Indian Journal of Experimental Biology
Vol. 47, December 2009, pp. 993-1000

Isolation and characterization of indole acetic acid (IAA) producing *Klebsiella pneumoniae* strains from rhizosphere of wheat (*Triticum aestivum*) and their effect on plant growth

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Received 16 December 2008; revised 4 September 2009

The present study was undertaken for isolation of *Klebsiella* strains from rhizosphere of wheat (*T. aestivum*), screening and characterization of these strains for *in vitro* indole acetic acid (IAA) production and studying the effect of these strains on plant growth under gnotobiotic conditions. Nine strains of *Klebsiella* were isolated from rhizosphere of wheat (var. Lokwan) and identified as *K. pneumoniae* by 16S rRNA gene sequencing. Six *K. pneumoniae* strains showed *in vitro* IAA production. Colorimetric analysis showed that K8 produced maximum IAA (27.5 mg l^{-1}) in the presence of tryptophan (1 mg ml^{-1}) at 72 h of incubation with optimum conditions as *p*H 8.0, 37°C and 0.5% (w/v) NaCl concentration. GC-MS analysis and IR studies confirmed presence of IAA in the cell filtrates of strain K8. Effect of six IAA producing *Klebsiella* strains on plant growth was studied by performing series of seed germination tests using moth bean seeds under axenic conditions and pot experiments using sterilized soil and wheat seeds (var. Lokwan). Strain K11 and K42 demonstrated increase in root length of inoculated moth beans (~ 92.71% over the control). Results of pot experiments indicated that almost all the six IAA producing *Klebsiella* strains significantly increased the root length and shoot height of inoculated wheat seedlings over the control. The results suggest that these are promising isolates from wheat rhizosphere and merits research on appliance of these strains in agriculture.

Keywords: Indole acetic acid, *Klebsiella,* Pot experiments, Rhizosphere, Seed germination tests, Wheat

Rhizosphere is a rich niche of microbes and should be explored for obtaining potential plant growth promoting rhizobacteria (PGPR), which can be useful in developing bio-inoculants for enhancement of growth and yield of crop plants. *Klebsiella* species have been found to be present in the rhizosphere and known to exhibit important PGP traits like nitrogen fixation. Although *Klebsiella* has been considered as a model system for intensive study on nitrogen fixation mechanisms[1], other plant growth promoting properties of *Klebsiella* species have not been explored in detail. *Klebsiella* species have been isolated and studied for IAA production from rhizosphere of sugarcane[2], soybean[3], rice[4], pearl millet[5] and kentucky bluegrass[3]. However, there is a lack of information on IAA production by *Klebsiella* species from rhizosphere of wheat.

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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 amino acid requiring bacteria exist in rhizosphere than in root free soil[7]. 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 promoting rhizobacteria (PGPR) [8]. These bacteria help in fixation of atmospheric nitrogen[9] production of siderophores[10], solubilization of minerals like phosphorus[11] and synthesis of phytohormones [indole-3-acetic acid (IAA)][12,13]. Some bacteria support plant growth indirectly by producing antagonistic substances or by inducing systemic resistance against plant pathogens[11].

Indole acetic acid (IAA), a phytohormone, is produced in young leaves, stems and seeds from transamination and decarboxilation reactions of tryptophan[14]. Effects of IAA on plants are significant

and some of them are- apical dominance (apex dominates lateral meristems), phototrophism, gravitropism, prevention of leaf and fruit abscission and induction of adventitious roots. Therefore, IAA has profound influence on crops. The present study was conducted to explore the rhizosphere of wheat for IAA producing *Klebsiella* strains and to study the effect of these strains on plant growth under controlled conditions.

Materials and Methods

Isolation of Klebsiella strains from wheat (var. Lokwan) cultivated fields — Nine isolates biotyped (API 32 GN identification system; BioMerieux Inc., France) as *Klebsiella pneumoniae* were isolated from wheat (*Triticum aestivum*) rhizosphere from local wheat (var. Lokwan) cultivated fields. These *K. pneumoniae* strains were designated as- *K. pneumoniae* K8, *K. pneumoniae* K11, *K. pneumoniae* K17, *K. pneumoniae* K20, *K. pneumoniae* K22, *K. pneumoniae* K23, *K. pneumoniae* K29, *K. pneumoniae* K30 and *K. pneumoniae* K42. These strains were screened for production of IAA by the method as described earlier[12].

Identification of IAA producing Klebsiella strains by 16S rRNA gene sequencing — Genomic DNA was extracted from the isolates by the standard phenol-chloroform extraction method[15]. Partial 16S rRNA gene was amplified from genomic DNA by PCR using the universal bacterial primers, corresponding to *E. coli* position 27f and 1525r[16]. Amplification was performed in GeneAmp PCR System 9700 (Applied Biosystems, USA) in 25 µl reaction volume containing 50 ng of DNA template, 0.2 m*M* each of dATP, dCTP, dGTP, and dTTP, 12.5 p*M* of each primer, 1X PCR buffer (Bangalore Genei, India) and 0.6U of Taq DNA polymerase (Bangalore Genei, India). The thermal cycling was performed as - initial denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were purified using QIA quick PCR purification kit (Qiagen, USA) according to the manufacturer's suggested protocol and nearly complete sequences of 16S rRNA genes were obtained using the primers 343r, 27f, 1525r[16], 530f[17], 946f[18], 704f, 685r and 907r[19]. Cycling sequencing was performed using Big Dye Terminator Cycle Sequencing Kit (v3.1, Applied Biosystems), according to the manufacturer's protocol, and analyzed in an Applied Biosystems 3730 DNA Analyzer. These sequences determined were compared with previously reported type strain sequences in GenBank database using EzTaxon server. The nomenclature used was as described in Bergey's Manual of Systematic Bacteriology[20]

Phylogenetic analysis — All 16S rRNA gene sequences were manually edited, assembled and analyzed, using the ChromasPro software (v1.41, Technelysium Pvt Ltd.). Identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity were achieved using EzTaxon server[21] (http://www.eztaxon.org/). Sequences of IAA producing *Klebsiella* strains were separately aligned using ClustalW2 at European Bioinformatics site[22] (http://www.ebi.ac.uk/Tools/clustalw2/) and similarity between the nucleotide sequences was computed using Kimura 2-parameter nucleotide substitution model[23]. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were 1386 positions in the final dataset. The reference sequences were downloaded in FASTA format, from NCBI database (http://www.ncbi.nlm.nih.gov) and phylogenetic tree was constructed by the Neighbor-joining method[24] using MEGA4 software[25]. Tree topologies were evaluated by performing bootstrap analyses[26] using 1000 re-samplings. Phylogenetic analysis was also performed with Minimum Evolution and Maximum Parsimony methods[25].

Sequence submission — The partial 16S rRNA gene sequences of IAA producing *K. pneumoniae* strains determined in this study were deposited in GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers; EU661373 (*K. pneumoniae* K11), EU661374 (*K. pneumoniae* K8), EU661375 (*K. pneumoniae* K17), EU661376 (*K. pneumoniae* K23), EU661377 (*K. pneumoniae* K30) and EU661378 (*K. pneumoniae* K42).

Characterization of IAA production by K. pneumoniae strains — IAA production by *Klebsiella* strains both in the presence and absence of tryptophan (1 mg ml^{-1}) was determined by colorimetric analysis. For this purpose, 100 ml of Luria Bertanii (LB) broth (containing g l^{-1}: tryptone-1, NaCl-1, yeast extract-0.5, *p*H 7.0 ± 2) were added in 250-ml Erlenmeyer flasks. The flask contents was inoculated by adding 100 µl of overnight grown

bacterial suspension adjusted to optical density 0.5 (10^6-10^7 CFU ml^{-1}) measured at 540 nm. The flasks were incubated at 28°C for 48 h with continuous shaking at 100 rpm. Non-inoculated broth was kept as control. After incubation, IAA production was determined using Salkowski method as described earlier[12]. Standard graph of IAA was constructed using standard IAA (HiMedia Laboratories Pvt. Ltd., Mumbai). The graph was plotted as absorbance at 540 nm against concentration of IAA (0.01 - 0.1 g $ml^{-1} \times 10^{-3}$).

IAA production by *Klebsiella* strains was quantified by Salkowski method[12] after 48 h of incubation at various *p*H (4.0-9.0) ranges, temperatures (4, 28, 37 and 42°C) and NaCl concentrations [0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v)]. For these characterization studies, 100 ml of LB broth containing tryptophan (1 mg ml^{-1}) was inoculated with 100 µl of overnight grown bacterial suspension adjusted to optical density 0.5 (10^6-10^7 CFU ml^{-1}) measured at 540 nm. To study the effect of *p*H on IAA production, buffered broths were prepared in the standard buffers such as acetate, phosphate and Tris HCl. Each experiment was performed in duplicates and the mean values were considered.

Time course of IAA production by K. pneumoniae strains — LB broth (100 ml) containing tryptophan (1 mg ml^{-1}) was inoculated with 100 µl of overnight grown bacterial suspension adjusted to optical density 0.5 (10^6-10^7 CFU ml^{-1}) measured at 540 nm and production of IAA was monitored at an interval of 24 h up to 168 h by the Salkowski method[12]. Optimum conditions obtained from previous experiments on *p*H, temperature and NaCl concentration were used for this study.

Purification and analysis of purified IAA from K. pneumoniae strain K8 — IAA produced by *Klebsiella* strain K8 was purified by the method as described elsewhere[13,27]. The purified compound was identified as IAA by gas chromatography- mass spectroscopy (GC-MS) after methylation of the compound and also by infrared (IR) spectroscopy. For the methylation of the purified compound, procedure was followed as described elsewhere[28] with modification. In a 500 ml round bottom flask, 50% potassium hydroxide solution (60 ml) in water was cooled to below 0°C and diethyl ether (200 ml) was added. To this cooled solution, N-nitroso methyl urea (20.6 g, 5 mole) was added in portions with vigorous stirring. Diazomethane, thus, formed was trapped in the ether layer. Separation of ether layer and subsequent washing of aqueous layer with cold ether gave bright yellow ether solution of diazomethane. The ethereal layer was kept still for 1 h on solid potassium hydroxide. The yellow ethereal solution containing diazomethane was directly used for further reactions. To an ice-cooled solution of standard IAA and purified compound (1 mole) in methanol, solution of diazomethane (5 mole) in ether was added for a period of 10 min. The mixture was stirred for 2 h during which temperature slowly attained the room temperature (30°C). The solvent was removed under reduced pressure and the residue was dissolved in ether. Ethereal solution was washed with $NaHCO_3$ (10%) followed by distilled water. Ether layer was dried over sodium sulphate and extracted under reduced pressure.

The methylated compound and standard IAA were subjected to gas chromatography - mass spectrometry (GC-MS – QP5050, Shimadzu Labsolutions, Japan). GC-MS was equipped with a DB-5 capillary column (30m, length, 0.25 µm thickness, 0.25 mm diam) and a high-resolution mass spectrometer. The operating conditions were - injector temperature, 220°C, detector temperature, 280°C, column temperature, 50°C for 1min to 280°C at 10°C min^{-1}; mass spectrometry scan, 200- to 500-s^{-1} interval; resolution, 1,000; electron impact, 70 eV; ionizing source temperature, 200°C[29]. The identity of purified compound, as IAA was determined on the basis of its retention time and characteristic fragmentation pattern as compared to the data of GC-MS analysis of methylated standard IAA.

Infrared spectrum of the purified compound was obtained using Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu FTIR 8400) and the spectra was recorded in nujol mull using KBr cells and expressed in wave number (cm^{-1})[12].

Seed germination test and pot experiments — To study the effect of IAA producing *Klebsiella* strains on growth of plant, seed germination test[30] and pot experiments[31] were performed. For seed germination test, moth bean (*Vigna aconitifolia*) seeds were surface sterilized by exposing to 95% ethanol and immersing in 0.2% $HgCl_2$ solution for 3 min. The seeds were then subjected to five washings with sterile distilled water. One milliliter of overnight grown bacterial suspension (10^7-10^6 cells ml^{-1}, 0.5 of O.D.540 nm) was applied on each seed for 10 min.

Seeds were dried and under axenic condition sown on soft agar (0.8%) plates. Plates were incubated at 30°C for 3 days. Six seeds were sown in each plate with two repeats. The sterile seeds were soaked in non-inoculated media for 10 min and sown on soft agar plate in case of control. The root length was recorded as it was considered the main parameter in determining the effect of IAA.

For pot experiments, soil samples were collected, air-dried, sieved (2-mm/10-mesh) and sterilized by repeated autoclaving before filling the pots. Wheat (var. Lokwan) seeds were surface sterilized by exposing to 95% ethanol and immersing in 0.2% $HgCl_2$ solution for 3 min. The seeds were then subjected to five washings with sterile distilled water. One milliliter of overnight grown bacterial suspension (10^7–10^6 cells ml^{-1}, 0.5 of O.D.540 nm) was applied on each seed for 10 min, seeds were dried and were transferred to pots containing sterile soil to a depth of 5 mm. Six seeds were sown in each pot and the experiment was performed in duplicate for each *Klebsiella* strain. Sterile seeds treated with non-inoculated media were used as control. The pots were kept in sunlight and were observed every day for 15 d. On 16th day the plant was carefully uprooted. The root lengths and shoot heights were measured.

Analysis of data — Statistical analysis was performed with the help of mean, SD, and LSD. Least significant difference (LSD) was calculated and means were separated by Tukey's test at the 1% level[32].

Results

Identification and phylogenetic analysis of IAA producing Klebsiella strains from rhizosphere of wheat — Nine isolates from rhizosphere of wheat were biotyped by API 32GN identification system as *Klebsiella pneumoniae.* It was found that out of nine *Klebsiella pneumoniae* strains, six isolates (K8, K11, K17, K23, K30 and K42) produced IAA. Further, 16S rRNA gene sequence analysis of IAA producing *Klebsiella* strains was performed. Almost entire 16S rRNA gene was sequenced using internal primers (≥ 90% of *E. coli* sequence) for all isolates. These sequences determined were compared with previously reported type strain sequences in GenBank database using EzTaxon server. 16S rRNA gene sequence similarity of 98% was used as the cut-off for positive identification of taxa.

Phylogenetic analysis of IAA producing *Klebsiella* strains (Fig. 1) revealed that these strains belonged to the cluster of *K. pneumoniae* and within this cluster there were three clades, indicating that these strains of *Klebsiella* were of different phylotypes. Phylogenetic analysis with Minimum Evolution and Maximum Parsimony methods also produced similar results.

Effect of tryptophan, pH, temperature and NaCl concentration on IAA production by K. pneumoniae strains — Strains K11 and K42 produced IAA when LB was supplemented with tryptophan. Production of IAA by the strains K8, K17, K23 and K30 was enhanced (up to 2.2 times) when tryptophan was added to the medium (Fig. 2a). No growth was observed at *p*H 4.0 and 5.0 for any of these strains. Maximum IAA (16.9 mg l^{-1}) production was seen for K8 at *p*H 8 (Fig. 2b). *Klebsiella* strains were not able to grow at 4°C. It was observed that maximum IAA (17.9 mg l^{-1}) was produced at 37°C by the strain K8 (Fig. 2c). *Klebsiella* strains K11 and K42 were not able to grow at salt concentration of 5% (w/v). K8 demonstrated maximum IAA (21.9 mg l^{-1}) production at 0.5 % (w/v) salt concentration. Interestingly, K42 showed optimum IAA (11.12 mg l^{-1}) production in absence of salt in the medium (Fig. 2d).

Time course of IAA production — Strains K8, K11, K17 and K23 showed maximum IAA production at 72 h of incubation at optimum conditions, whereas strains K30 and K42 showed maximum IAA production at 48 h. Strain K8 produced 22.7 mg l^{-1} of IAA under optimum conditions at 72 h and was the

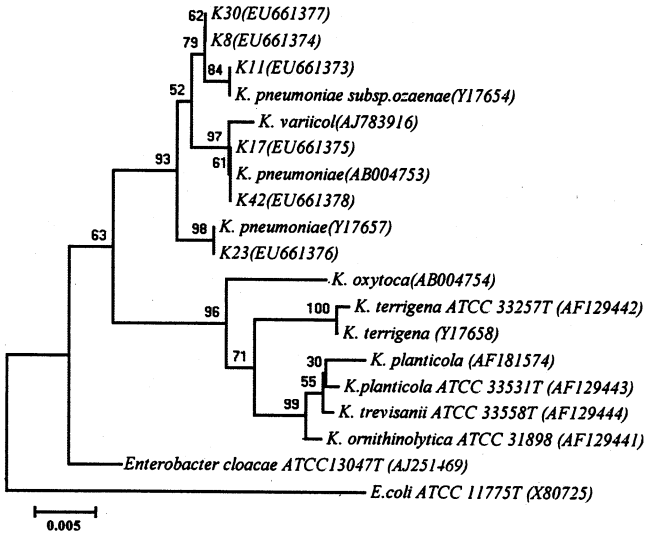

Fig. 1—Phylogenetic tree from partial 16S rRNA gene sequences of *Klebsiella* strains Bootstrap values (in percent) calculated from 1,000 resamplings are shown at the nodes. *E. coli* and *Enterobacter cloacae* were taken as an out-group. Scale bar shows number of nucleotide substitutions per site

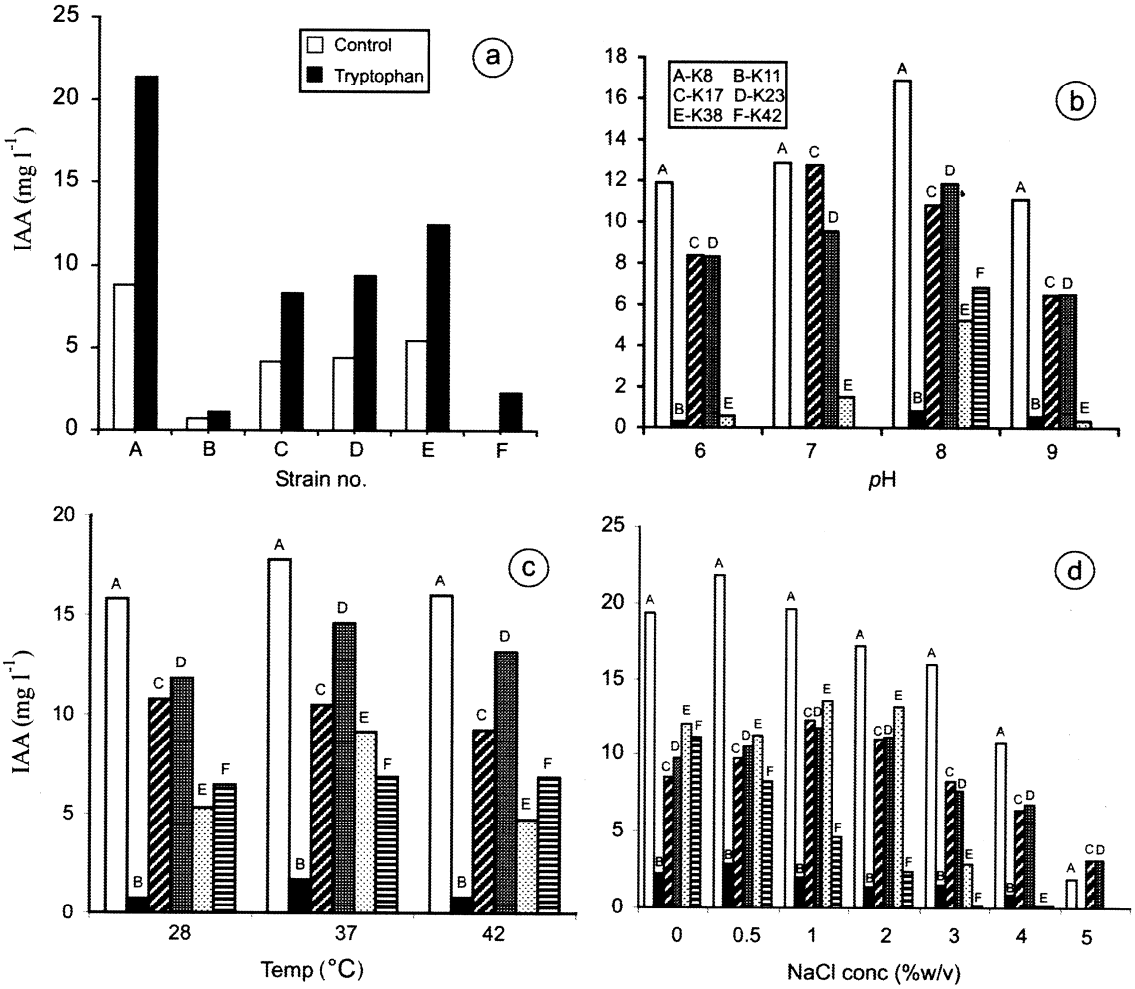

Fig. 2—Effect of (a) tryptophan; (b) *p*H; (c) temperature; and (d) NaCl on IAA production by *Klebsiella* strains. [Each value is the mean of two replicates]

best IAA producing strain among the *Klebsiella* strains used in this study (Fig. 3).

Confirmation of IAA production by K. pneumoniae K8 — Purification of IAA was performed by preparative thin layer chromatography. The purified compound was identified as IAA by GC-MS analysis. The retention time (11.83 min) and fragmentation pattern of the methylated compound was comparable to reference standard (methyl ester of standard IAA) (Fig. 4a, b). The molecular weight of the methylated compound was found to be 189 Da further confirming its identity. The IR spectrum of the purified compound showed OH frequency at 3389 cm^{-1} and a C=O frequency at 1698.4 cm^{-1} (Fig. 5). The IR spectrum of the standard IAA also showed the same results.

Effect of IAA producing Klebsiella strains on plant growth — In seed germination test, maximum effect on root elongation was demonstrated by strain K11 and K42. These strains increased root length of inoculated moth beans ~92.71% over the control. Analysis of the data obtained from the pot experiments revealed that all the six *Klebsiella* strains had significant effect on root length and shoot height as compared to control (Table 1). Strain K42 had the most favorable effect on root length (~1.59 folds over the control), as well as shoot height (~1.56 folds over the control) whereas strain K17 and K23 had optimum effect on root length (~1.67 folds increase over the control).

Discussion

IAA production by bacteria present in the rhizosphere is an important property contributing to plant growth[33]. Thus, the purpose of this research was to investigate IAA producing *Klebsiella* strains from wheat rhizosphere. We could isolate nine *Klebsiella* strains from the rhizosphere of wheat (var. Lokwan)

out of which six strains demonstrated *in vitro* IAA production.

There are several reports on enhancement of IAA production by rhizobacteria in the presence of tryptophan[13,34-36]. Tryptophan increased IAA production in the *Klebsiella* strains in this study indicating that it might be the precursor for IAA biosynthesis by these bacterial strains. *Klebsiella* strains produced IAA in the *p*H range 6-9. However, optimum IAA production for most of the strains was detected at *p*H 8. The probable reason could be the alkaline nature (*p*H 7.8-8.0) of the wheat rhizosphere (clay soil) from where these strains were isolated. *Klebsiella* strains in this study were able to grow and produce IAA at varied NaCl concentrations (0-5% w/v). It has been shown that foliar spray of indole-3-acetic acid can restore the metabolic alterations imposed by NaCl salinity in *Vigna radiata* (L.) Wilczek[37]. Thus, these IAA producing *Klebsiella* strains can be used as plant growth promoters under salinity stress conditions. From the characterization studies, it was revealed that strain K8 was the best IAA producer among the *Klebsiella* strains of this study and it produced 22.7 mg l^{-1} of IAA under optimum conditions at 72 h of incubation.

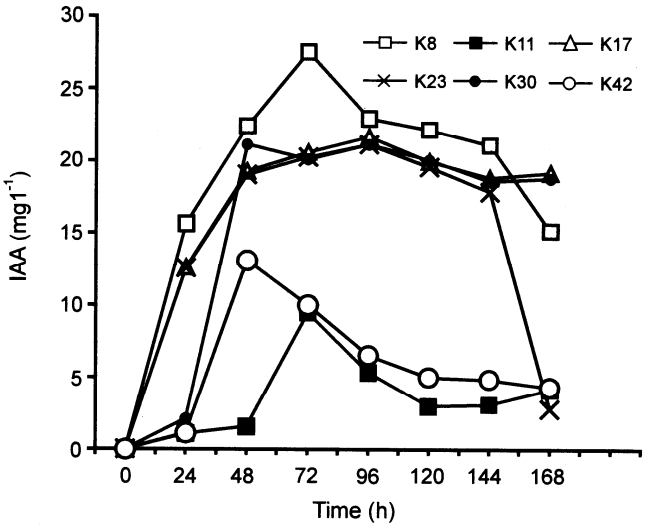

Fig. 3—Time course of IAA production by *Klebsiella* strains. Each value is the mean of two replicates]

The property of synthesizing indole acetic acid is considered as an effective tool for screening beneficial microorganisms as there have been reports suggesting that IAA producing bacteria have profound effect on plant growth[2,38]. Thus, these IAA producing *Klebsiella* strains were further studied for their effect on plant growth under controlled conditions. Data obtained from the seed germination

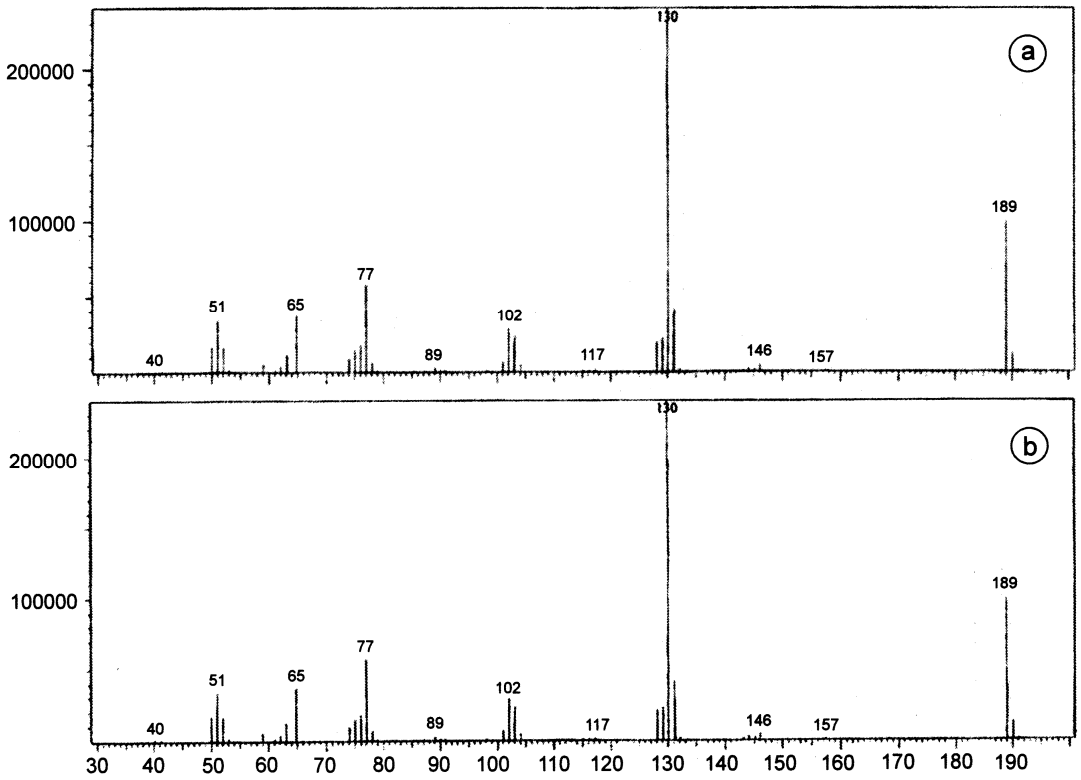

Fig. 4—Fragmentation pattern of (a) methylated purified compound from *K. pneumoniae* K8 and (b) methylated standard IAA

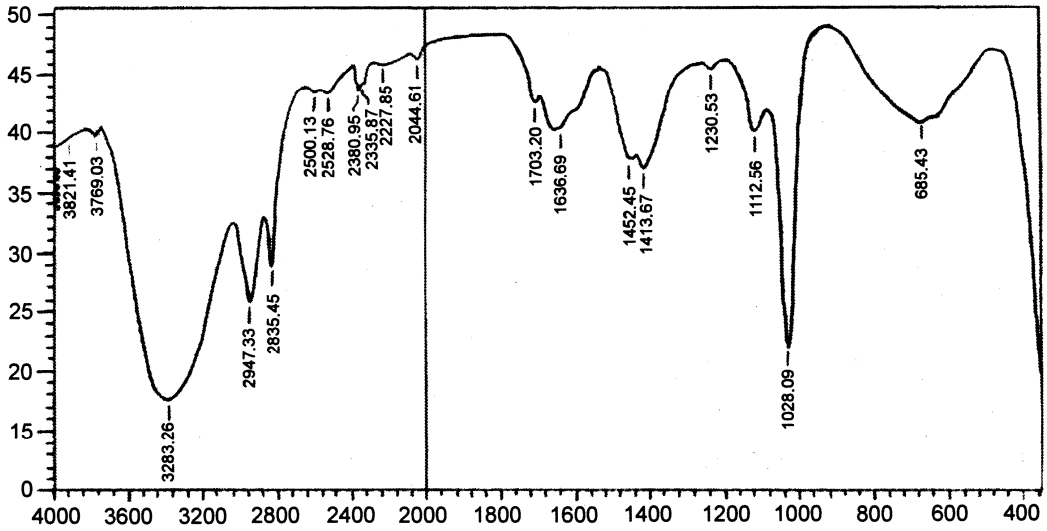

Fig. 5—IR spectrum of purified IAA from *K. pneumoniae* K8

Table 1—Root length (R) and shoot height (S) of wheat plant treated with IAA producing *Klebsiella* strains
[Each value is a mean of 12 plants]

Treatment	Mean (cm)		Standard error		Difference from control		LSD[§] value at 0.01	
	R	S	R	S	R	S	R	S
Control	12.45	8.0	0.873	1.032	-	-	-	-
K8	19.75	9.46	0.872	0.2027	7.3**	1.46**	3.515	0.817
K11	16.36	10.83	0.733	0.244	3.91**	2.83**	2.955	0.983
K17	20.66	11.58	0.231	0.1511	8.21**	3.58**	0.930	0.6092
K23	20.66	11.66	0.231	0.170	8.21**	3.66**	0.930	0.6854
K30	18.35	10.4	0.181	0.1493	5.9**	2.40**	0.727	0.6019
K42	19.86	12.55	0.528	0.2069	7.41**	4.55**	2.132	0.8342

[§]LSD – Least significant difference; **Significant at 1% level of probability

test and pot experiments demonstrated positive effect on root elongation of treated plants over the control. This indicated that these *Klebsiella* strains can improve the root development and thus can be considered as plant growth promoters.

These *Klebsiella* strains had also demonstrated *in vitro* PGP traits such as nitrogen fixation, phosphate solubilization and antifungal activity (Data not shown) in addition to IAA production. Hence, these strains have a potential of being developed as bio-inoculants. Overall, the plant growth promoting properties of these *Klebsiella* strains and their effect on wheat plant growth under axenic conditions suggests that these are promising strains for application in agriculture.

Acknowledgement

The authors are thankful to the Department of Biotechnology (DBT), Govt. of India, New Delhi (Project Sanction No. BT/PR6454/AGR/05/302/2005) for financial assistance. We are also thankful to Dr Y S Shouche, Scientist, National Centre for Cell Sciences (NCCS), Pune, India, for identification of isolates by 16S rRNA gene sequencing.

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