The Journal of Animal & Plant Sciences, 30(4): 2020, Page: 1024-1036
ISSN (print): 1018-7081; ISSN (online): 2309-8694

EXTRACTION AND EVALUATION OF INDOLE ACETIC ACID FROM INDIGENOUS AUXIN- PRODUCING RHIZOSPHERE BACTERIA

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ABSTRACT

Auxins are the phytohormones which play vital role in the growth and development of plants. Among the plant growth promoting rhizobacteria, some bacterial strains can synthesize auxins and help in plant growth stimulation. The present work deals with auxin production potential of the bacterial strains. Eighteen auxin-producing rhizobacteria were isolated and phylogenetically analyzed through 16S rDNA sequencing for identification. The isolated bacterial strains had shown similarity to various genera including *Enterobacter*, *Bacillus*, *Halomonas*, *Cronobacter*, *Exiguobacterium*, *Kushneria* and *Arthrobacter* spp. Isolates were also characterized morphologically, biochemically and physiologically. Most of the bacterial strains synthesized higher amounts of auxin during stationary phase of their growth. Bacterial auxin synthesis was monitored by varying concentrations of the precursor i.e., L-Tryptophan ranging from 200 to 1200 μgml^{-1}. The present study revealed variation in the auxin production potential of bacteria belonging to various genera with different phases of their growth and varying amount of Tryptophan available, thus, improved our insight regarding microbial auxin synthesis with reference to their growth and phylogeny which will be helpful in better exploitation of bacterial indole acetic acid (IAA) for phyto-stimulation.

Key words: Auxin, Tryptophan, 16S, rhizobacteria, Indole acetic acid.

https://doi.org/10.36899/JAPS.2020.4.0117 *Published online April 25, 2020*

INTRODUCTION

A wide range of bacterial species are found associated with the plants in the rhizospheric region exhibiting a wide variety of traits which affect growth and development of plants. Generally, symbiotic association is found to exist between the plants and the associated microbes. Knowing the mechanisms involved in improvement of plant growth by the rhizospheric bacteria would certainly be helpful in utilization and exploitation of these natural tools for growth enhancement in plants(Santoyo *et al*., 2016). IAA production by the bacteria is one of the primary mechanisms involved in plant growth enhancement associated with PGPR since it is the master hormone which controls a variety of metabolic activities in plants either directly or indirectly(de Souza *et al*., 2015; Berg *et al*., 2015). The optimization of auxin synthesis by bacteria will not only be helpful in improving plant growth in lab and field studies but will support and stimulate plant growth in tissue culture experiments and will reduce the cost of plant tissue culture technology as well. Isolation of plant growth promoting rhizobacteria from the local environment is the key point in this regard since local isolates will provide a cost-effective alternative to the local farmers for plant growth improvement. The present study deals with the screening of auxin-producing bacterial isolates from the indigenous environment, their phylogenetic analysis and evaluation of their auxin synthesis potential.

MATERIALS AND METHODS

Screening of auxin-producing bacteria: Various plant species i.e., *Launea nudicaulis, Populus sp., Euphorbia prostrata, Cassia occidentalis, Malvestrum tricuspidatum, Sonchus oleraceae, Solnum nigram, Amaranthus viridus, Oxalis corniculata*, etc. were selected from the local environment i.e., University of the Punjab, Lahore, Pakistan and then bacterial strains were isolated from the rhizoplane, rhizosphere and histo-plane of the selected plants through serial dilution method using LB medium at 37°C (Table 1). The isolates were analyzed for auxin biosynthesis via colorimetric method. Only auxin-producing bacterial isolates were selected for further study. Five already isolated and characterized auxin-synthesizing bacterial strains (AHA, APA, AHT, AST and AMP2)were also used in the present study.

Phylogenetic analysis of bacterial isolates: Phylogenetic analysis of the selected bacterial isolates was also carried out through 16S rDNA sequencing using Applied Biosystem, Model 3100 (Ahmed and Hasnain, 2010). The National Centre for Biotechnology Information (NCBI) database (http:// www.ncbi.nlm.nih. gov/BLAST) was used to search the homology of the

sequences through BLAST and the sequences obtained were submitted to GenBank for accession numbers. MEGA 4 software was used to construct the phylogenetic tree using Neighbour-joining algorithm.

Characterization of the bacterial isolates: For morphological studies of the isolates, colony and cell morphology of the isolates was studied by following Gerhardt *et al*. (1994). Biochemical characterization of the isolates was carried out by performing various biochemical tests i.e., catalase, urease, cytochrome oxidase, phenylalanine deaminase, acid production from glucose, mannitol, sucrose, lactose, maltose by bacteria, voges-proskauer, methyl red, nitrate reduction, gelatin hydolysis, arginine hydrolysis, malonate utilization, lysine hydrolysis, citrate utilization, ornithine hydrolysis, H_2S production, indole production(Cappuccino and Sherman, 2007). For physiological characterization of the isolates, the isolates were incubated at varying temperatures (25, 37 and 42° C) and growth of the isolates recorded. Similarly, the isolates were also grown using LB-medium adjusted to varying pH values ranging from 5 to 9 at 37°C and growth of the isolates monitored. Impact of various metals on the growth of the isolates was studied by growing the isolates in the presence of varying concentrations (100, 500 and 1000 μgml^{-1}) of different metals [Cr^{+++} ($K_2Cr_2O_7$), Hg^+ ($HgCl_2$), Cu^{++}($CuSO_4$), Zn^{++} ($ZnSO_4$), Mn^{++} ($MnSO_4$), Fe^{++}($FeSO_4$), Co^{++}($CoCl_2$), Ba^{++}($BaCl_2$), Ni^{++}($NiSO_4$)] on L-Agar plates for 24 hours at 37°C. Discs of various antibiotics [Ampicillin (SAM), Cefazolin (CZ), Trimethoprim (TMP), Doxycycline (D), Cefuroxime (CXM), Clarithromycin (CLR), Ceftizoxime (ZOX), Cefixime (CFM), Rifamycin (RF), Lincomycin (L), Neomycin(N), Streptomycin (S), Gentamicin (CN), Chloramphenicol (C)]were placed on the lawn of bacterial isolates on L-Agar medium and growth of the isolates after incubation of 24 hours in the presence of various antibiotics recorded at 37°C.

Bacterial growth and auxin biosynthesis: Bacterial auxin synthesis was analyzed through colorimetric analysis (Ahmed and Hasnain, 2010). Effect of time of incubation on bacterial auxin production was studied by growing the isolates for varying incubation periods i.e., 0 to 80 hours at 150*g* and auxin production by the isolates was observed by recording the optical density at 535nm. Simultaneously, growth of the isolates was also monitored by growing the isolates for varying incubation periods i.e., 0 to 80hours at 150*g* by recording the optical density at 600nm. Impact of precursor on bacterial auxin synthesis was studied by growing the isolates in the presence of varying concentrations of tryptophan i.e., 0, 200, 400, 600, 800, 1000, 1200 μgml^{-1}while auxin production monitored at 535nm using Spectrophotometer [Cecil, UK(CE-7200)]. The amount of IAA produced was determined using standard IAA curve.

Statistical Analysis: All the data of bacterial growth and auxin synthesis were subjected to Correlation Analysis using software SPSS v. 16.

RESULTS

Screening of auxin-producing bacteria: Thirty bacterial isolates were obtained from the rhizoplane, rhizosphere and histoplane of the selected plants. The isolates were analyzed for auxin biosynthesis. Out of thirty bacterial isolates, thirteen rhizospheric bacterial strains [AAL1, AL2, A3E, P4, A5C, S6, A7B, AB8, A9G, AM10, A11E, A12G, A13G] were able to synthesize auxin (Table 1). Five already isolated and characterized auxin-synthesizing bacterial strains [APA, AHA, AST, AHT, AMP2]were also used in the present study.

Phylogenetic analysis of bacterial isolates: For the identification of the bacterial isolates, 16S rDNA sequencing is the most reliable method. The sequences obtained for the bacterial strains AAL1, S6, P4, AMP2 and AB8 had shown maximum similarity with the genus *Bacillus*. Similarly, the isolates A3E, A7B, A9G, A11E, AL2, A5C, AM10 and A12G had shown maximum homology with the genus *Enterobacter*. The isolates AST and APA were closely related to the genes *Halomonas*. The isolate AL2 showed similarity to the genus *Cronobacter muytjensii* while AHT, AHA and A13G had shown similarity to the genera *Kushneria avicenniae*, *Arthrobacter mysorens* and *Exiguobacterium* sp., respectively (Table 2). All the eighteen isolates can be categorized into three groups in the phylogenetic tree (Fig 1). Majority of the bacterial strains in the first group had shown resemblance with the genus *Bacillus*. The high bootstrap values of the internal nodes justified the grouping of the isolates into various groups. Three isolates were included in the second group i.e., AHT, AST and APA with two isolates [AST and APA] showing close resemblance to the genus *Halomonas* while AHT closely related to *Kushneria avicenniae*. The third group comprised of eight isolates with seven of them belonging to the genus *Enterobacter*. The isolate AL2 belonged to the genus *Cronobacter muytjensii*. The high bootstrap value of 98% strongly supported the groups (Fig 1).

Characterization of the bacterial isolates: For morphological studies, cell and colony morphology of the isolated bacterial strains was recorded (Table 3). Four of the isolates i.e., AB8, S6, P4 and A13G were gram-positive with rod shaped cells while cells of all the other isolates (AL2, AAL1, A5C, A3E, A9G, A7B, A12G, A11E, AM10) were gram –ve rods. All the isolates were mobile except AB8. Only four isolates i.e., S6, P4, AAL1 and AB8 were spore-formers (Table 3). The results of biochemical tests were shown in Table 3. Variations were

recorded in the results of biochemical tests of various isolates.

For the growth of most of the isolates, optimum temperature was recorded to be 37°C. Growth of some of the isolates i.e., AL2, AM10, A11E and A12G was almost same at 25 and 37°C while most of the strains exhibited reduced cell density at high and low temperature i.e., 25 and 45°C. Thus, all the isolated bacterial strains can be categorized as mesophilic. Another physiological factor studied was pH of the growth medium which affected bacterial growth. For the bacterial isolates, AAL1, A3E, A7B, A9G and AM10, pH7 was found to be the best for maximum growth. P4 and A5C grew best in the medium with pH adjusted to 5. Optimum pH value for the isolates AL2, S6, AB8 and A12G was recorded to be 6.The most effective antibiotic was found to be neomycin (N) which worked against all the bacterial isolates. Two other antibiotics i.e., Gentamicin (CN) and Doxycycline (D) also proved to be effective against all the isolates except for A13G (Table 4). The isolates were also grown in the presence of various metal salts. Presence of mercury (Hg) badly affected the growth of all the isolates resulting in no growth. Table 5 showed the growth results of isolates in the presence of various metals.

Bacterial growth and auxin biosynthesis: With increase in the growth of the bacterial strains, auxin biosynthesis by the bacteria was recorded to be increased exhibiting strong positive correlation between the two (Fig 3). Auxin production by the bacterial strains was maximum during the stationary growth phase of bacterial isolates. Some bacteria produced high concentration of auxin during early stationary phase, some at the mid and some at the later stage of stationary phase (Fig 3). Highest concentration of auxin was synthesized by the isolate *Enterobacter cloacae* A9G i.e., 114 µg/ml after 48 hours of incubation. Among the various *Enterobacter* spp., A3E produced maximum auxin (70 µg/ml) after 48 hours of incubation, A5C produced maximum auxin (71 µg/ml) after 56 hours of incubation while A7B and A11E synthesized maximum auxin (59 µg/ml & 65 µg/ml) after 72 hours of incubation. *Enterobacter cloacae* AM10 produced maximum auxin (70 µg/ml) after 80 hour of incubation and A12G synthesized high concentration of auxin (89 µg/ml) after 64 hours of incubation. The isolates AL1 and P4 produced highest quantity of auxin (38 µg/ml and 59 µg/ml) after 64 and 48 hours of growth respectively. Similarly, maximum amount of auxin produced by S6 was found to be 86µg/ml. Maximum concentration of auxin synthesized by *Bacillus* sp. AB8, *Bacillus* sp. AMP2 and *Exiguobacterium* sp. A13G was 37 µg/ml, 19µg/ml and 45.2 µg/ml, respectively. Maximum amount of auxin produced by *Kushneria avicenniae* AHT was recorded after 72 hours of incubation i.e., 27 µg/ml. A positive correlation was recorded between the growth of *Halomonas* sp. AST , *Arthrobacter mysorens* (AHA) and *Halomonas venusta* APA and *Cronobacter muytjensii* AL2 and amount of auxin produced by these isolates till 80 hours of incubation (late stationary phase) (Fig 3). Similarly, A13G, A7B, AB8, A3E and S6 had also shown increased auxin biosynthesis with increase in the bacterial densities. However, in some isolates such as A9G, P4 and A5C, highest amount of auxin was produced during stationary growth phase which was followed by decrease in auxin biosynthesis inspite of further increase in bacterial densities (Fig 3).

Generally, most of the auxin producing PGPR synthesize auxin through tryptophan-dependent mechanisms. The availability and amount of the precursor i.e., tryptophan, was of prime importance in tryptophan-dependent mechanisms of auxin production. Most of the isolates had exhibited increase in the amount of auxin produced with increasing concentration of tryptophan showing significant positive correlation ranging from 0.864^{*} (P= 0.05) to 0.983^{**} (P=0.01) (Table 6; Fig 2). In most of the isolates, increase in the concentration of auxin synthesized by the bacterial strains was recorded with increasing tryptophan concentration upto 1200 µg/ml while in *Cronobacter muytjensii* AL2, *Arthrobacter mysorens* AHA and *Halomonas venusta* APA, highest amount of auxin was synthesized upto 1000 µg/ml tryptophan. The isolate *Enterobactercloacae* A9G produced maximum concentration of auxin at tryptophan concentration of 1200 µg/ml. Among the *Enterobacter* spp., A3E, A12G, A5C and A11E synthesized 75 µg/ml, 89 µg/ml, 49.5 µg/ml, 73.5 µg/ml and 77 µg/ml auxin in the presence of tryptophan (1200 µg/ml), respectively while among the *Bacilli,* the isolates AAL1, P4, S6, AMP2 and AB8 produced 41 µg/ml, 103.5 µg/ml, 53 µg/ml, 16 µg/ml and 34.5 µg/ml auxin, respectively, at the concentration of 1200 µg/ml tryptophan. The isolates *Halomonas* sp. AST, *Exiguobacterium* sp. A13G and *Kushneria avcenniae* AHT produced 31 µg/ml, 43 µg/ml and 23.8 µg/ml auxin with 1200 µg/ml tryptophan. Some isolates produced maximum quantity of auxin in the presence of 1000 µg/ml tryptophan such as *Halomonas venusta* APA (46.8 µg/ml), *Arthrobactermysorens* (34 µg/ml) and *Cronobacter muytjensii* AL2 (107 µg/ml) (Table 6).

Table 1. List of the isolated bacterial strains and their auxin production potential recorded colorimetrically.

S. #.	Plant	Family	Source	Isolates	Auxin production
1.	*Populus* sp.	Salicaceae	Rhizoplane	P1	-
				P2	-
2.	*Populus* sp.	Salicaceae	Rhizosphere	P4	++++
3.	*Populus* sp.	Salicaceae	Histoplane	P3	-
4.	*Cassia occidentalis*	Fabaceae	Rhizoplane	AC1	-
				AC2	-
				A3C	-
5.	*Cassia occidentalis*	Fabaceae	Rhizosphere	A5C	++++
				A13G	++
6.	*Cassia occidentalis*	Fabaceae	Histoplane	A4C	-
7.	*Amaranthus viridus*	Amaranthaceae	Rhizoplane	Av1	-
8.	*Amaranthus viridus*	Amaranthaceae	Rhizosphere	Av2	-
				Av3	+
9.	*Euphorbia prostrata*	Euphorbiaceae	Rhizosphere	Eu1	-
				Eu2	+
				Eu3	+
10.	*Sonchus oleraceae*	Asteraceae	Rhizosphere	S6	++++
11.	*Solanum nigram*	Solanaceae	Rhizosphere	A7B	+++
				AB8	++
12.	*Launea nudicaulis*	Compositae	Rhizosphere	AAL1	++
				AL2	++++
				A11E	++++
				AM10	++++
13.	*Launea nudicaulis*	Compositae	Rhizoplane	L3	-
14.	*Oxalis corniculata*	Oxalidaceae	Rhizosphere	OC	-
15.	*Malvestrum tricuspidatum*	Malvaceae	Rhizoplane	AMT1	-
16.	*Malvestrum tricuspidatum*	Malvaceae	Rhizosphere	A3E	+++
				A9G	++++
				A12G	++++
17.	*Malvestrum tricuspidatum*	Malvaceae	Histoplane	AMT2	-

Table 2. Identification of the bacterial isolates using 16S rDNA gene sequencing.

S. #.	Isolates	Identification	Acession no.	Homology
1.	AAL1	*Bacillus* sp.	HQ844259	99 %
2.	AL2	*Cronobacter muytjensii*	HQ844262	99 %
3.	A3E	*Enterobacter* sp.	HQ179966	99 %
4.	P4	*Bacillus flexus*	FJ356233	97 %
5.	A5C	*Enterobacter* sp.	HQ179967	99 %
6.	S6	*Bacillus* sp.	FJ356234	99 %
7.	A7B	*Enterobacter* sp.	HQ441562	99 %
8.	AB8	*Bacillus* sp.	HQ844261	98%
9.	A9G	*Enterobacter cloacae*	HQ202888	99 %
10.	AM10	*Enterobacter cloacae*	HQ533176	99 %
11.	A11E	*Enterobacter* sp.	HQ533177	99 %
12.	A12G	*Enterobacter* sp.	HQ202889	99%
13.	A13G	*Exiguobacterium* sp.	HQ202890	98%
14.	APA	*Halomonas venusta*	EF115298	98%
15.	AHA	*Arthrobacter mysorens*	EF102871	100%
16.	AST	*Halomonas* sp.	HQ883967	99 %
17.	AHT	*Kushneria avicenniae*	EF115299	94%
18.	AMP2	*Bacillus* sp.	HQ144214	98 %

Table 3. Morphological and biochemical characteristics of the bacterial isolates.

S.#.	Characters	Bacterial isolates						
		AAL1	AL2	A3E	P4	A5C	S6	A7B
1.	Colony colour	Cream	Cream	Offwhite	Offwhite	Offwhite	Cream	Offwhite
2.	Colony shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular
3.	Colony margin	Undulate	Entire	Entire	Entire	Entire	Undulate	Entire
4.	Colony elevation	Raised	Convex	Convex	Raised	Convex	Raised	Convex
5.	Colony size (mm)	3-5	1-2.5	1-3	2.5-4	1.5-3	2.5-3.5	0.5-1
6.	Cell shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods
7.	Gram staining	G-ve	G-ve	G-ve	G+ve	G-ve	G+ve	G-ve
8.	Spore formation	+	-	-	+	-	+	-
9.	Motility	+	+	+	+	+	+	+
10.	Catalase	+	+	+	+	+	+	+
11.	Urease	-	-	-	-	-	-	-
12.	Citrate	-	+	+	+	+	+	+
13.	Oxidase	+	-	-	+	-	+	-
14.	Gelatin	-	-	-	-	-	-	-
15.	Nitrate	-	+	+	+	+	+	+
16.	Voges-Proskauer	-	+	+	+	+	+	+
17.	Methyl Red	+	-	-	+	-	+	-
18.	Indole	-	+	-	-	-	-	-
19.	H_2S	-	-	-	-	-	-	-
20.	Lysine	-	-	+	-	+	+	-
21.	Arginine	-	+	+	+	+	+	+
22.	Ornithine	-	+	-	-	-	-	-
23.	Glucose	-	+	+	+	+	+	+
24.	Sucrose	-	+	+	+	+	+	+
25.	Mannitol	-	+	+	+	+	+	+
26.	Lactose	-	+	+	+	+	+	+
27.	Malonate	-	+	+	+	+	+	+

Table 3. (Contd.) Morphological and biochemical characteristics of the bacterial isolates.

S.#.	Characters	Bacterial isolates					
		AB8	A9G	AM10	A11E	A12G	A13G
1.	Colony colour	Lightpink	Offwhite	Offwhite	Offwhite	Offwhite	LightOrange
2.	Colony shape	Circular	Circular	Circular	Circular	Circular	Circular
3.	Colony margin	Entire	Entire	Entire	Entire	Entire	Undulate
4.	Colony elevation	Convex	Convex	Convex	Convex	Convex	Convex
5.	Colony size (mm)	2.5-5	0.5-1.5	0.5-3	0.5-1	0.5-3	0.5-2
6.	Cell shape	Rods	Rods	Rods	Rods	Rods	Rods
7.	Gram staining	G+ve	G-ve	G-ve	G-ve	G-ve	G+ve
8.	Spore formation	+	-	-	-	-	-
9.	Motility	-	+	+	+	+	+
10.	Catalase	+	+	+	+	+	+
11.	Urease	-	-	-	-	-	-
12.	Citrate	+	+	+	+	+	-
13.	Oxidase	-	-	-	-	-	+
14.	Gelatin	-	-	-	-	-	-
15.	Nitrate	+	+	+	+	+	+
16.	Voges-Proskauer	+	+	+	+	+	-
17.	Methyl Red	+	-	-	-	-	+
18.	Indole	-	-	-	-	-	-
19.	H_2S	-	-	-	-	-	-
20.	Lysine	-	-	-	-	-	-

21.	Arginine	+	+	+	+	+	-
22.	Ornithine	-	+	+	-	+	-
23.	Glucose	+	+	+	+	+	+
24.	Sucrose	+	+	+	+	+	+
25.	Mannitol	+	+	+	+	+	-
26.	Lactose	+	+	+	+	+	-
27.	Malonate	+	+	+	+	+	-

Table 4. Antibiotic resistance profile of bacterial isolates.

Sr#	Isolates	Antibiotics													
		N	CN	AM	L	S	RF	C	D	CXM	ZOX	CZ	CFM	TMP	CLR
1.	AAL1	-	-	-	+	-	-	-	-	-	-	-	+	-	-
2.	AL2	-	-	+	+	-	+	+	-	+	-	+	+	w+	-
3.	A3E	-	-	+	+	-	+	-	-	+	-	+	+	w+	+
4.	P4	-	-	-	+	+	-	-	-	-	-	-	+	+	-
5.	A5C	-	-	+	+	-	+	-	-	+	-	+	+	+	+
6.	S6	-	-	-	-	-	-	-	-	-	-	-	+	+	-
7.	A7B	-	-	+	+	-	-	-	-	+	-	+	+	+	-
8.	AB8	-	-	-	+	-	-	-	-	-	-	-	+	w+	-
9.	A9G	-	-	+	+	-	+	-	-	+	-	+	+	+	+
10.	AM10	-	-	+	+	-	+	-	-	+	-	+	+	w+	+
11.	A11E	-	-	+	+	-	+	-	-	+	-	+	+	+	+
12.	A12G	-	-	+	+	-	+	-	-	+	w+	+	+	w+	+
13.	A13G	-	w+	-	-	w+	w+	-	w+	+	w+	w+	+	+	w+

Resistance: + ; Sensitive: - ; Weakly resistant: w+
Ampicillin (AM), Cefazolin (CZ), Trimethoprim (TMP), Doxycycline (D), Cefuroxime (CXM), Clarithromycin (CLR), Ceftizoxime (ZOX), Cefixime (CFM), Rifamycin (RF), Lincomycin (L), Neomycin (N), Streptomycin (S), Gentamicin (CN),Chloramphenicol (C)

Table 5. Heavy metal resistance profile of bacterial isolates

Sr #	Bacterial Isolates	Heavy Metals														
		Cr			Mn			Co			Ni			Cu		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	AAL1	+	-	-	+	+	-	+	-	-	+	w+	-	+	+	w+
2	AL2	+	-	-	+	+	+	+	-	-	+	w+	-	+	+	+
3	A3E	+	-	-	+	+	+	+	-	-	+	+	-	+	+	+
4	P4	+	w+	-	+	+	-	+	-	-	+	w+	-	+	+	+
5	A5C	+	-	-	+	+	+	+	-	-	+	+	-	+	+	+
6	S6	+	-	-	+	+	-	+	-	-	+	w+	-	+	+	w+
7	A7B	+	-	-	+	+	w+	+	-	-	+	+	-	+	+	-
8	AB8	+	-	-	+	+	-	+	-	-	+	+	-	+	+	-
9	A9G	+	-	-	+	+	+	+	+	-	+	+	-	+	+	+
10	AM10	-	-	-	+	+	+	+	-	-	+	+	-	+	+	+
11	A11E	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+
12	A12G	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+
23	A13G	+	-	-	+	+	-	+	-	-	+	+	-	+	+	-

Table 5: (Contd.) Heavy metal resistance profile of bacterial isolates.

Sr#	Bacterial Isolates	Heavy Metals											
		Zn			Hg			Fe			Ba		
		1	2	3	1	2	3	1	2	3	1	2	3
1.	AAL1	+	-	-	-	-	-	+	+	-	+	+	+
2.	AL2	+	-	-	-	-	-	+	+	-	+	w+	w+
3.	A3E	+	-	-	-	-	-	+	+	-	+	+	+
4.	P4	w+	-	-	-	-	-	+	+	-	+	+	+
5.	A5C	+	-	-	-	-	-	+	+	-	+	+	+
6.	S6	+	-	-	-	-	-	+	+	-	+	+	+
7.	A7B	+	-	-	-	-	-	+	w+	-	-	-	-
8.	AB8	w+	-	-	-	-	-	+	+	-	+	+	+
9.	A9G	+	-	-	+	-	-	+	+	+	+	+	+
10.	AM10	+	-	-	+	-	-	+	+	-	+	+	+
11.	A11E	+	-	-	-	-	-	+	+	+	+	+	+
12.	A12G	+	+	-	+	-	-	+	+	+	+	+	+
13.	A13G	+	-	-	-	-	-	+	-	-	+	+	+

1 = 100 µg ml^{-1}; 2 = 500 µg ml^{-1}; 3 = 1000 µg ml^{-1}
Resistance: + ; Sensitive: - ; Weakly resistant: w+

Table 6. Effect of varying concentrations of L-tryptophan on auxin production by the bacterial isolates.

Bacterial Isolates	Amount of L-tryptophan (µgml^{-1})							Correlation Coefficient (r)
	0	200	400	600	800	1000	1200	
	Auxin production (µgml^{-1})							
Bacillus sp. AAL1	8.0±0.5	19.5±0.2	30.0±1.5	35.5 ±0.7	37.7±1.2	40.5±0.5	41.0±1	0.929**
Cronobacter muytjensii AL2	19.0±0.5	45.0±1.5	62.5±1.5	89.0±0.6	96.0±0.5	107.0±0.5	98.0±2.5	0.934**
Enterobacter sp. A3E	35.0±1.0	39.5±1.6	64.0±0.5	68.5±1.3	69.0±1.7	70.0±1.5	75.0±0.5	0.896**
Bacillusflexus P4	37.0±1.5	51.0±1.1	66.2±1.7	66.1±1	72.0±1.1	95.0±0.5	103.5±1.8	0.975**
Enterobacter sp.A5C	15.3±1.4	51.5±1.0	52.5±1.8	63.0±1.5	67.5±1.0	73.0±2.0	73.5±1.1	0.886**
Bacillus sp.S6	34.5±1.3	36.0±0.2	37.0±0.1	41.0±0.5	46.0±0.3	52.0±1.0	53.0±0.5	0.974**
Enterobacter sp. A7B	13.3±0.5	16.5±0.4	29.0±0.7	30.8±0.6	33.0±0.5	35.5±0.8	49.2±0.8	0.959**
Bacillus sp. AB8	13.4±0.4	16.0±0.7	24.5±0.05	27.0±0.2	30.0±0.2	34.0±0.4	34.5±0.2	0.974**
Enterobacter cloacae A9G	16.0±0.3	37.0±0.8	66.0±1.7	44.5±0.6	111±1.1	124±1.0	136±1.0	0.947**
Enterobacter cloacae AM10	17.0±0.5	30.0±1.0	44.0±1.1	59.0±0.6	78.0±1.5	80.0±0.6	86.0±1.3	0.982**
Enterobacter sp. A11E	12.5±0.3	25.0±0.03	43.0±0.4	52.5±1.3	65.5±0.8	68.0±0.5	77.0±0.7	0.983**
Enterobacter sp.A12G	18.5±0.2	50.2±1.4	64.5±1.0	73.3±0.8	87.0±2.0	86.8±0.5	89.0±0.5	0.923**
Exiguobacterium sp.A13G	9.0 ± 0.1	9.5± 0.5	12.2±0.1	16.5±0.6	19.5±0.5	42.5±1.4	43.0±1.0	0.912**
Halomonas venusta APA	14.0±0.3	13.0±0.1	16.2±0.1	20.2±0.3	31.0±1.0	46.8±1.1	33.0±1.0	0.864*
Arthrobacter mysorens AHA	11.8±0.4	15.0±0.1	17.5±0.4	23.0±1.0	28.5±0.3	34.0±0.5	33.5±0.8	0.982**
Halomonas sp. AST	12.0±0.4	18.5±0.3	19.0±0.3	20.0±0.5	21.0±0.6	24.5±0.2	31.0±0.4	0.941**
Kushneria avicenniae AHT	10.3±0.4	12.6±0.5	13.5±0.2	16.0±0.7	16.5±0.7	17.0±0.4	23.8±1.1	0.948**
Bacillus sp. AMP2	6.0±0.5	10.0±0.5	13.2±0.6	14.5±0.2	15.0±0.5	16.0±0.6	16.0±0.5	0.925**

Mean ± S.E. of three replicates; ** P = 0.01; *P = 0.05

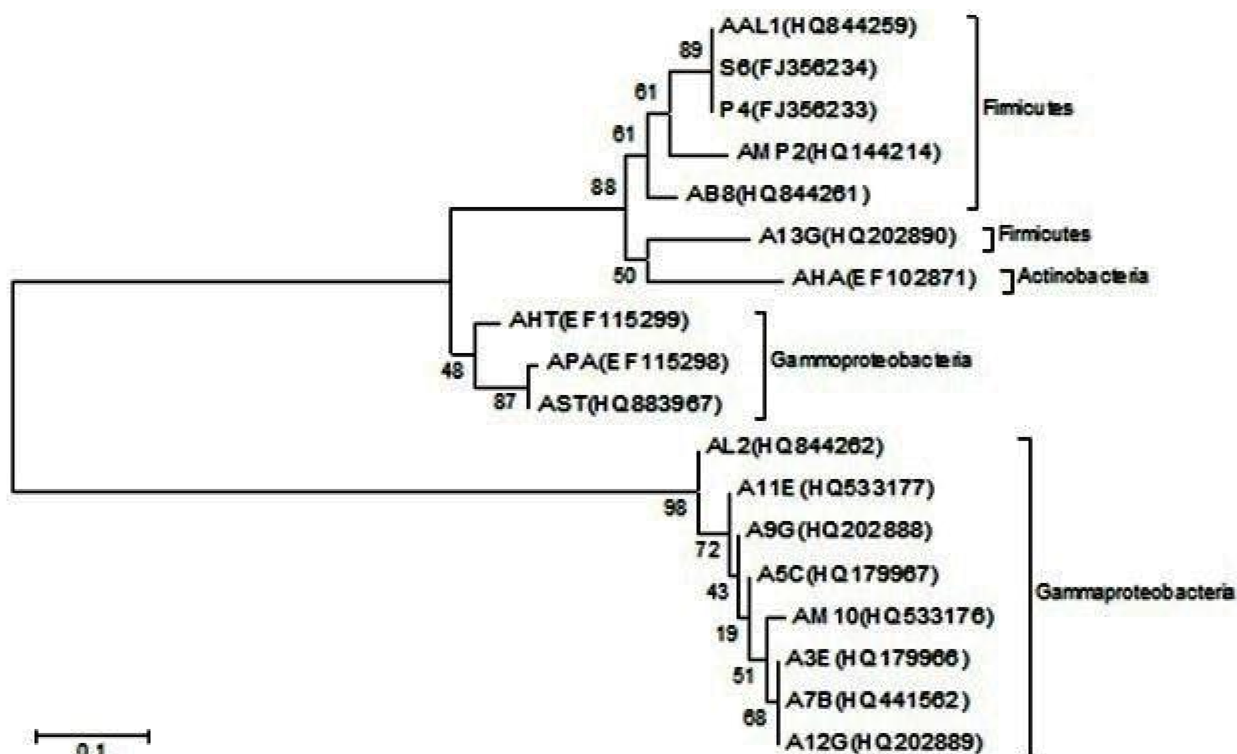

Fig 1. Phylogenetic relationship among the eighteen bacterial isolates The scale bar represents substitutions per nucleotide position. Neighbour-joining method was used to construct the trees using MEGA4 software. Bootstrap values expressed as a percentage of 500 replicates are shown at the nodes. Sequence accession numbers are given in parantheses. Affiliations: AAL1, *Bacillus* sp.; P4, *Bacillus flexus*; S6, *Bacillus* sp.; AMP2, *Bacillus* sp.; AB8, *Bacillus* sp.; A13G, *Exiguobacterium* sp.; AHA, *Arthrobacter mysorens*; AHT, *Kushneria avicenniae*; APA, *Halomonas venusta*; AST, *Halomonas* sp.; AL2, *Cronobacter muytjensii*; A11E, *Enterobacter* sp.; A5C, *Enterobacter* sp.; A9G, *Enterobacter cloacae*; AM10, *Enterobacter cloacae*; A7B, *Enterobacter* sp.; A3E, *Enterobacter* sp.; A12G, *Enterobacter* sp.

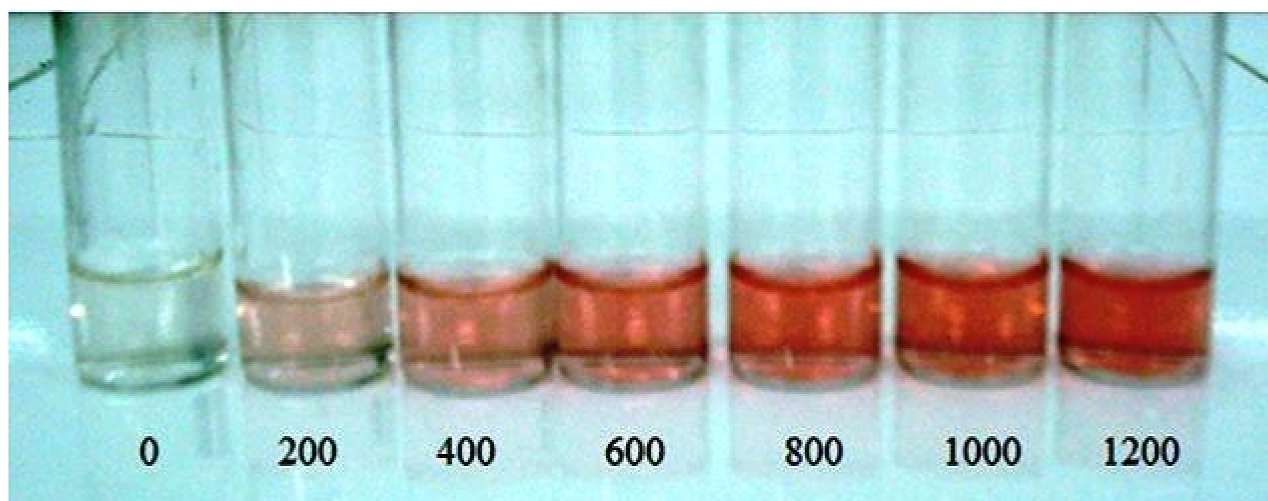

Fig. 2: Effect of different concentrations of L-tryptophan (0, 200, 400, 600, 800, 1000, 1200µgml^{-1}) on auxin production by *Enterobacter* sp. (A5C)

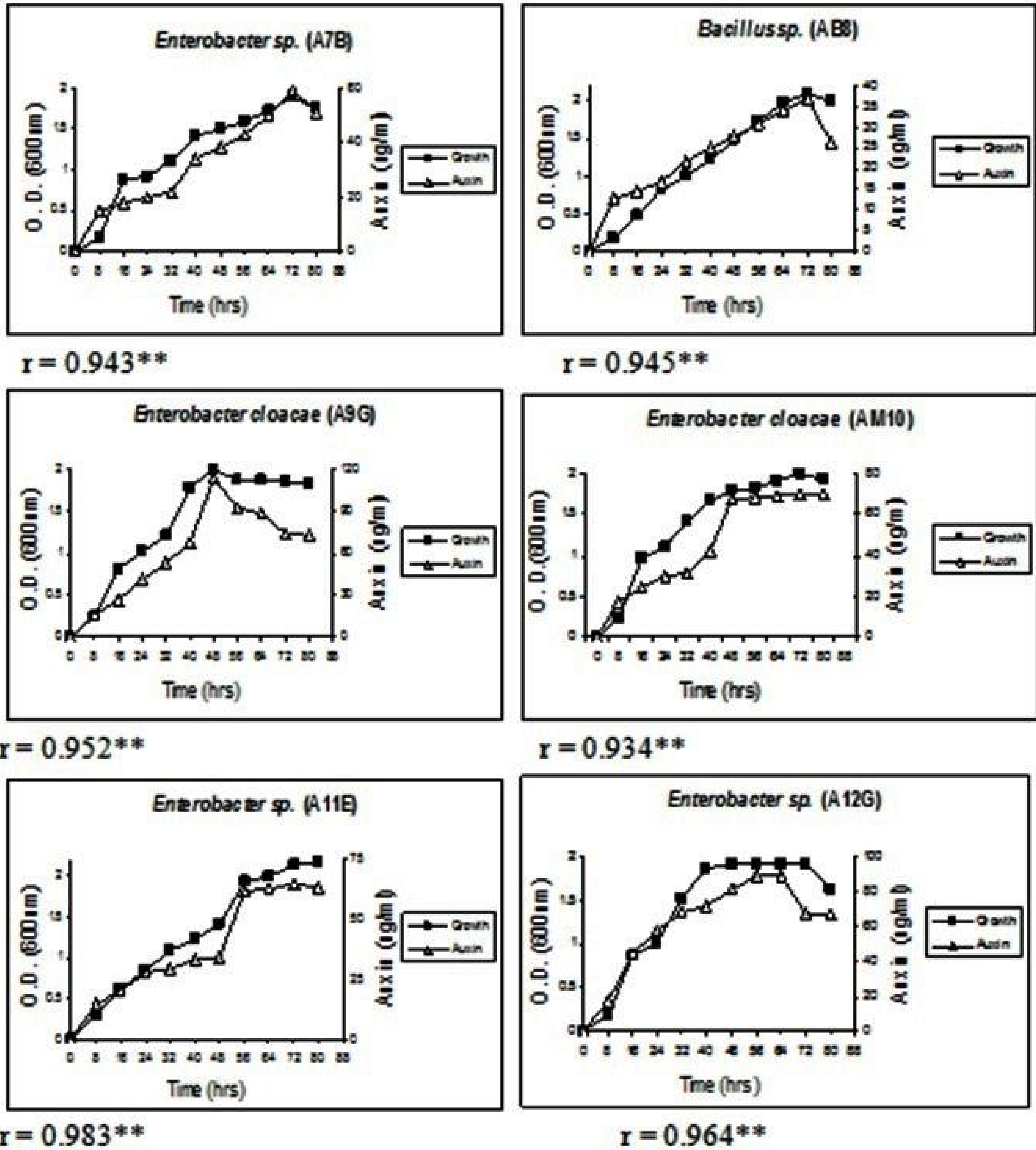

Fig. 3. Auxin biosynthesis by the bacterial isolates estimated through colorimetric analysis. "r" stands for coefficient of correlation showing correlation between auxin production by the bacteria isolates and their cell densities ($^{**}P = 0.01$; $^{*}P = 0.05$).

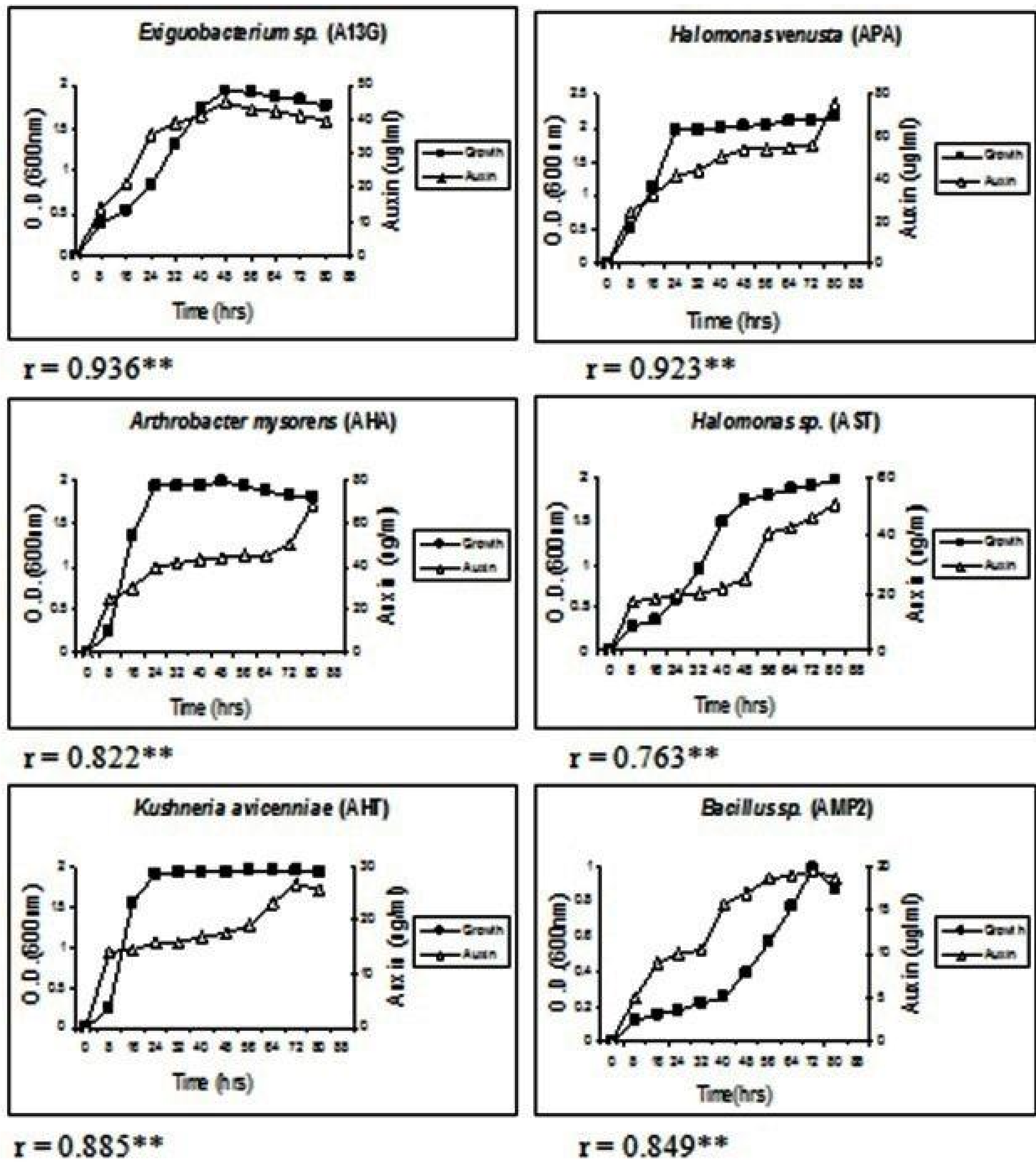

Fig. 3 (contd.). Auxin biosynthesis by the bacterial isolates estimated through colorimetric analysis. "r" stands for coefficient of correlation showing correlation between auxin production by the bacteria isolates and their cell densities (P = 0.01; * P = 0.05).**

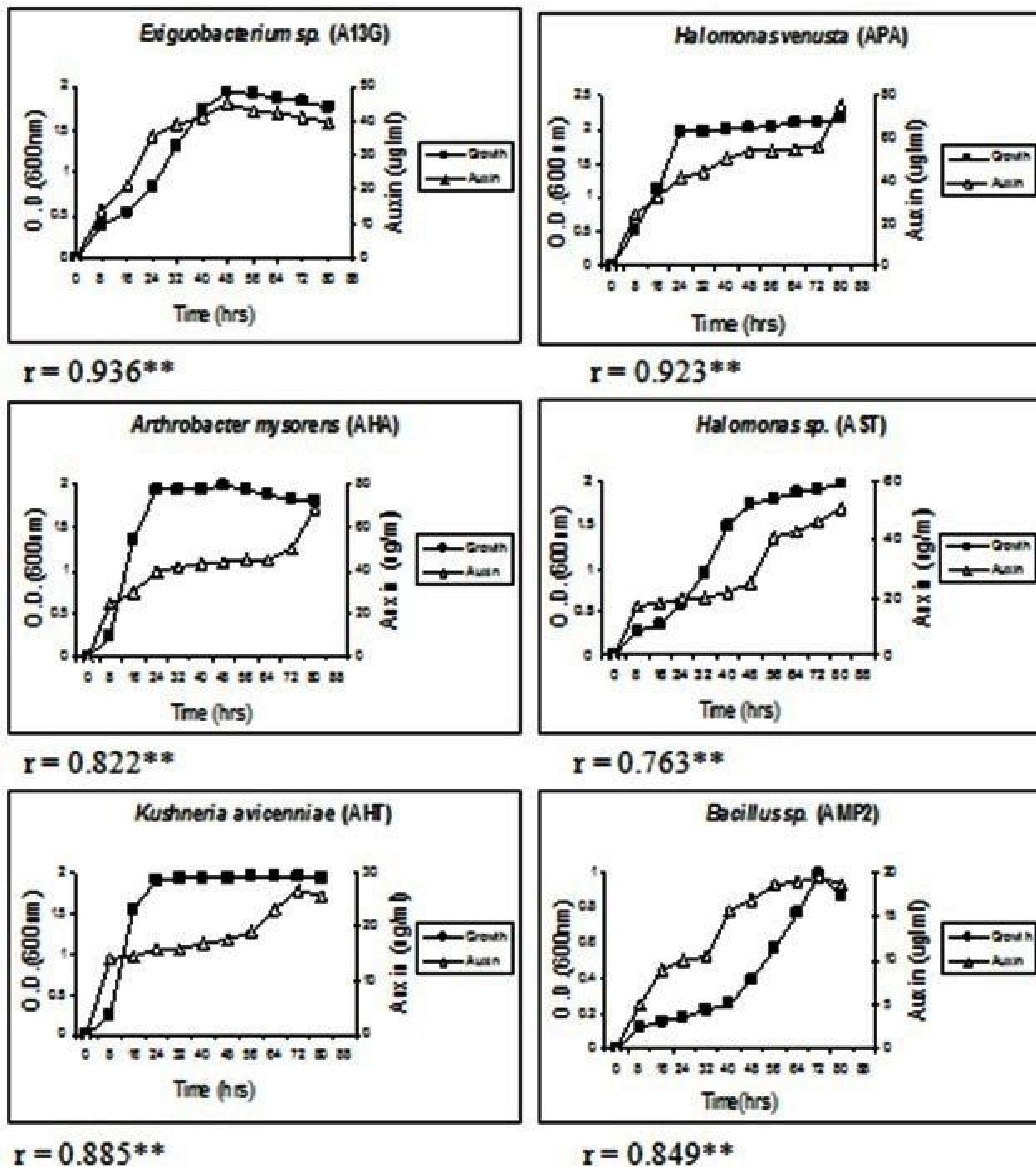

Fig. 3 (contd.). Auxin biosynthesis by the bacterial isolates estimated through colorimetric analysis. "r" stands for coefficient of correlation showing correlation between auxin production by the bacteria isolates and their cell densities (**P = 0.01; * P = 0.05).

DISCUSSION

Plants and bacteria are inseparable entities. Majority of the plant growth promoting bacteria are found to be residing in the rhizospheric region where these colonize plant roots, thereby, developing association with plants for the mutual exchange of metabolites including growth improving products such as auxins, cytokinins etc. Other mechanisms speculated to be involved in plant growth were nitrogen fixation, siderophore production and suppression of diseases.

Many workers have associated two major mechanisms i.e., phytohormone production and nitrogen fixation with plant growth improvement by PGPR(Shokri and Emtiazi, 2010; Berg *et al*., 2015; de Souza *et al*., 2015). To get benefit from the plant growth promoting rhizobacteria, the first step is to isolate these bacteria from the environment followed by their identification and further study.

Auxin synthesized by the bacteria is assumed to act as signaling molecule among various bacteria as well as between the plant roots and bacteria where signaling crosstalk occurs between the root exudates and bacteria. Bacteria in response to root exudates release IAA as signals to support and promote plant-bacteria interaction(Ahmed and Hasnain, 2014; Boivin *et al*., 2016). Bacteria utilize tryptophan, the precursor for IAA synthesis, present in the root exudates for the biosynthesis of IAA in case of tryptophan-dependent mechanism of IAA production whereas in tryptophan-independent pathways, bacteria do not rely on the availability of tryptophan for IAA synthesis(Normanly, 2010; Ahmed and Hasnain, 2014).Five different tryptophan-dependent pathways exist in nature for bacterial auxin synthesis (Spaepen and Vanderleyden, 2010; Tivendale *et al*., 2010). Majority of the PGPRs are reported to follow Indole-3-pyruvic acid pathway (IPyA) for IAA synthesis while Indole-acetamide pathway (IAM) is generally followed by most of the phytopathogenic bacteria(Ahmed and Hasnain, 2014). Bacterial IAA production is speculated to be a pathway for the detoxification of tryptophan, however, existence of multiple mechanisms including tryptophan-independent pathways working in a very coordinate manner indicates the importance and programmed synthesis of IAA which, later, via various metabolic activities may also strengthen plant-bacteria relationship (Tivendale *et al*., 2010). The quantity of tryptophan plays key role during auxin biosynthesis via tryptophan-dependent pathways(Lambrecht *et al*., 2000). In the present work, strong positive correlation ranging from 0.864^{*} (P = 0.05)to 0.983^{**} (P = 0.01) was recorded between the amount of available tryptophan and auxin synthesized(Table 6). Thus, the amount of auxin produced by the isolates was found to be directly proportional to the concentration of tryptophan upto 1200ug/ml. Among the isolates, maximum concentration of IAA was recorded to be synthesized by *Enterobacter cloacae* A9G i.e., 136 ug/ml when the amount of tryptophan was 1200ug/ml. Similarly, strong positive correlation was recorded between the amount of auxin synthesized and bacterial densities (Fig 3). Majority of the bacteria have synthesized maximum concentration of auxin during stationary phase of bacterial growth. In the current study, the amount of auxin produced by various isolates was ranging between 5ug/ml to 114 ug/ml as determined colorimetrically. Ouzari *et al*. (2008) had also recorded auxin production by the bacteria ranging from 3.09 ug/ml to 63.7ug/ml. We can conclude that significant positive correlation exists between the quantity of the precursor i.e., tryptophan and amount of bacterial auxin synthesized upto a certain level during tryptophan-dependent auxin synthesis in rhizospheric bacteria. Similarly, strong positive correlation also exists between the bacterial density and the amount of auxin manufactured by that bacterium. However, this works upto the stationary growth phase where optimum auxin production occurs by most of the bacteria. Among the various genera, higher concentrations of auxin were synthesized by the isolates belonging to the genus *Enterobacter*. Isolate belonging to the genus *Cronobacter* (AL2) and one isolate belonging to the genus *Bacillus* (P4) also produced high amounts of auxin. The auxin-production ability of these isolates can be further exploited for utilization of bacterial auxin in the fields of plant-microbe interaction and plant biotechnology such as plant tissue culture.

Conclusion: The present work revealed about the auxin production potential of indigenous *Enterobacter*, *Bacillus*, *Halomonas*, *Cronobacter*, *Exiguobacterium*, *Kushneria* and *Arthrobacter* spp. with special emphasis on the mechanism of auxin synthesis via tryptophan dependent pathway. Optimization of bacterial auxin synthesis showed that maximum amount of bacterial auxin synthesized during stationary growth phase of bacteria which will be helpful to get maximum quantity of bacterial auxin for use in plant tissue culture and plant growth experiments in a cost- effective way.

Acknowledgement: University of the Punjab is acknowledged for financial support to complete this work.

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