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Vol. 9(16), pp. 1139-1149, 22 April, 2015
DOI: 10.5897/AJMR2015.7456
Article Number: 7E820E052661
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Biosynthesis of indole-3-acetic acid by plant growth promoting rhizobacteria, *Klebsiella pneumonia, Bacillus amyloliquefaciens* and *Bacillus subtilis*

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Received 28 February, 2015; Accepted 13 April, 2015

Indole-3-acetic acid biosynthetic activity of *Klebsiella pneumonia*, strain MR-M1, *Bacillus amyloliquefaciens* strain MR-AI, four *Bacillus sp*. strain MR-SP, RR-R2, WR-W2 and MR-Z1 were investigated. Here, the authors demonstrated the effect of carbon sources, amino acids, vitamins and abiotic stress on the indole-3-acetic acid (IAA) production level under *in vitro* condition by the six strains. The culture medium was exogenously supplemented with L-tryptophan (200 µg/ml) and incubated for 96 h. IAA biosynthesis was further confirmed by indole-3-pyruvate decarboxylase encoding *ipdc* gene amplification. Succinate followed by acetate followed by malate was observed as the most preferred carbon source for IAA production. Stimulation of IAA production at pH 6.0, 0.1% salinity and 32°C temperature after 96 h of growth in presence of L-tryptophan was observed. The highest amount of IAA production was observed in strain MR-M1 followed by WR-W2. Decreasing trend of IAA levels was observed in the presence of vitamins and amino acids as compared to the control. The amplicon of 250 bp was observed in all the six rhizospheric strains. Taken together, the result shows that L-tryptophan stimulates IAA production and strain MR-M1 was observed as the most efficient IAA producing rhizospheric bacteria.

Key words: L-tryptophan, indole-3-acetic acid, plant growth promoting rhizobacteria, indole pyruvate decarboxylase.

INTRODUCTION

Rhizospheric colonization of cereal crop plants and plant growth promoting activity involving indole-3-acetic acid (IAA) production by *Klebsiella* sp. and B*acillus* sp. has been well documented (El-khawas and Adachi, 1999; Mishra and Kumar, 2012; Saharan and Nehra, 2011). Phytohormone, especially auxin indole-3-acetic acid plays important role in the plant growth regulation as cell enlargement, cell division, cell elongation and root initiation by tryptophan dependent and independent pathways (Woodward and Bartel, 2005). Rhizosphere is a site with complex interactions between the root and associated microorganisms with high microbial diversity (Saharan and Nehra, 2011). Microbial population in rhizospheric soil is physiologically more active, as

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the nutritional substance is released by the root and its surfaces. Rhizospheric microorganisms are considered as labile source of nutrients such as B-vitamins, amino acids, tryptamine, soluble root exudates, carbon sources and organic matter (Saharan and Nehra, 2011; Sainio et al., 1996; Ahemad et al., 2013; Barraquio et al., 2000). The role and metabolism of IAA in Gram-negative bacteria is well documented, but little is known about IAA biosynthesis and regulation in Gram-positive bacteria (Saharan and Nehra, 2011; Sainio et al., 1996; Ahemad et al., 2013,). L-tryptophan serves as a physiological precursor for biosynthesis of IAA in plants and in microbes (Woodward and Bartel, 2005). IAA is a secondary metabolite produced in early stationary phase of growth. Synthesis of IAA depends on environmental conditions, availability, uptake and rate of deamination of precursor, the types of soil nutrients, metabolites released and the preferred pathways involved (Spaepen et al., 2007; Woodward and Bartel, 2005). Soil pH, temperature and salinity condition are crucial for attachment and spread of the microbes (Strzelczyk et al., 1994; Dobbereinere et al., 2001). Moreover, L-tryptophan dependent biosynthesis of IAA involving *ipdc,* indole-3-acetamide (*iam*) and indole-3-acetonitrile (*ian*) gene has been well documented in plant growth promoting rhizobacteria (PGPR) (Duca et al., 2014). Although, the complete mechanism involved in the biosynthesis of IAA is poorly understood.

Reports suggest that most plant associated microorganism might have cellulase activity for adoption or establishment of a plant microbe interaction. Cellulase activities have seen in many N2 fixing bacteria such as *Bacillus spharricus*, *Bacillus circulans*, *Paenibacillus azotofixans* and *Azospirillum* (Emtiazi et al., 2007). Moreover, recent study reveals cellulase production by the *Bacillus subtilis* (Femi-Ola and Aderibigbe, 2008). Indole pyruvate decarboxylase encoding *ipdc* gene acts as key modulator for IAA production, which catalyses formation of indole-3-acetaldehyde from indole-3-pyruvic acid in *Klebsiella pneumonia* and *Bacillus* sp. (El-khawas and Adachi, 1999; Idris et al., 2002). The 16S rRNA gene (1.5 Kb) sequence involves identification of bacteria upto genus level on the basis of highly conserved sequences (Elgaml et al., 2013). Literature showed that the hypervariable regions V2 and V3 contain the maximum degree of nucleotide heterogeneity and the maximum discriminatory power for the bacterial species identification (Baker et al., 2003; Chakravorty et al., 2007). After thorough review of literature, it appears that the rhizospheric bacteria are of great relevance in terms of agricultural productivity. The present study was aimed to demonstrate IAA production capability of most efficient isolates under various physiological conditions. To replace costlier and hazardous chemically synthesized fertilizers, application of efficient PGPR as biofertilizer can bridge the gap. Many workers have acknowledged the role of PGPR and significant advancements have been made in this interesting area of research but there are still gaps left especially on molecular aspect, which needs further study.

MATERIALS AND METHODS

Sample collection, culture media and incubation condition

To isolate most efficient IAA producing PGPR, soil samples were collected from rice, (RR-R2) wheat (WR-W2) and maize (MR-M1, MR-SP, MR-MZ and MR-AI) agricultural field of Banaras Hindu University, Varanasi, Uttar Pradesh, India. 1 kg rhizospheric soil sample were brought to the laboratory in the sterile polythene bags. Isolation of bacteria from rhizospheric soil was performed, following the standard microbiological methods of Barraquio et al. (2000). One gram of rhizospheric soil of each sample were suspended in 10 ml sterilized double distilled water (DDW) separately and serially diluted in 50 ml Borosil glass tubes, up to 10 dilutions with three replicates. The soil suspension obtained was used to pour onto dextrose, yeast, glutamate medium (DYGS), agar-agar solid plate containing dextrose 1.0 g/L, yeast extract 2.0 g/L, glutamate 1.5 g/L, peptone 1.5 g/L, K_2HPO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, pH 6.0 (Kirchhof et al., 2001). 100 µl inoculums were taken from 10^{-1} to 10^{-10} dilutions and plated onto both solid culture plates in duplicate set, incubated at 32°C for 24 h. Pure culture was isolated from rice, wheat and maize soil samples by streaking three to four times repeated subculture on the fresh slightly modified Johanna Nitrogen fixing bacteria (JNFb) agar-agar medium containing malic acid 5.0 g/L, K_2HPO_4 0.60 g/L, KH_2PO_4 1.80 g/L, $MgSO_47H_2O$ 0.20 g/L, NaCl 0.10 g/L, $CaCl_2.2H_2O$ 0.20G g/L, $Na_2MoO_4.2H_2O$ 0.002 g/L, KOH 4.5 g/L, Fe.-EDTA (1.4%) 4.0 ml and NH_4Cl, 2.5 mM at pH 5.8 (Dobereiner, 1995). pH was maintained at 5.8 with the help of KOH and HCl by cyberscan ph ion 510 bench pH/ion/mv emter (Eutech instruments Pvt Ltd, Singapore). Six most efficient IAA producing rhizospheric bacteria were selected for plant growth promoting activity study namely MR-M1, MR-SP, MR-Z1, MR-AI, WR-W2 and RR-R2. All the selected strains were repeatedly subcultured and maintained onto JNFb solid plate.

Quantitative estimation of IAA

Quantitative estimation of IAA was performed according to the colorimetric assay of Gordon and Weber (1951). Culture was grown in JNFb liquid medium additionally supplemented with tryptophan (200 µg/ml). Equal volume of inoculation was made (1/10th) in inoculums coming from the same medium grown culture and incubated in shaking conditions at 100 rev/min for 72-96 h. 1.5 ml culture was harvested via centrifugation at 8000 rpm for 5 min. 1.0 ml supernatant were collected and added with 2 ml IAA test reagent, that is, Salkowski reagent containing 1.0 ml of 0.5 M of $FeCl_3$ in 50 ml of 35% $HClO_4$ (Yamada et al., 1990). Appearance of pink color indicates presence of IAA and absorbance was recorded at 530 nm in UV spectrophotometer. Culture medium without tryptophan was maintained as control for each isolate. The concentration of IAA produced was estimated using a standard prepared separately with pure IAA (Loba, India). Estimation of IAA was carried out after the incubation of 96 h at 32°C in triplicate set.

Effect of carbon sources on IAA production

Estimation of IAA production was checked in JNFb medium, where malate was replaced by eleven different carbon source such as ribose (0.1%), mannitol (1%), malate (0.5%), sucrose (0.5%), glucose (0.5%), succinate (0.5%), sorbitol (1.0%), maltose (0.5%), fructose (1.0%), sodium acetate (0.5%), dextrose (0.5%) exoge-

nously supplemented with tryptophan (200 µg/ml). In control set, malate (0.5%) was used as carbon source. Quantity of IAA was estimated after the incubation of 96 h at 32°C in triplicate set.

Effect of vitamins on IAA production

IAA production in the presence of five vitamins such as vitamin B6 (Pyredoxin: 100 µg/ml), vitamin B5 (nicotinic acid: 100 µg/ml), vitamin B_2 (riboflavin: 100 µg/ml), vitamin B_{12} (cyanocobalamin: 50 µg/ml) and vitamin H (biotin: 100 µg/ml) were checked. Isolates were grown in JNFb medium exogenously supplemented with tryptophan (200 µg/ml). Control set was prepared without tryptophan for each isolate.

Effect of amino acids on IAA production

IAA producing capacity were checked in the presence of five amino acids like L-phenylalanine (50 µg/ml), L-glutamic acid (100 µg/ml), L-tyrosine (100 µg/ml), L-serine (100 µg/ml) and L-lysine (100 µg/ml) in JNFb medium with tryptophan (200 µg/ml). Control set lack tryptophan for each test strains. On the other hand, culture tube containing tryptophan (200 µg/ml) only without amino acids was also maintained for each isolate.

Time course study of IAA production

To further examine the effect of time duration, all the six strains were grown in JNFb culture medium added with L-tryptophan (200 µg/ml) and incubated for 0, 24, 48, 72 and 96 h.

Effect of abiotic stress on IAA production

To explore the effect of abiotic stress on the bioproduction of IAA, strains were grown in JNFb medium containing 0, 0.1, 1.0 and 5.0% sodium chloride added with L-tryptophan (200 µg/ml). To check the effect of pH on the production of IAA level, strains were cultured in JNFb medium exogenously supplemented with L-tryptophan (200 µg/ml). pH of the culture test tubes were adjusted with KOH and HCl upto 6, 7, 8 and 9 separately for each strain. To study the effect of temperature on the production level of IAA, strains were grown in JNFb medium with L-tryptophan (200 µg/ml) and incubated at 20, 30 and 40°C separately for each strains. All the quantitative estimations were performed after 96 h of growth.

Extra cellular cellulase assay

Extra cellular cellulase assay was carried out according to the method adopted by Park et al. (1997) with slight modification. In brief,celluloase production was determined in JNFb agar-agar solid medium amended with carboxy methyl cellulose (0.4%). 10 µl of 48 h grown JNFb cultures of the 6 isolates were used to spot inoculation onto solid plate. Qualitative estimation plate based cellulase activity was performed after 4 days of incubation at 32°C. The plates were stained with 0.1% Congo red solution for 30 min, rinsed with DDW, washed twice with 1 M NaCl and then stained with 0.1 N HCl.

Thin layer chromatography of IAA

Extraction of IAA from each strain was performed according to the modified method of Manulis et al. (1994) Briefly, 10 ml of stationary phase grown and tryptophan added (200 µg/ml) (Loba, India) culture was harvested via centrifugation followed by the addition of equal volume of ethyl acetate to the collected supernatant. The above-prepared solution were mixed properly and incubated for 1 h. The upper acetate phase was collected and the extraction procedure was repeated thrice. Furthermore, the ethyl acetate phase was evaporated by air-drying. The dried pellet was collected in 1.5 ml methanol and again evaporated to the 0.5 ml of IAA in methanol. 10.0 µl of above collected supernatant were loaded onto thin layer plate. The running solvent used was: N-butanol: glacial acetic acid: DDW in ratio 12:3:5. After complete running, the TLC plate was activated at 60°C in oven followed by spraying with Salkowski reagent. After 10 min, appearance of pink spot clearly indicates the production of IAA, as same color of spot also shown by the standard IAA (Sigma, India). R_f value was calculated as the distance traveled by the compound divided by the distance traveled by the solvent.

Determination of dry weight

1.0 ml of grown culture was harvested at 10,000x rpm for 5 min, pellets were washed with phosphate buffer saline (PBS) thrice and suspended in the 500 µl PBS and filtered through 0.45 µM (Millipore Intertech Inc., Bedford, MA, USA) filter paper employing vacuum filtration instrument. Thus, all the cells were carefully collected onto filter paper and allowed to dry in hot air oven for 1 h. Therefore, the weight of cells containing filter paper was measured and previously recorded weight of fresh filter paper was deducted and expressed in mg dry wt.

Amplification of *IPDC* gene

The PCR primers used to amplify the *ipdc* gene were designed by Patten and Glick (2002), forward primer: 5`-GAA GGA TCC CTG TTA TGC GAA CC-3` and reverse primer: 5`-CTG GGG ATC CGA CAA GTA ATC AGG C-3`. Amplification condition used was denaturation at 94°C for 30 s, annealing at 45°C for 30 s elongation at 72°C for 2 min and final extension was kept 72°C for 5 min for 35 cycles.

Genomic DNA isolation and polymerase chain reaction of 16S rDNA

Whole cell genomic DNA was extracted following the standard protocol of Sambrook et al. (1989). PCR amplification of 16S rDNA was performed following the method of Eckert et al. (2001) with some modification using universal primer in a final volume of 50 µl. The PCR reaction mix included; 1.5 U of Taq DNA polymerase (Banglore Genei, India), 1X PCR assay buffer, 25 mM $MgCl_2$, 20 pmol each forward and reverse primers (Integrated DNA Technologies, Inc, CA, USA), each dNTPs:200 µM (Banglore Genei, India) template DNA:50 ng. Primer pair was forward 5'-AGA GTT TGA TYM TGG CTC AG-3' and reverse 5`-CTA CGG CTA CCT TGT TAC GA-3`). Amplification was performed in PTC-100 Thermal Cycler (MJ Research, Inc, Walthon, MA, USA), using initial denaturation at 94°C for 30 s, annealing at 57°C for 1 min, elongation at 72°C and final extension at 72°C for 5 min and finally, storage at 4°C. 5 µl of amplified reaction mixture was analyzed by agarose (2% w/v) gel electrophoresis in TAE buffer (40 mM Tris, 1 mM EDTA, pH -8.0). After, running at 50 V for 3 h, the gel was stained with ethidium bromide (0.5 µg/ml) and photograph was taken in Gel-documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

Molecular identification

The 16S rDNA insert was sequenced by the dideoxy-chain

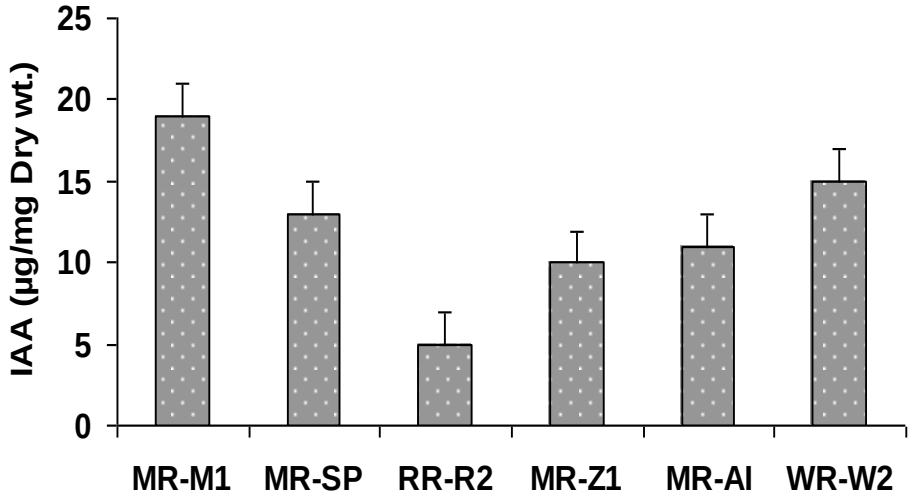

Figure 1. Quantitative estimation of IAA production.

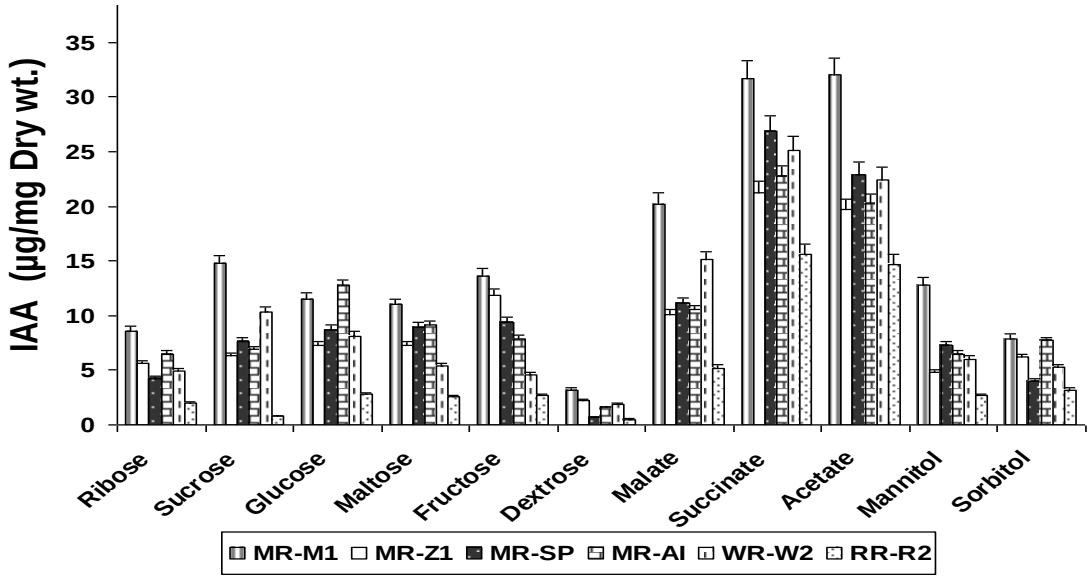

Figure 2. Test of IAA production in the presence of different carbon sources.

termination method using an automated DNA sequencer (ABI Prism; Model 3100). To identify on genus level, PCR amplification and partial sequencing of 1.5 Kb gene sequence and 400 bp of hypervariable region between V2-V3 regions of 16S rRNA of the six strains were carried out. Highest percent similarity sequences obtained were searched through online available Basic Local Alignment Search tool (BLAST) tool of National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences were submitted to the NCBI gene bank on http://www.ncbi.nlm.nih.gov.

Statistical analysis

Values were expressed as means of ± SD for triplicate samples. Differences were considered to be significant at the $P<0.05$ level.

RESULTS AND DISCUSSION

Result presented in Figure 1 shows that the highest amount of IAA was produced by MR-M1 (19.36 µg/mg dry wt.) followed by WR-W2 (15.84 µg/mg dry wt.). Isolate RR-R2 shows poor amount of IAA producing ability (5 µg/mg dry wt). On the other hand, isolate MR-SP, MR-AI and MR-Z1 shows more or less similar amount of IAA producing activity. Literature survey show that the highest accumulation of IAA occurs in the presence of L-tryptophan after 96 h of growth (Bhattacharya and Basu, 1992), which is in accordance with the present study. It is evident from data presented

Table 1. Effect on pH during IAA production in the presence of various carbon sources.

Carbon source	pH					
	MR-M1	MR-Z1	MR-SP	MR-AI	WR-W2	RR-R2
Sucrose	3.42±3	3.82±2	3.20±3	4.00±3	3.60±3	4.14±2
Glucose	3.62±2	3.32±3	3.02±3	2.54±3	4.42±3	4.02±2
Maltose	3.73±4	3.63±2	2.43±3	2.60±3	3.75±3	3.74±2
Fructose	3.16±3	3.45±2	4.02±2	3.00±3	2.95±2	4.16±2
Dextrose	3.31±3	3.54±3	3.96±2	4.01±3	3.68±2	3.21±2
Succinate	8.80±2	8.85±3	9.02±4	8.95±3	8.60±2	8.74±2
Malate	9.18±2	8.62±3	8.73±3	8.51±3	8.76±2	8.98±2
Acetate	3.25±3	3.64±4	2.50±2	4.10±3	2.91±2	3.75±2

in Figure 2 that isolate MR-M1 proved to be the most efficient strain considering its capability to produce IAA, utilizing a wide range of carbon sources, whereas others showed variable preferences to carbon sources. Malate, succinate and acetate were found to be the best carbon source. On the other hand sucrose, glucose, mannitol, sorbitol, maltose and furctose were found to be intermediate. However, dextrose and ribose were identified as poor carbon sources for the IAA production. In the presence of sucrose, glucose, mannitol and sorbitol, more or less similar amount of IAA production was observed. The highest amount of IAA production was observed in MR-M1, that is, 32 µg/gm dry wt. in the presence of succinate. IAA production was not observed without tryptophan. Effect of various carbon compounds on IAA production has been well documented in *Rhizobia* (Bhowmik and Basu, 1984, Frankenberger and Arshad, 1995). To our knowledge, this is the first report demonstrating highest amount of IAA production, when organic acid salts succinate, acetate and malate acts as a sole carbon source in the strains studied. Previous studies suggest malate as the best carbon source for microorganism mediate IAA production (Bhowmik and Basu, 1984). In the present experiment, the change in pH after 96 h of growth was recorded to study whether IAA production is accompanied with the production of other organic acid in the culture medium. It is evident from the result presented in Table 1 that a continuous decrease in pH 5.8 to 3.0 to 4.0 takes place, which showed the production of organic acid. However, more or less similar pattern of pH change was observed. Interestingly, when malate and succinate was used as carbon source a continuous increase in pH was observed from 5.8 to 9.18 and 8.88, respectively. The pH has a significant effect on the amount of IAA produced (Kirchhof et al., 2001). Since pH of the culture medium directly influences the growth of the strain, studies suggest either release of IAA under *in vitro* condition is the major cause of decrease in pH of the culture medium or accumulation of IAA is directly proportional to decrease in pH. However, increase in pH up to 8 to 9 after 96 h of growth was observed when malate and succinate were used as sole carbon source. The increase in pH might be due to release of other alkaline metabolites or other unknown compounds. Therefore, decrease in the culture medium pH could not always be correlated with accumulation of IAA. However, the drop in pH and IAA content in the tryptophan supplemented medium at different time varies according to the type and genotype of the strain. Result presented in Figure 3 shows differential IAA production efficiency in response to various amino acids. Tryptophan followed by glutamic acid proved to the most suitable amino acid considering its capability to produce IAA. The production of IAA was inhibited in the presence of L-phenylalanine and L-lysine as compared to control. Production of IAA was not observed in presence of L-tyrosine (100 µg/ml), L-serine (100µg/ml) and L-methionine (25 µg/ml) even in the presence of tryptophan, which might be due to the fact that, these amino acids may act as competitive inhibitor of tryptophan. Reports shown indole and serine are involved in biosynthesis of tryptophan (Tatum and Bonner, 1944). (data not shown). Therefore, L-tyrosine, L-serine and L-methionine could be considered as inhibitor of IAA biosynthesis. Biosynthesis of IAA was also checked in the presence of 5 vitamins namely, vitamine B12, B5, B6, B2 and biotin. All the vitamins studied were found to inhibit the IAA production by all the isolates in the presence of tryptophan (Figure 4). Vitamins showed differential influence on IAA production by different isolates. Biotin showed weak inhibitory effect whereas B2 and B12 showed strong inhibition of IAA production. IAA production was not observed in the presence of vitamins without tryptophan. There were also productions of IAA in above vitamins containing (without tryptophan) JNFb medium. Effect of vitamin and amino acids on IAA production has been demonstrated by *Azospirillum brasilense* and various other microbes (Zakharova et al., 2000). Vitamins and amino acids may play a key role in the production of IAA by microorganism. Although, the literature related to the PGPR mediated IAA production in presence of vitamins and

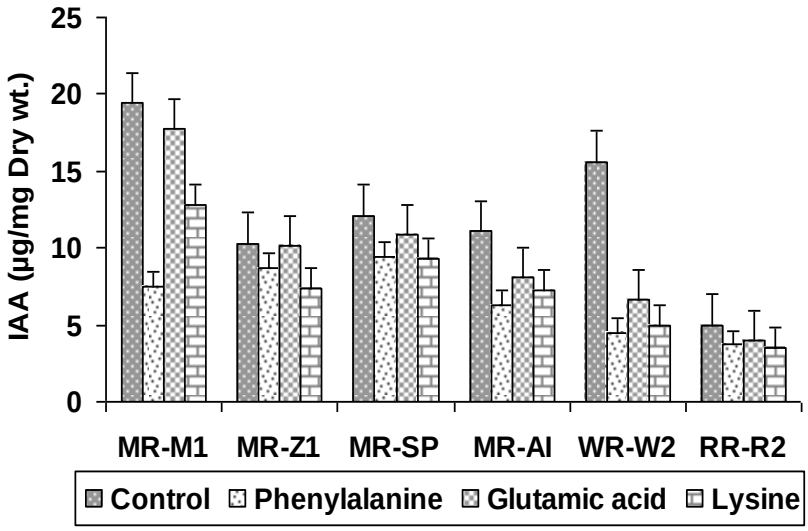

Figure 3. Test of IAA production in the presence of different amino acids.

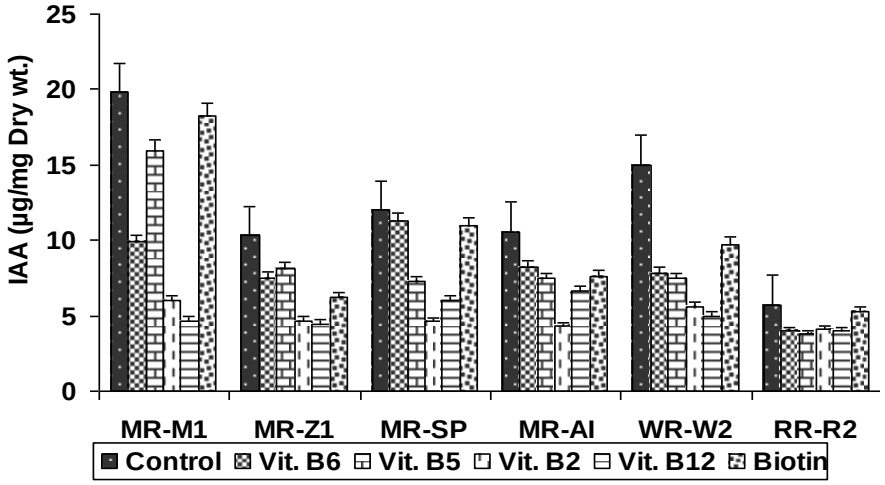

Figure 4. Test of IAA production in the presence of various vitamins.

amino acids are limited. Suppression of IAA production in presence of vitamin and amino acids as compared to control, might be due its high molecular weight, which make it inaccessible to microorganisms, even in the presence of tryptophan also. Production of IAA was not observed in the presence of L-tyrosine, L-serine and L-methionine, even in the presence of tryptophan. Appearance of single pink spot after spray of Salkowaski reagent on the TLC plate shows release of IAA in the culture medium. The Rf value of MR-M1: 0.919; MR-SP: 0.957; RR-R2: MR-Z1 and MR-AI: 0.987 and WR-W2 was 0.979 which corresponded to the standard IAA (0.953) (data not shown). The highest accumulation of IAA level was observed after 96 h of growth by the six strains as shown in Figure 5.

Biosynthesis of IAA was observed with the beginning of exponential phase of the growth. In the present experiment, the change in pH after 0, 24, 48 72 and 96 h of growth was recorded. The continuous increase in pH was observed from initial pH 5.8 to 9.11 as presented in Table 2 which clearly shows release of alkaline compounds or formation of any byproduct reaction complex and require further study. Results of this study showed that pH 5.8-6.0, (Figure 6.) 0.1% salinity condition (Figure 7) and 30±2°C temperature (Figure 8) are the most optimal condition for IAA bioproduction. Poor or no growth of the isolates was observed at pH above 9.0 or below pH 3.0 and hence no IAA production was observed. Similarly, in the presence of 7.5% or higher sodium chloride concentration, growth of the

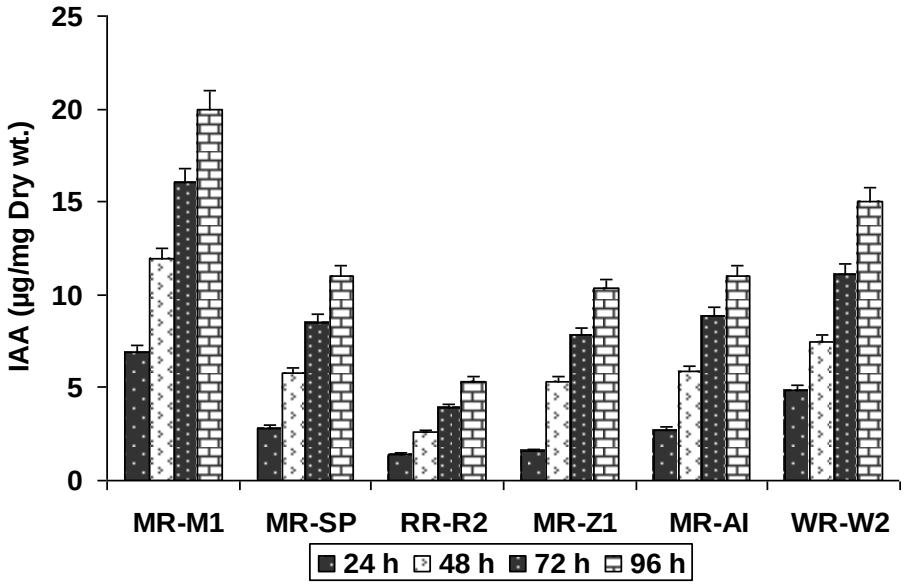

Figure 5. Time course study of the IAA production.

Table 2. Effect on pH during time course study of IAA production.

Isolate	Time						
	0 h	6 h	18 h	24h	48h	72 h	96 h
MR-M1	5.96±2	7.23±3	8.25±3	8.35±2	8.62±3	8.91±2	8.97±3
MR-SP	5.95±2	6.06±3	7.42±3	7.62±2	8.39±3	8.77±2	8.86±3
RR-R2	5.97±2	6.91±3	7.81±3	8.29±2	8.71±3	8.98±2	9.11±3
MR-Z1	5.95±2	6.49±3	6.74±3	7.16±2	7.59±3	8.64±2	8.80±3
MR-AI	5.94±2	6.42±3	6.73±3	6.80±2	7.07±3	7.30±2	7.35±3
WR-W2	5.94±2	6.17±3	6.59±3	7.08±2	8.43±3	8.84±2	8.91±3

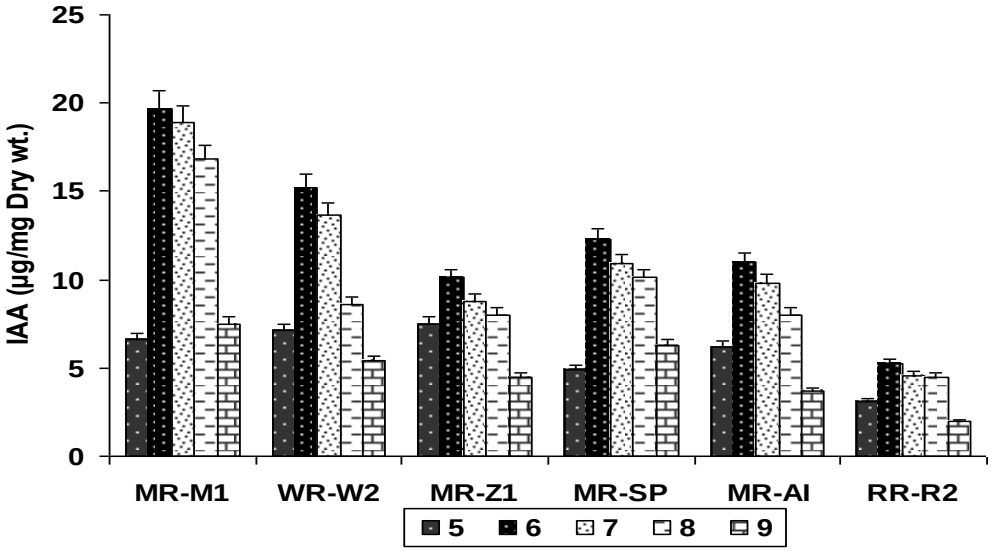

Figure 6. Effect of pH on IAA production.

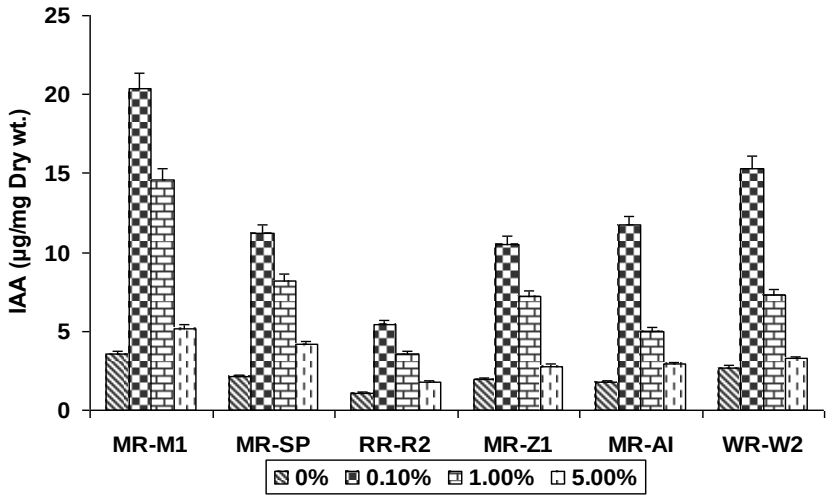

Figure 7. Effect of salinity (NaCl) on IAA production.

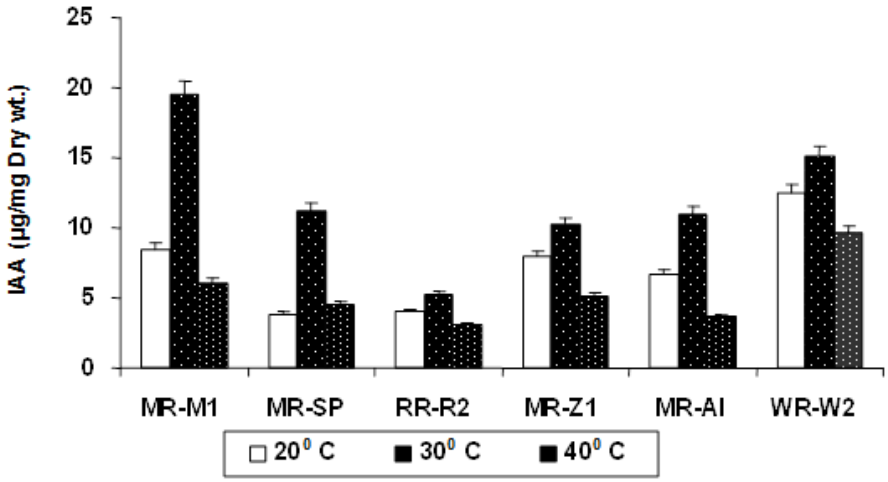

Figure 8. Effect of temperatures on IAA production.

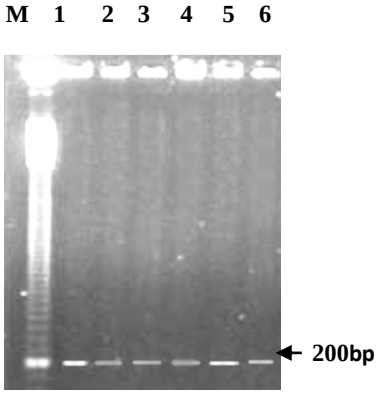

Figure 9. Amplification of ipdc gene of the six isolates where 1.MR-M1, 2. MR-SP 3. RR-R2 4. MR-Z1.5.MR-AI 6.WR-W2.

strains were not observed and hence IAA production was not observed. Strain MR-M1 proved to be most efficient strains in terms of IAA production under abiotic stress conditions. Similarly, buffered (pH 6.9 with 21 mM K_2HPO_4 and 11 mM KH_2PO_4) rich L.B medium containing no salt was used to study of *Escherichia coli* and *B. subtilis* osmoregulation (Boylan et al., 1993). Poor growth of rhizobacteria and decreased level of IAA production under salinity stress condition was reported in *Rhizobium* sp. by Ikeda et al. (1989). Environmental stress some time leads to hormonal changes to PGPR (Strzelczyk et al., 1994). Cellulase production was determined in nitrogen free medium amended with carboxy methyl cellulose (CMC) (0.4%). Cellulose, a β-1,4-linked polymer of glucose, represents approximately half of the dry weight of plant cell walls, which also contain xyloglucan and xylan (Coughlan and Mayer, 1992). Moreover,

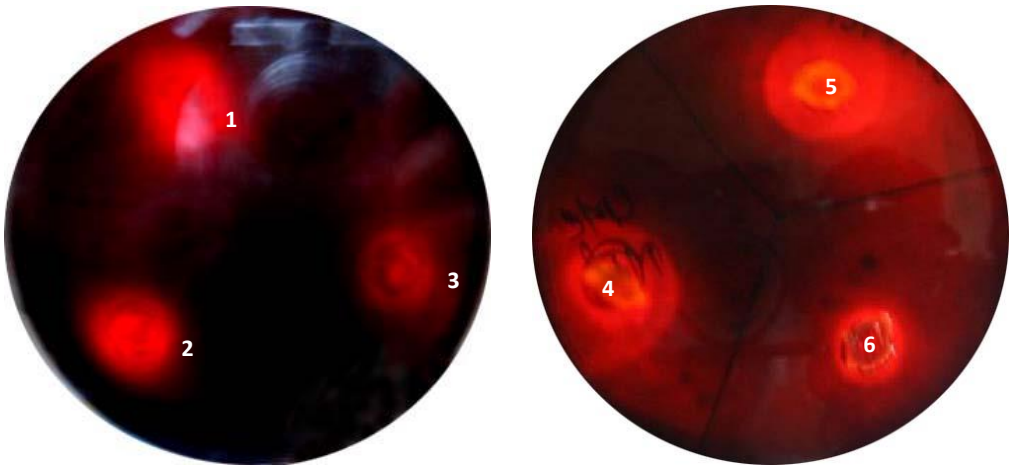

Figure 10. Halo zone production by extra cellular cellulase activity, where 1. MR-M1, 2. RR-R2, 3. MR-Z1,4. MR-SP, 5. MR-AI and 6. WR-W2.

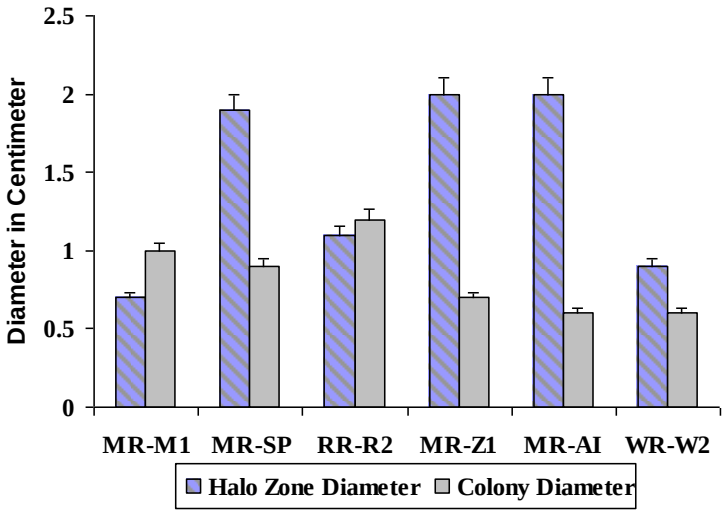

Figure 11. Colony and halozone diameter (cm) of cellulose assay.

cellulolytic enzymes released by rhizospheric bacteria are the important factors involved in enzymatic hydrolysis of fungal cell walls and in preventing plant pathogens. Transient appearance of yellow/orange halo zone of various diameters onto CMC added agar-agar plate and against a dark/blue background ensures biosynthesis of cellulase. Cellulase production was observed in all the six strains as shown in Figure 10. On the basis of halo zone production, isolate MR-AI followed by MR-Z1 followed by MR-SP was observed to be the most efficient cellulase producer. On the other hand, strain MR-M1 followed by RR-R2. Colony diameter and halo zone diameter production was measures as represented in Figure 11. Diverse level of cellulose activity has been demonstrated in the six strains. To further examine IAA production by the six isolates, amplification of *ipdc* gene was performed (Figure 9). Indole pyruvate decarboxylase, a key enzyme for IAA biosynthesis, was observed in all the isolates. The enzyme catalyzes the decarboxylation of indole-3-pyruvic acid to yield indole-3-acetaldehyde and carbon dioxide. Moreover, Koga et al. (1991) firstly reported the identification, purification and characterization of indolepyruvate decarboxylase, *ipdc* which is a novel enzyme for IAA biosynthesis in *Enterobacter cloacae.* Amplicons of 200 bp was observed in all the six isolates. *Ipdc* gene was identified as a key gene involved in IAA biosynthetic pathway in the decarboxylation step of the indole-3-acetaldehyde as shown in *Bacillus* sp. (Idris et al., 2002). Sequence analysis of the 16S ribosomal RNA (rRNA) gene has been widely used to identify bacterial

species and to perform taxonomic studies (Chakravorty et al., 2007). Nucleotide sequence accession numbers of 16S rRNA gene sequences of the six strains are MR-AI (1460 bp), identified as *Bacillus amyloliquefaciens* [FJ222551]. Strain MR-M1 (1468 bp) was revealed as *K. pneumonia*, [FJ222552]; Strain WR-W2, (1473 bp) identified as *B. subtilis* [FJ222553]; Strain MR-Z1 (1459 bp) identified as *B. subtilis* [FJ269243]; Strain RR-2 (393 bp) identified as *B. subtilis* [EU327502] and Strain MR-SP (384 bp) identified as *B. subtilis* [EU327504]. Reports suggest that human pathogenic bacteria are inhabitants of plants, which may be due to beneficial relationship between each other (Tyler and Triplett, 2008).

Conclusion

The present research investigations proved that the strains could serve as an excellent model to study the physiological and biochemical mechanism of IAA production and provide tremendous opportunities in environmentally sustainable approach to increase crop production. The present study need further research so that the strains could be directly applied to crop fields in different formulations for sustainable agriculture.

Conflict of interests

The authors did not declare any conflict of interest.

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