diameter filter paper immediately after inoculation, and then incubated until formation of 0.5 to 2 mm colonies. After removing the filter papers, the plates were treated with Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and kept at room temperature (30°C) until the development of color. IAA producing bacteria were identified by the formation of a characteristic pink to red halo surrounding the colony. Diameter of each halo was recorded after 30 min. The strains distinguished with large pink color halo on agar plates were screen for the further studies.

Identification of the selected bacterial strain

The partial sequencing of 16S rRNA for the bacterial strains was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The online program BLAST was used in identifying the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using CLUSTAL X program (Thompson *et al.*, 1997), which involved sequence alignment by neighbor joining method (Saitou and Nei, 1987) and maximum parsimony using the MEGA4 program (Kumar *et al.*, 2001). Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Assay of inorganic phosphate solubilization

The selected bacterial strain was grown in sterilized liquid NBRIP medium (20 ml) at 30°C for 2 days with continuous shaking at 150 r min^{-1}. Aliquots of culture (1 ml) was then transferred to a

500 ml flask (n=3 per strain) containing sterilized liquid medium (250 ml) and incubated with continuous shaking at 30°C. Sterilized uninoculated medium served as a control. A sample (10 ml) of each cultured and control were taken 2, 5 and 7 days after the inoculation and centrifuged at 8000 rpm for 15 min. The clear supernatant was used in determining the pH and amount of phosphorous released into the medium.

Optimization of IAA production

Effect of incubation period on IAA production

The selected strain was cultured in sterilized 100 ml of liquid nutrient broth (NB) contained 0.2% tryptopan and incubated for 60 hrs with continuous shaking at 30°C. A sterilized uninoculated medium was served as the control. Sample of cultured and control were taken into centrifugation tube with different time intervals and centrifuged 10 min at 10000 rpm. The clear supernatant was used to determine IAA production as described by Gutierrez *et al*. (2009).

Clear supernatant of 1 ml was mixed with 4 ml of the Salkowski's reagent (50 ml of 35% perchloric acid and 1 ml of 0.05 M $FeCl_3$ solution). The mixture was incubated in the dark at 37°C for 30 minutes. With the development of pink color, which indicated the IAA production, the optical density was measured at 535 nm using UV spectrophotometer. The IAA production was quantified with the help of standard graph of IAA obtained in the range of 10-100 μgml^{-1}.

Effect of different media on IAA production

The strain was cultured in 250 ml erlenmeyer flasks containing 100 ml of different media such as NB, TSB (Tryptic Soy Broth), LB (Luria-Bertani), and NBRIP medium (National Botanical Research Institute Phosphorus) supplemented with 0.2% tryptophan to study the effect of media on IAA production. Sample of cell culture and control were taken into centrifugation tube after 12 hrs of incubation and centrifuged for 15 min at 10,000 rpm at 4°C. The clear supernatant was subjected to detection of IAA production as described by Gutierrez *et al*. (2009).

Effect of different temperature on IAA production

The effect of temperature on the IAA production was determined by incubating culture medium (NB medium with 0.2% tryptophan) at different temperatures ranging from 25-40°C. After incubation, IAA production was assayed as described by Gutierrez *et al*. (2009).

Effect of different tryptophan concentrations on IAA production

The optimum tryptophan concentration for the maximum IAA production was assayed by adding different tryptophan concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1%) to culture medium. The IAA production was assayed as described by Gutierrez *et al*. (2009).

In each experiment, optical density of culture supernatant was measured at 660 nm using UV spectrophotometer to obtain the cell growth.

Extraction of crude IAA and thin layer chromatography (TLC)

Single bacterial colony was inoculated to 200 ml of nutrient broth containing 0.6% of tryptophan and incubated at 30°C for 12 hours on a shaker. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5-3.0 with 1 N HCl and extracted twice with ethyl acetate. Extracted ethyl acetate fraction was evaporated in a rotator evaporator at 40°C. The extract was dissolved in 1 ml of methanol and kept at -20°C. Extracted methanol fraction (100 µl) were taken to TLC plates (Silica gel G_f 254, thickness 0.25 mm) and developed by reagent spray n-butanol: ethyl acetate: ethanol: water – 3:5:1:1. Spots with R_f values identical to authentic IAA were identified by spraying the plates with Ehmann's reagent (Ehmann, 1977).

Determination of IAA production by HPLC

The used column was C18, 5 µm 25 × 0.46 cm and a UV detector set to 280 nm at 40°C. Mobile

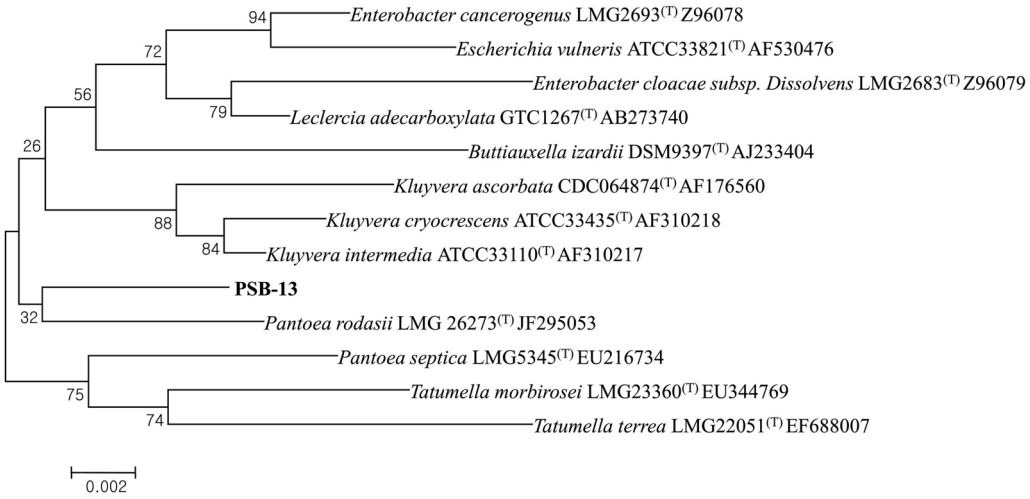

Fig. 1. Phylogenetic tree based on 16S rDNA gene sequences, showing the position of *Pantoea rodasii* (PSB-13) with respect to related species. The scale bar indicates 0.02 substitutions per nucleotide position and accession numbers are given in parenthesis.

phase consisted of methanol and water (80:20 v/v) run at a flow rate of 1 ml/min. HPLC profiles of the culture filtrate was analyzed by comparison with the elution profiles of those of authentic standards IAA injected separately.

Plant growth promotion bioassay with mung bean (*Vigna radiata*)

Mung bean (*Vigna radiate* var. paiyur 1) seeds were surface sterilized by immersing in 0.1% sodium hypochlorite solution for 10 minutes followed by washing thrice with distilled water. Seeds of mung bean were soaked in bacterial culture suspensions prepared in nutrient broth about 30 min prior to plant. The soil from 15 mm depth was removed from earthen pots and five seeds were placed at equal distance. Control plants received mung bean seeds soaked in nutrient broth without bacteria. Pots were watered daily to maintain the water holding capacity of the soil during the study period. After one week of germination, plants were thinned out allowing 3 plants per plot to remain. Growth promotion effects of bacterial treatment were assessed by measuring shoot and root length of mung bean plants.

Statistical analysis

Values were given as means ± SD for triplicate samples. The data were subjected to analysis of variance (ANOVA) using SAS package (SAS, 1999). The Duncan's Multiple Range Test (DMRT) was applied to test the significance of treatment means at $P \leq 0.05$.

Results

Isolation of phosphate solubilizing bacterial strains and screening for IAA production

Thirty five bacterial strains (PSB 1 to PSB 35) which exhibited clear zones on the NBRIP agar plates were selected as phosphate solubilizing organisms and they were screened for IAA production. All the tested phosphate solubilizing bacterial isolates showed positive response for IAA production with different concentrations as identified by characteristic red-pink halo. Out of 35 strains, 3 strains showed high production ability, 20 strains showed moderate while the rest showed low production ability of IAA. A strain with distinguished performance was selected, based on the results of IAA production and phosphate solubilization, for further studies

Identification of the bacterial strain

According to 16S rRNA sequence analysis, the strain showed close proximity with *Pantoea rodasii*

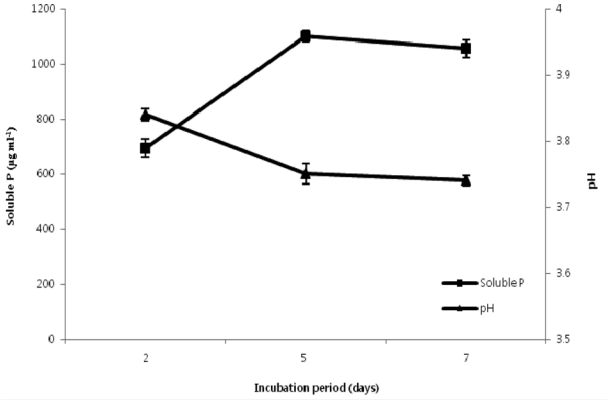

Fig. 2. Insoluble Phosphate solubilization (µg ml^{-1}) and changes of pH in NBRIP culture medium containing *Pantoea rodasi*. Values given here are the means (n = 3) ± standard deviation.

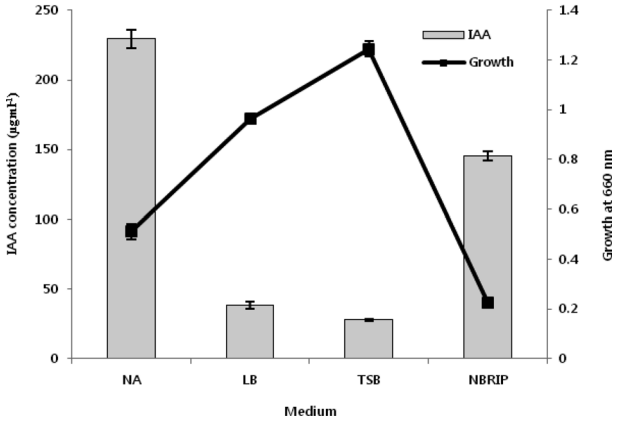

Fig. 3. Effect of different medium on growth and IAA production of *Pantoea rodasii*. Values given here are the means (n = 3) ± standard deviation.

LGM 26273. Phylogenetic tree (Fig. 1) shows the position of isolated bacterial strain with respect to the related species.

Assay of inorganic phosphate solubilization

As shown in Fig. 2, it was apparent that bacterial isolate significantly increased available phosphorus content in the medium. The increment was shown to be pronounced during the first 4-5 days (1100 µg ml^{-1}) of the incubation. However, as incubation progressed, a significant drop in soluble phosphorus level was observed. As depicted in Fig. 2, the bacterial inoculation resulted in acidification of the culture medium also. The pH of the medium was reduced to 3.75 at the end of 5th day of incubation.

Optimization of IAA production

Effect of different media on IAA production

As revealed by the results, NB medium was the best medium for IAA production (229.54 µg ml^{-1}) followed by NBRIP medium (145.57 µg ml^{-1}). The strain showed very low IAA production with LB and TSP medium (Fig. 3), though higher bacterial growth was recorded with those two medium also.

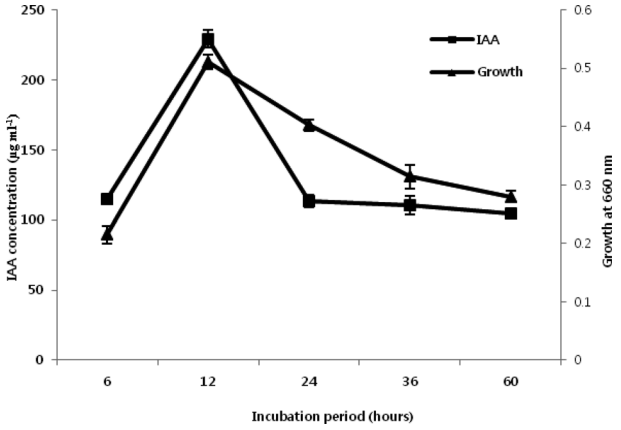

Fig. 4. Effect of incubation period on growth and IAA production of *Pantoea rodasii*. Values given here are the means (n = 3) ± standard deviation.

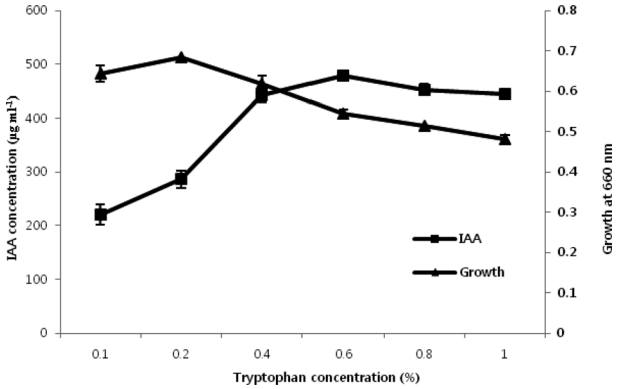

Fig. 5. Effect of tryptophan concentration on growth and IAA production of *Pantoea rodasii*. Values given here are the means (n = 3) ± standard deviation.

Effect of incubation period on IAA production

As shown in Fig. 4, the growth of the bacterial strain was found to be parallel to IAA production which reached to the maximum (229.54 µg ml^{-1}) at the stationary phase (after 12 hrs) of the growth.

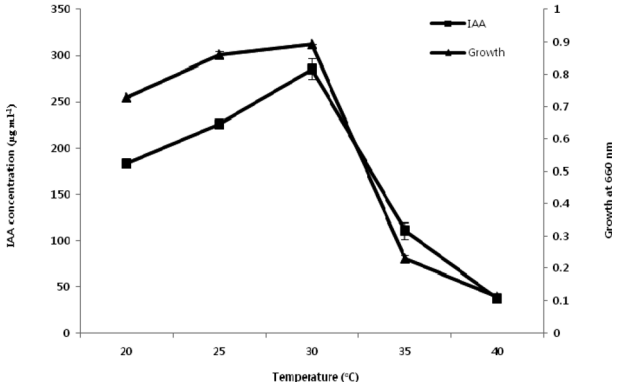

Fig. 6. Effect of incubation temperature on growth and IAA production of *Pantoea rodasii*. Values given here are the means (n = 3) ± standard deviation.

Effect of different temperature on IAA production

The highest IAA production (229.54 µg ml^{-1}) was recorded at 30°C followed by 25°C (201 µg ml^{-1}). The IAA production was significantly decreased when temperature increased from 30 to 35°C. The growth (after recording the maximum at 25°C) was found to be decreased with the increasing temperature. No growth or IAA production was recorded at the temperature over 40°C (Fig. 5).

Effect of different tryptophan concentration on IAA production

To determine the effect of tryptophan concentration on the IAA production, various tryptophan concentrations ranging from 0.1% to 1% were added to the culture medium. As depicted in Fig 6, IAA production was dramatically increased with increasing amounts of tryptophan up to 0.6%, thereafter a slight reduction was observed. The growth of the strain was increased with increasing tryptophan concentrations up to 0.2%, however, decreased thereafter.

TLC and HPLC analysis

The stain' ability to produce IAA was further confirmed by TLC and HPLC analysis. As shown in Fig. 7, when TLC plate was treated with Ehmann's reagent, the ethyl acetate extracts from culture filtrate showed a clear pink color spot on the TLC plate at the R_f value corresponding to standard IAA (0.93). HPLC analysis was in fact conducted to identify and quantify the IAA production precisely. As shown in Fig 7, culture filtrate of the strain and corresponding reference authentic standard showed peak at the similar retention time (2.99 min.). The results of HPLC are in close agreement with those assessed by the Salkowski reagent (data not shown).

Plant growth promotion bioassay with mung bean (*Vigna radiata*)

According to the results of plant growth promo-

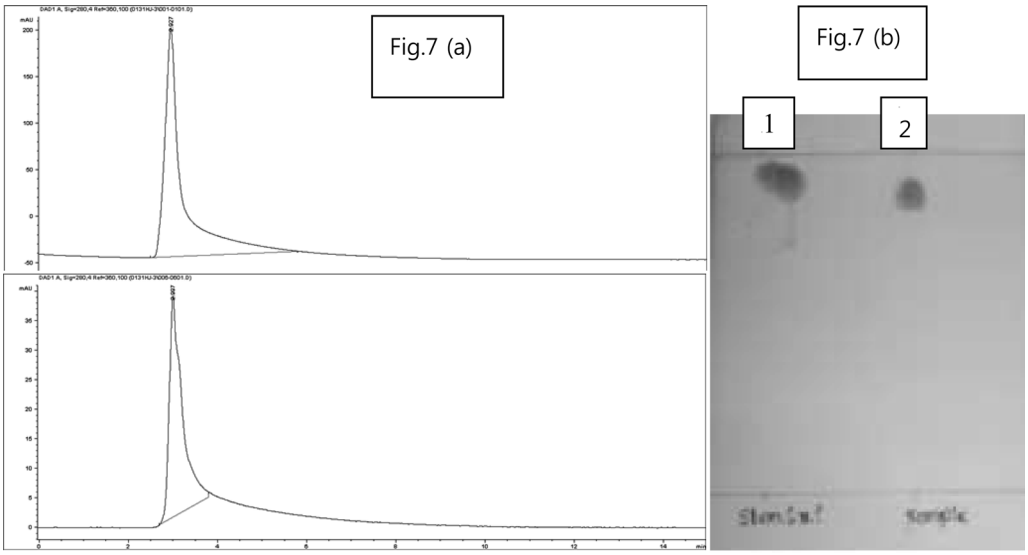

Fig. 7. Results of HPLC and TLC analysis. (a) HPLC chromatograms of the authentic IAA (upper) and the culture supernatant of *pantoea rodasii* (lower) using a C18 reverse HPLC column (retention time 2.99 min). (b). TLC chromatograms of the authentic IAA (lane 1) and the ethyl acetate extracted fraction of culture supernatant of *pantoea rodasii* (lane 2).

Table 1. Shoot length and root length of mung bean seedlings after inoculation of *Pantoea rodasii*. Values given here are the means of three replicates (n = 3)

	Inoculated	Un-inoculated
Shoot length (cm)	37.58	32.31
Root length (cm)	21.22	18.15

tion assay, the strain could significantly promote the shoot and root growth of mung bean as compared with uninoculated seeds. As shown in Table 1, the inoculated seedlings recorded 14.16% and 16.36% higher shoot and root lengths respectively compared to uninoculated control.

Discussion

Apart from releasing available phosphorous to plants, phosphate solubilizing microorganisms (PSMs) are known to be involved in plant-growth promotion through biological nitrogen fixation, iron chelation, producing plant hormones such as auxins, cytokinins, and gibberellins, producing enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which lowers plant ethylene levels, antagonism against phytopathogenic microorganisms by producing siderophores, hydrogen cyanide, enzymes and/or fungicidal compounds such as chitinase, cellulose, protease etc (Wani *et al*., 2007; Lipping *et al*., 2008; Mittal *et al*., 2008). Therefore, it is worth to isolate and screen phosphate solubilizing bacteria for other plant growth promoting substances also in order to use them as effective bio-inoculants.

In the present study, thirty five PSB were isolated and screened for their IAA production. Based on the results of phosphate solubilization and IAA production, one strain with outstanding performances was selected for further studies. The strain was identified as *Panotea rodasii* according to the 16S rRNA sequence analysis. There are some previous reports about *Pantoea* spp. as efficient IAA producers and also as efficient phosphate solubilizers (Sergeeva *et al*., 2002; Manulis and Barash, 2003; Dastager *et al*., 2009).

The present strain, once inoculated, decreased the pH in culture medium which indicates the production of organic acid and phosphatase enzyme, the main mechanism responsible for phosphate solubilization (Chaiharn and Lumyong, 2011). The slight decrease in phosphate solubilization after 5 days of incubation may be due to increased concentration of soluble phosphorus in the culture medium, which has an inhibitory effect on further phosphate solubilization (Varsha-Narsian *et al*., 1994). However, as suggested by some researchers, depletion of nutrients in the culture medium, especially carbon source for the production of organic acids may also contribute at least in part, to decreased rate of phosphate solubilization at the later stages of the incubation (Kang *et al*., 2002; Kim *et al*., 2005; Chaiharn and Lumyong, 2009).

The strain released greater quantities of IAA in the presence of L-tryptophan as physiological precursor. This suggests that the present strain is capable of synthesizing IAA from L-tryptophan through the indole-3-pyruvic acid pathway (Patten and Glick, 1996). Under natural conditions, some plants exudates containing amino acid L-tryptophan can be utilized by the microorganisms for IAA biosynthesis in the rhizosphere (Ahmad *et al*., 2005). IAA production greatly varies with incubation period, medium composition, temperature and tryptophan concentration.

As reported previously, different general mediums with different compositions have been used for IAA production. In this regard, Shahab *et al*. (2009) and Chaiharn and Lumyong (2011) have used NB media and Medipour Moghaddam *et al*. (2012) have used LB medium as base medium for IAA production. For the present strain, NB medium was shown to be the best medium for IAA production.

A significant positive correlation was observed between IAA production and bacterial growth in NB medium. The stationary phase of the growth began to appear just 12 hours after the commencement of the incubation. IAA production commenced right from the beginning of the incubation was found to reach the maximum at the stationary phase of the growth, which is analogous to Unyayar *et al*. (2000), Hansan (2002) and Swain *et al*. (2007) who also observed the maximum IAA pro-

duction during stationary phase of the growth. This may be due to availability of maximum tryptophan concentration from the dead bacterial mass (Swain *et al*., 2007). Reduction in IAA production at the later stages might be due to release of IAA degrading enzymes such as IAA oxidase, peroxidase by the bacteria as reported by Hunter (1989).

As revealed by the results, L-tryptophan at 0.6% is the best concentration for the maximum production of IAA. Concentrations over 0.6% of tryptophan might be detrimental thus results in slight reduction in IAA production (Mandal *et al*., 2007). Similar to our findings, Patil *et al*. (2011) reported that IAA production by *Acetobacter diazotrophicus* L1 increased when tryptophan concentration increased from 0.2 to 1.0 g L^{-1} remained static up to 1.2 g L^{-1} and then declined from 1.2 to 1.6 g L^{-1}. According to Swain *et al*. (2007), tryptophan at the concentration of 1g L^{-1} could increase the IAA production by *Bacillus subtilis* CM5, though decrease at higher concentrations. Bacteria are known to use several different biosynthesis pathways for IAA production and sometimes single strain uses more than one biosynthesis pathway, which explain the species-dependent manner of IAA production (Patten and Glick, 1996).

It is well known that the growth of bacteria is affected by high temperature (Malboodi *et al*., 2009), which is further confirmed by the present results with decreased bacterial growth and IAA production when incubated at high temperature over 30^{o}C.

The sensitivity of TLC to identify indole compounds is found to be lower as compared to that of HPLC, however, combinations of TLC and chromogenic reagents can provide another means for verification of indole derivatives (Chung *et al*., 2003). There are many chromogenic reagents with different specificities and sensitivities which can recognize and react with indole derivatives. Therefore, use of them could ensure easy visualization and differentiation of indole derivatives. Ehrlich reagent reacts with IAA to give pink color, which gradually turns to blue color. In this study, we were benefited by the chromogenic stains, which further confirmed the results obtained from HPLC analysis. Our TLC results are in agreement with Sudha *et al*. (2012), who observed the same R_f value (0.93) for IAA produced by *rhizobium* sp. and *Bacillus* sp.

As proved by the increased growth performances of mung bean plant, the isolated strain through its ability to produce IAA, may form a beneficial association with the host plants. Though bacteria are known to exhibit several plant growth promoting characteristics, the production of phytohormones such as IAA and gibberellins is considered to be one of the most commonly reported direct plant growth promotion mechanism (Chanway, 2002). IAA stimulates a rapid response (e.g. increased cell elongation) as well as a long-term response (e.g. cell division and differentiation) in plants (Ahmad *et al*., 2008). Furthermore, it involves in development of root structures such as lateral roots and root primordia which in turn could facilitate high root surface area for better uptake of water and nutrients from soil (Compant *et al*., 2010). Sergeeva *et al*. (2007) observed 15-37% enhanced root length, root weight or shoot weight in canola, lentil and pea plants inoculated with IAA producing five different *Pantoea* sp. Chaiharn and Lumyong (2011) also isolated high IAA producing *Klebsiella* (291.97 ppm) SN 1.1 and demonstrated stimulatory effects on bean and maize seedlings.

The findings of the present investigation highlighted that the isolated strain has great potential to enhance soil fertility and plant growth through phosphate solubilization and IAA production. However, assessment of other plant growth promoting characteristics and further studies under field conditions would be ideal in confirming the present findings and also in recommending the strains as bio-inoculants.

Acknowledgment

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