Int.J.Curr.Microbiol.App.Sci (2018) Special Issue-7: 4849-4859

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-7 pp. 4849-4859**
Journal homepage: http://www.ijcmas.com

Original Research Article

Isolation and Characterization of Pesticide Tolerant Bacteria from Brinjal Rhizosphere

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Keywords

Pesticide Tolerant Bacteria, Brinjal Rhizosphere, Isolation

A B S T R A C T

Pesticides are the substances for preventing, destroying, repelling any pest. Due to bulk handling or accidental release, they are accumulated in soil which leads to occasional entry into ecosystem that shows lethal effect on living system. An enrichment culture technique was used to isolate bacterial strains from brinjal rhizospheric soil tolerating high concentration of the selected pesticides. Nineteen pure bacterial cultures were isolated. All nineteen isolates were characterized on the basis of biochemical features like IAA and ammonia production, phosphate solubilization and screened for pesticide, pH, and salt tolerance. The rhizobacterial isolates were also tested for quantitative IAA production. Production of IAA from the isolates ranged between 93.15 µg/ml to253.34 µg/ml. The screening of pesticide tolerance was done at 50 µg/ml to 50000 µg/ml for fungicides and 100 ppm to 2000 ppm for insecticides.

Introduction

The global area under brinjal cultivation has been estimated at 1.85 million ha with a total production of about 32 million tonnes (FAO 2005). India accounts for about 8.7 million tonnes with an area of 0.53 million hectares under cultivation. West Bengal is the highest producer of brinjal (26%) followed by Orissa (20%) and Bihar (10%). The brinjal cultivated suffers from several diseases and pests. The most common pests are the Stem and Fruit Borer Worms (SFBW) along with fungal and viral diseases. To get good crop yield, a large quantity of pesticides and fungicides various pesticides or combination of pesticides are sprayed in controlling pests in brinjal fields. However, over the years due to their indiscriminate usage and their bioaccumulation had resulted in acute toxicity to mammals, other non-target organisms (Sogorb *et al.*, 2004) and vegetables (Bhattacherjee, 2013) which may have profound effect on the environment and health. Ideally a pesticide must be lethal to the targeted pests but not to non-target species. Unfortunately, this is not the case, so the controversy of use and abuse of pesticides has surfaced. The rampant use of these chemicals will be havoc with human and other life forms. Thus, there is a need to explore microbial diversity which can either transform toxic pesticides to non-toxic

forms or could survive in presence of high concentrations. In this regard, the consortium based on stable association of microbes, belonging to different groups, is an attractive option to achieve mineralization of pesticides and its toxic intermediates. PGPR are known to improve plant growth in many ways when compared to synthetic fertilizers, insecticides and pesticides. They enhance crop growth and can help in sustainability of safe environment and crop productivity. Therefore, considering the above facts in view; we have isolated pesticide tolerant bacteria from rhizosphere of brinjal and characterized the plant growth promoting and other traits.

Materials and Methods

Collection of Soil samples

Soil samples were collected from the rhizosphere of brinjal field of vegetable farm of Narendra Deva University of Agriculture and Technology, Kumarganj, Faizabad. The brinjal plants were uprooted from the field and rhizosphere soil was pooled and filled in sterile polythene bags**.**

Isolation of pesticide tolerant rhizobacteria

Nutrient broth (100 ml in 250-Erlenmeyer flask) supplemented with 10mg, 20 mg, 30 mg and 40 mg of fenvalerate 20% EC was used for enrichment of microbial populations capable of growing in presence of fenvalerate. The respective flasks were inoculated with 1% (w/v) rhizospheric soil samples collected from brinjal and incubated at 30°C on an environmental shaker at 150 rpm. Serial dilutions were made up to 10^{-3} from all four soil samples and 10^{-3} dilutions were taken for spread plating on nutrient agar medium having 5ppm, 20 ppm, 50 ppm, 100ppm fenvalerate and pH 7.0. Plates were incubated at 30 ^{0}C for 24 hours. After incubation, plates were observed for different isolates based on morphological traits. Morphologically variable colonies picked up and purified on nutrient plates. Pure cultures of the soil isolates were made and preserved on the nutrient agar slants.

In-vitro evaluation of brinjal rhizosphere isolates against common pesticides

Nutrient agar medium was used for screening of rhizobacterial isolates for various pesticide tolerances. The one litre medium contained beef extract (3.0 gm), peptone (5.0 gm), agar (18.0 gm) pH 7.0 ± 0.2. For screening against Mancozeb 75 WP and Carbendazim 50 WP, the nutrient agar medium was supplemented with variable concentration (50, 125, 500, 1000, 2000, 5000, 10000, 20000 and 50000 µg/ml), Chlorpyriphos 20% EC and Fenvalerate 20% EC(100, 500, 1000 and 2000 ppm) and poured in petri plates. Inoculums of Rhizobacterial culture isolates was spotted on the medium plate and incubated for 48 hours at 30^{0}C.

Screening of soil bacterial strains for plant growth promoting traits

Different media were used for the qualitative screening of isolates for salt tolerance, pH, ammonia, indole acetic acid (IAA) and phosphorus solubilization.

Salt tolerance

For screening of rhizobacteria for salt tolerance, the nutrient agar medium supplemented with variable NaCl concentration was used. The medium had beef extract (3.0 gm), peptone (5.0 gm), agar (18.0 gm), NaCl (2%-12%) per litre of distilled water and pH 7.0 was maintained.

The sterilized medium with different concentration of NaCl (2%, 5%, 7%, 10% and 12%) was poured in petri plates. Inoculums of Rhizobacterial culture isolates were spotted on the medium plate. The plates were incubated at 30^0C for 24 hours and observed the growth of rhizobacterial isolates.

pH

The nutrient agar medium with pH range (5 to 9) was made and poured in petri plates. Inoculums of Rhizobacterial culture isolates were spotted on the medium plate and incubated the plates at 30^0C for 48 hours. The plates were observed for the growth of rhizobacterial isolates.

Ammonia production

Peptone water (Peptone 5.0 g, Beef Extract 3.0 g per litre of distilled water, pH 7) was used for screening of brinjal rhizosphere isolates for ammonia production. The isolates were grown in peptone water medium in bigger test tubes. The test tubes were incubated at 30^0C for four days. One ml of Nessler's reagent (Qualigens make) was added to each test tube. Then test tubes observed for the presence of faint yellow colour, deep yellow to brownish colour for ammonia production.

IAA production

For evaluation of rhizobacteria for IAA production, the nutrient broth (Peptone 5.0 g, Beef Extract 3.0 g, per litre of distilled water and pH 7.0) was prepared. Loopful of culture was inoculated in 25 ml nutrient broth and incubated at 28^0C for 24 hour on rotary shaker. The broth was centrifuged at 10000 rpm for 15 minute. Two ml of supernatant was taken and added 2-3 drops of orthophosphoric acid and 4ml freshly prepared solkouski's reagent (0.5M $FeCl_3$ 1ml and 35% perchloric acid 50 ml) was added to the aliquot. Then samples were incubated for 25 minutes at room temperature. Pink colour was observed and recorded optical density at 530nm.

Phosphorus solubilization

Pikovskaya medium (Glucose10g, Yeast extract0.5g, Ammonium sulphate 0.5g, KCl 0.2g, NaCl 0.2g, $MgSO_4.7H_2O$ 0.1g, $FeSO_4.7\ H_2O$ and $MnSO_4.7H_2O$ in traces, $Ca_3(PO_4)_2$ 5g, Agar 18g per litre of distilled water and pH 7.0) was prepared and poured in petriplates. Inoculums of rhizobacterial culture were spotted on the medium and incubated the plates for 72 hours at 30^0C. The cleaning zone around the culture spot was observed for phosphorus solubilization

Results and Discussion

Isolation of pesticide tolerant bacteria from brinjal rhizosphere

In the present study nineteen rhizobacteria soil isolates were obtained from brinjal rhizospehere by using nutrient agar medium containing variable concentration of pesticide at 28^0C and pH7.0. Organophosphorus insecticide degrading bacteria were also isolated inoculated by Baishya *et al.*, 2014 as they used two pesticides, *i.e.* malathion and quinalphos using mineral salt medium (MSM) and similarly Kumar., 2011 also obtained chlorpyriphos degrading bacterial isolates from soil samples collected from cultivated field with MIC of 100 mg/l. whereas in the present study isolates could tolerate as maximum as concentration of Mancozeb 75 WP (20000 µg/ml), carbendazim 50 WP (50000 µg/ml), chlorpyriphos 20 EC (2000 µg/ml) and fenvalerate 20 EC (2000 µg/ml) respectively. Another piece of work also

supported the current investigation as Mohan and Naveen 2015 obtained organophosphorus degrading bacteria from paddy field. Similar results on the occurrence and isolation of bacterial isolates from rhizosphere of brinjal including other vegetable cereal and legume crops obtained by El-Bestawy *et al.*,2013;Akhter and Laz 2013; Naphade *et al.,* 2012; Sarat and Barathi 2013.

In-vitro evaluation of brinjal rhizosphere isolates against common pesticides, pH and salt

The present study said that all nineteen isolates were screened against mancozeb. At 5000 µg/ml of concentration of mancozeb, isolate no.4 and 10 (high) 1, 2, 5, 6, 11 and 13 9 (medium) while isolates 3, 7, 8, 9, 12, 16 and 18 had shown poor tolerance on nutrient agar medium whereas 14, 15, 17 and 19 did not show tolerance. Only isolates 3, 4, 5, 6 and 10 shown tolerance at 20000µg/ml (Table 1).

The research work Drouin *et al.,* (2010) has supported the present study as they isolated 122 strains of Rhizobia from different geographical regions, and belonging to the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* were tested against four fungicides and five insecticides. Sensitivity to the pesticides was measured by using the filter paper disk method at four concentrations, 0.45, 4.5, 45 and 450 µg per disk. Mancozeb inhibited the highest number of strains. Arya and Sharma, 2015 also reported that the various concentrations of Carbendazim (25µg/ml, 50µg/ml and 500µg/ml) were well-tolerated by the strain even up to 500µg/ml concentration. Our study also said that screening of all the isolates against carbendazim tolerance was done in nutrient agar medium @ 50, 125, 500, 2000, 5000, 10000, 20000, 50000µg/ml concentration and pH-7.0 and all bacterial isolates were able to grow at 50 to 500 µg/ml concentration but at 2000µg/ml and 5000 µg/ml only seventeen isolates shown their tolerance and only sixteen isolates were able to grow at 10000 µg/ml whereas only 14 and 10 isolates could grow at 20000 and 50000 µg/ml of carbendazim respectively. Kuperberg *et al.,* 2000, screened the isolates for their ability to resist chlorpyrifos at different concentrations ranging from 2 to 20 mg/ml. While in present study, Screening of all the isolates for tolerance was done in nutrient agar medium with 100, 500, 1000 and 2000 ppm concentration and pH-7.0. The observation was taken after 48 hrs and found almost all rhizosphere isolates tolerant at 100 and 500 ppm concentration but at 1000 ppm and 2000 ppm concentration of chlorepyriphos only 16 and 13 shown light to medium tolerance. Rashmi and Joseph 2005 also isolated chlorpyriphos tolerating bacteria from soil using enrichment culture technique and Kumar., 2011also isolated bacteria of growing in the concentration of chlorpyriphos in the range to 60- 100 mg/l.

Latifi *et al.,* 2011 obtained isolates which could grow at concentration of chlorpyriphos up to 2000 mg/l and *Azotobacter* strains from paddy soil found tolerant to chlorpyriphos. Baishya *et al.,* 2014 isolated 7 malathion and quinalphos tolerating bacterial isolates by using miniral salt medium (MSM) and identified as different *Bacillus* strains which were found tolerant towards Dichlorovas. Our invstigation also showed that Fenvelarate @ 100, 500, 1000 and 2000 ppm per ml in nutrient agar medium medium (pH 7.0) was used to screen all the isolates and observation were taken after 48 hrs of inoculation. All bacterial soil isolates were found tolerant light to high at 100, 500 and 1000 ppm of fenvelerate except isolate 12.

Table.1 Screening of brinjal rhizosphere isolates for pesticide tolerance

Concentration of Mancozeb 75 WP. µg/ml	Isolates																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
50	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
125	+++	++	+++	+++	+++	++	+++	++	++	+++	++	++	+++	+++	++	+++	++	+++	+++
500	+++	++	++	+++	+++	++	+++	++	++	+++	++	++	+++	+++	++	+++	++	++	++
1000	+++	++	++	+++	+++	++	+++	++	++	+++	++	++	+++	++	++	++	++	++	++
2000	+++	++	++	+++	+++	++	+++	++	++	+++	++	++	+++	++	+	++	+	++	++
5000	++	++	+	+++	++	++	+	+	+	+++	++	+	++	-	-	+	-	+	-
10000	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	-
20000	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-
50000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Concentration of Carbendazim 50 WP µg/ml																			
50	+++	+++	+++	+++	+++	++	+++	+++	++	+++	++	++	+++	+++	+++	+++	+++	+++	+++
125	+++	+++	+++	+++	+++	++	+++	+++	++	+++	++	++	+++	+++	+++	+++	+	+++	+++
500	+++	+++	++	+++	+++	++	+++	+++	++	+++	++	++	++	++	++	++	+	+++	+++
2000	++	+	++	++	+++	++	++	++	++	++	++	-	++	+	++	++	-	++	++
5000	+	+	+	++	++	++	+	+	++	+	++	-	++	+	++	++	-	++	++
10000	+	+	++	++	++	++	-	+	+	+	+	-	+	+	+	+	-	+	+
20000	-	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	-	+	+
50000	-	+	+	+	+	+	-	+	+	-	+	-	-	-	-	+	-	+	-
Concentration Chlorpyriphos 20 EC in ppm																			
100	+++	++	++	+++	+++	+	+++	+++	+++	+++	++	++	+++	+++	+++	+++	++	+++	+++
500	+++	++	++	+++	+++	++	+++	+++	++	+++	++	++	+++	++	++	++	++	++	+
1000	++	++	++	++	++	-	++	++	+	++	+	-	+	+	+	+	-	++	+
2000	-	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	-	+	-
Concentration Fenvelarate 20 EC.in ppm																			
100	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
500	+++	+++	++	+++	++	++	++	++	+	++	++	-	++	+	++	++	+	++	++
1000	+	++	++	++	++	++	++	++	+	++	+	-	+	+	+	++	+	+	+
2000	+	++	++	+	+	-	-	+	-	+	+	-	+	+	-	+	+	+	+

+++ = High growth ++ = Medium growth + = low growth - = No growth

Table.2 Screening of brinjal rhizobacterial isolates for variable NaCl and pH tolerance

Isolates number	NaCl concentration				pH		
	5%	7%	10%	12%	5	7	9
1	++	++	-	-	+++	+++	+++
2	++	++	+	-	+++	+++	+++
3	++	++	+	-	+++	+++	++
4	++	+	-	-	+++	+++	+++
5	++	++	-	-	-	+++	++
6	++	++	-	-	-	+++	++
7	++	++	++	+	-	+++	++
8	++	++	++	-	-	+++	++
9	++	++	-	-	+++	+++	++
10	++	++	-	-	+++	+++	++
11	++	+	-	-	+++	+++	++
12	+	+	++	-	+++	+++	++
13	++	++	+	-	++	+++	+++
14	++	++	-	-	+++	+++	+++
15	++	++	+	-	++	+++	++
16	++	++	-	-	+++	+++	++
17	++	++	-	-	+++	+++	+++
18	++	++	-	-	+++	+++	+++
19	++	++	-	-	+++	+++	+++

+++ = High growth, ++ = Medium growth, + = Poor growth, - = No growth

Table.3 Morphological characterization of pesticide tolerant brinjal rhizosphere bacterial isolates

Isolate	Morphological character of colonies						
	Form	Elevation	Margin	Appearance	Optical density	Pigment	Texture
1	Circular small	Flat	Round	Shiny	Opaque	Cream	Smooth
2	Circular small	Flat	Round	Shiny	Opaque	Cream	Smooth
3	Circular medium	Raised	Round	Shiny	Opaque	Dark cream	Smooth
4	Circular small	Flat	Round	Shiny	Opaque	Light cream	Smooth
5	Circular small	Flat	Round	Shiny	Opaque	Yellow	Smooth
6	Circular small	Raised	Round	Shiny	Opaque	Cream	Smooth
7	Circular large	Flat	Round	Shiny	Opaque	Cream	Smooth
8	Circular large	Flat	Round	Shiny	Opaque	Cream	Smooth
9	Circular small	Flat	Round	Shiny	Opaque	Cream	Smooth
10	Circular medium	Flat	Round	Dull	Opaque	Cream	Smooth
11	Circular large	Flat	Round	Dull	Opaque	Light yellow	Smooth
12	Circular large	Flat	Round	Shiny	Opaque	Cream	Rough
13	Circular small	Flat	Irregular	Shiny	Opaque	Dark cream	Smooth
14	Circular small	Flat	Round	Dull	Opaque	Dark yellow	Smooth
15	Circular small	Raised	Round	Shiny	Opaque	Cream	Rough
16	Circular small	Raised	Round	Shiny	Opaque	Light yellow	Rough
17	Circular large	Raised	Irregular	Dull	Opaque	Yellow	Smooth
18	Circular medium	Flat	Irregular	Shiny	Opaque	Cream	Rough
19	Circular medium	Flat	Round	Shiny	Opaque	Cream	Smooth

Table.4 Quantitative estimation of brinjal rhizosphere bacterial isolates for Indole acetic acid production

Isolates	Quantity of IAA (µg/ml)
1	93.56
2	104.76
3	93.16
4	93.19
5	102.56
6	102.45
7	103.28
8	253.34
9	101.38
10	102.59
11	87.03
12	87.25
13	103.56
14	101.87
15	93.81
16	94.17
17	91.45
18	92.36
19	91.59

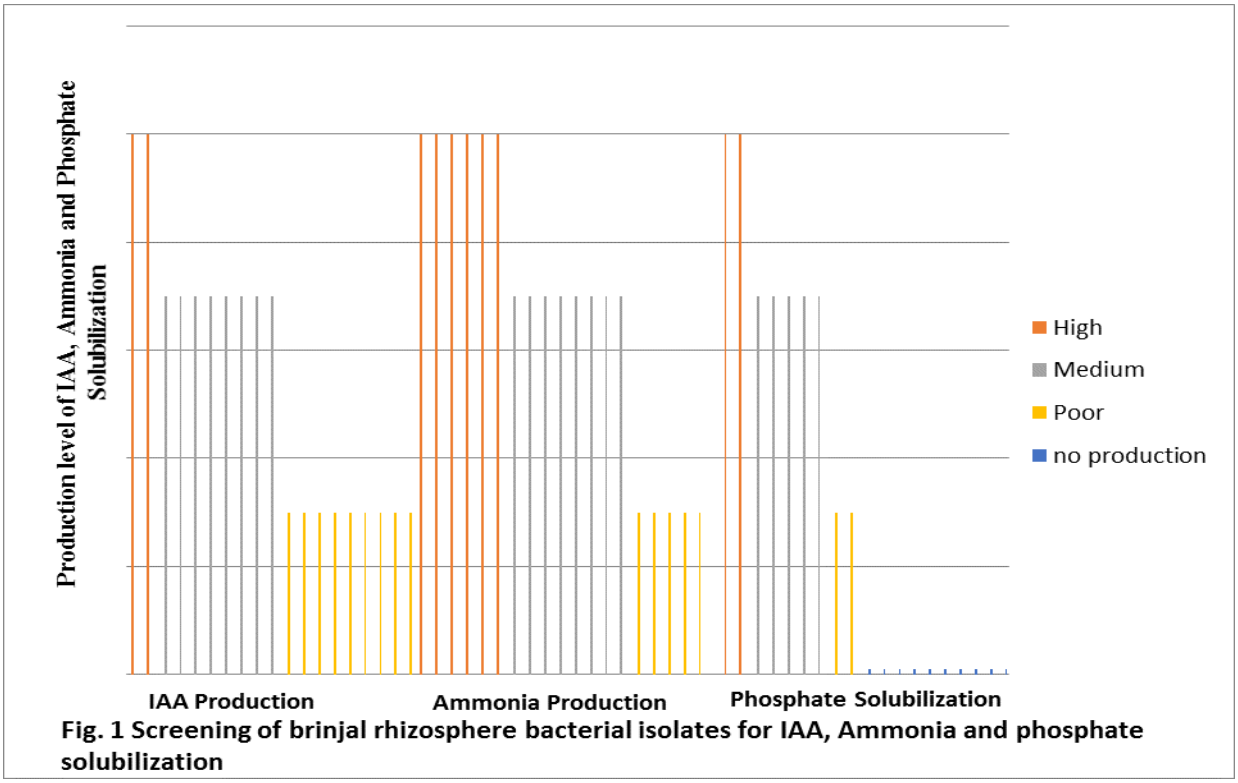

Fig. 1 Screening of brinjal rhizosphere bacterial isolates for IAA, Ammonia and phosphate solubilization

While at 2000 ppm concentration, only isolate 2 and 3 shown (medium) whereas isolate no. 1, 4, 5, 8, 10, 11, 13, 14, 16, 17, 18 and 19 shown poor tolerance. Isolates no. 6, 7, 9, 12 and 15 did not show tolerance against fenvalerate concentration @ 2000 ppm/ml. The present study is in concurrence with works done other researchers also.

Naphade *et al.,* 2012 screened five isolates for ability to grow a wide range of pH and isolates showed growth in range of pH 3.0 to 11.0 with optimum pH found 7.0, while in present study, screening of all the isolates showing pH tolerance was done in nutrient agar medium at 5, 7 and pH 9. All isolates have shown their growth at pH 7 and pH 9. But at pH 5, only fifteen (79%) isolates able to grow. Isolates no. 1, 2, 3, 4, 9, 10, 11, 12, 14, 16, 17, 18 and 19 showed higher growth while isolates no. 13 and 15 showed medium growth and isolates 5, 6,7 and 8 shown negative growths. All nineteen isolates showed higher growth at pH-7 and only eight (42%) isolates showed higher growth at pH-9 while 11 isolates have shown medium growth (Table 2). Pawar and Mali (2014) also studied eight different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) were used in optimization experiment. Optimum pH value was 7.0 for degradation of Dichlrovas by *Bacillus*. Similar findings were also reported by Rashmi *et al.,* 2015, Thabit *et al.,* 2013, Akhtar and Laz 2013, El-bestway *et al.,* 2013, Ravi *et al.,* 2010 and Shivaramaiah *et al.,* 2006.The findings of the present investigation are well matched with previous work done.

Upadhyay *et al.,* 2009 screened rhizospheric bacterial strains for salt tolerance, out of 133 stains, only 24 strains could grow at 8% NaCl concentration and no strains was able to grow at 9% NaCl concentration. Whereas present study showed that out of nineteen rhizobacterial isolates, seven rhizobacterial isolates could grow at 10% NaCl while only one strain could grow at 12% NaCl (Table 2). The findings of Nashaw and Mansour 2012 also supported the present study as they observed rhizobacterial strains isolated from root nodules of *Medicago sativa*, *Phaseolus vulgaris* and *Vicia faba* found as maximum as up to 100 mM to 300 mM NaCl concentration tolerance.

Morphological and biochemical characterization

Baishya *et al.,* (2014) isolated 4 bacterial cultures based on colony morphology and two different colonies were observed on nutrient agar medium from soil sample of vegetable farm but in present study, all nineteen soil isolates were characterized on the basis of morphological characters like margin, appearance, texture, form, elevation and pigment *etc* on nutrient agar medium. In relevance of our study Naphade *et al.,* (2012) isolated five morphologically distinguishable bacterial colony on agar plate and biochemical studies carried out also, so the result of the present investigation are in accordance with the earlier works.

Similarly, almost all bacterial isolates showed light to high production of indole acetic acid while the isolate 1 and 8 showed high production of indole acetic acid and other isolates appeared to be poor to medium producer of Indole acetic acid (Table3). PGPRs such as *Azotobacter, Bacillus, Azospirillum, Pseudomonas, Serratia, Klebsiella* isolated from wheat rhizosphere produced IAA reported Mapelli *et al.,* 2013 while *Pseudomonas fluorescens* and *Bacillus subtilis* from rhizosphere of onion produced in-vitro indole acetic acid as studied by Reetha *et al.,* 2014. In accordance to the present study, Ahmed *et al.,* 2010 assessed fungicide tolerant rhizospheric

bacteria of Mustard for plan IAA and ammonia production, Phosphate solubilization and Bharucha *et al.,* (2013) also tested quantitative production of IAA in L-trypophan containing IAA production medium and they found IAA production in the range of 13.53 to 126 µg/ml by the isolates, while our investigation gave higher value (253.34 µg/ml) of IAA (Table 4). As far as ammonia production in concerned, the isolates of present study showed low to high production of ammonia (Fig 1). The finding of Ahmad *et al.,* 2008 also support as they evaluated 72 bacterial strains from rhizosphere of brinjal, wheat, mustard, sugarcane *etc*. and found to be producer of ammonia in peptone water and Joseph *et al.,* 2007 also evaluated 150 bacterial strains and found positive for ammonia production as indicated by light yellow to brown colour development. Out of nineteen, only nine rhizobacterial isolates found positive for phosphate solubilization on Pikovaskaya medium as shown in Fig1.Yasmin *et al.,* (2009) analyzed 15 PGPR isolates for phosphate solubilizing capability, only six isolates were able to soulbilize insoluble phosphate as evident by producing of clearing zone on calcium phosphate medium. The findings of present study are in accordance with the available reports. Rani *et al.,* 2012 also screened 65 isolates of pigeon pea rhizosphere for phosphate solubilization and only six isolates found positive for phosphate solubilization. The isolates of PGPR of mustard rhizosphere having tolerance toward fungicides have been screened for phosphate solubilizing by Ahmad *et al.,* 2010

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