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CHARACTERIZATION OF *PSEUDOMONAS* ISOLATES FROM DIVERSE AGROECOLOGIES FOR THEIR PLANT GROWTH PROMOTING TRAITS

Sravani Pinisetty*[1], Suseelendra Desai[1], Uzma Sultana[1,2] and Meenakshi Taduri[1]
[1]Central Research Institute for Dryland Agriculture (ICAR), Santoshnagar, Hyderabad, India
[2]Telangana Social Welfare Residential Degree College, Kamareddy, India
*Corresponding author-mail: sravanipinisetty@gmail.com

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ABSTRACT

Microorganisms contribute to soil health by improving growth and productivity of crops. The huge diversity of the genus *Pseudomonas* for their plant growth promoting traits is still under-exploited. Hence, 50 isolates of *Pseudomonas* from various agro-ecologies were assessed for their plant growth promoting traits. All the isolates were screened for production of growth hormones, exo-polysaccharides, siderophores, hydrolytic enzymes and bio-film. Among the 26% of IAA producers, P84 has shown the highest activity (39.73 µg mL^{-1}). The highest EPS production was exhibited by P105 (60.58µg mL^{-1}) and the least recorded was 2.93 µg mL^{-1} by P103. In case of ammonia production, ten isolates were strong, eighteen were moderate, fifteen isolates were weak and seven isolates were non producers of ammonia. The ability to produce HCN was exhibited by six isolates. Out of 50 isolates, nine isolates have shown clear zones for chitin/chitosan modifying enzymes. The efficient isolates were further assessed for growth promotion and disease suppression.

Keywords: *Pseudomonas* spp, PGPR, PGP traits, Plant growth

Introduction

The future food-and nutritional security challenges of the growing world population demands for enhanced vertical farm productivity as the land will be a limiting resource. A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050 suggested that the total global food demand is expected to increase by 35% to 56% between 2010 and 2050, while population at risk of hunger is expected to change by –91% to +8% over the same period (van Dijk *et al*., 2021). As the population continues to rise, there is a need to increase agricultural production and meet food demand with sustainable practices while maintaining the human and animal health due to soil, water and air pollution. The vigorous usage of non-sustainable agricultural practices for satisfying the global food supply is leading to degradation of agro-ecosystems (de los Santos-Villalobos *et al*., 2021). The prolonged use of these chemicals has deteriorated the soil health, polluted natural resources, strengthened pesticides and released greenhouse gases, there by making the environment hazardous to life. Thus, in recent years, the focus towards the development of sustainable alternatives has increased (Zain *et al*., 2019). Research interest has diverted to restore the soil fertility and improve plant productivity by involving free living microbes in the rhizosphere known as Plant Growth Promoting Rhizobacteria (PGPR).The plant-microbe interactions play a vital role in plant health. A wide range of microorganisms like, *Azotobacter, Azospirillum, Bacillus, Pseudomonas, Rhizobium, Brady rhizobium, Sino rhizobium, Aspergillus* and *Arbuscular mycorrhizae* (AM) and nematodes used for pest and disease management were employed in crop production and crop protection. PGPR achieves growth promotion either directly by production of phytohormones and nitrogen fixation or indirectly by preventing the phytopathogens (Brown, 1974; Howell *et al*., 1979; Glick, 1995). These PGPR influences the plant growth by the production of auxins. Of these, Indole Acetic Acid (IAA) is a well-known plant growth regulating compound (Wanek *et al*., 2010). PGPRs induce the growth regulation by production of phytohormones (Dey *et al*., 2004). The synthesis of antibiotic enzymes, siderophores and fungicidal compounds by growth promoters enables the disease suppression (Patten *et al*., 1996). The enhancement of plant growth and grain yield of the treated crops is found to be effective with *Pseudomonas* spp. among various bacterial isolates tested (Leveau *et al*., 2005; Kochar *et al.,* 2011; Weller, 2007). The ability of rhizobacteria to persistently colonize the roots is helpful to the crops (Ali *et al.,* 2010). Keeping in view, the vast diversity of the microorganisms for sustainable agricultural production, this study was undertaken to explore various agro-ecologies to identify efficient strains of *Pseudomonas* spp. endowed with plant growth promoting traits.

Material and Methods

Source of culture, maintenance and evaluation of PGPR traits

Several isolates of *Pseudomonas* spp. were collected from various agro-ecologies of India and stored in the ICAR-CRIDA culture bank, Hyderabad, India. Out of them, 50 isolates were used in this study. From the stock cultures working culture slants were prepared and used throughout the study. All the isolates of *Pseudomonas* were characterized for the presence of PGPR traits by following standard procedures both qualitatively and quantitatively.

Qualitative estimation of Indole acetic acid (IAA) and Gibberellic acid (GA_3)

The ability of the isolates to produce IAA and GA_3 was assessed using minimal medium containing 0.1% L-tryptophanas described by Gordon and Weber (Gordon *et al.,* 1951). The tubes containing10 ml of the medium was inoculated with 100 µL bacterial culture and incubated for 48 h at 28°C. The medium was centrifuged for 15 min at 10000 rpm after incubation. The supernatant was transferred to a fresh tube and the pellet was discarded. The culture filtrate was mixed well with equal volume of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and evaporated. The obtained crystals were dissolved in acetone. Along with standard phytohormones the sample was spotted on silica gel-60 F_{254} ready-to-use TLC plates (Merck) and run using a solvent mixture of ammonia: water: isopropanol (1:1:10 ratio) for 30 min. The plates were sprayed with a reagent of 3% H_2SO_4 in methanol with 50 mg of $FeCl_3$ and are air dried. The spots were observed for orange-coloured spots (IAA) and greenish spots (GA_3) under UV light.

Quantitative estimation of IAA

IAA was estimated quantitatively by using minimal medium amended with 0.1% L-tryptophan (Gordon *et al.,* 1951). An aliquot of 100 µL culture suspension was inoculated into 10mL sterile broth and incubated at 30°C for 48 h. The medium was centrifuged for 15 min at 10000 rpm after incubation. The supernatant was transferred to a fresh tube and the pellet was discarded. IAA standards were prepared from 100 μL^{-1} stock solution. An aliquot of 0.5mL of the standards and supernatant of the sample were taken in test tubes to which two mL of Salkowski's reagent was added and incubated in dark for 20 min. The optical density was recorded at 530 nm in a UV-Visible spectrophotometer.

Quantitative estimation of GA_3

GA_3 was quantified by colorimetric method using minimal medium amended with 0.1% L-tryptophan (Holbrook *et al*., 1961). 10mL of sterile broth was inoculated with100 µL of bacterial suspension and incubated at 30°Cfor 48 h. The culture broth was centrifuged for 15 min at 10000 rpm and the supernatant was transferred to a fresh tube. To 10 mL of the supernatant 2mL each of potassium ferrocyanide and zinc acetate were added and centrifuged for 15 min. The supernatant was collected and to 5mL of the aliquot, 5mL of 30% HCl was added and incubated at 20°Cfor 75 min. Blank sample treated with 5% HCl served as control. The absorbance was measured at 254 nm using a UV-VIS spectrophotometer (Elico, India) andGA_3content was calculated from standard curve.

Detection of ammonia production

To test the production of ammonia, 50 *Pseudomonas* isolates were grown in 10 mL of sterile peptone broth and incubated for 72h (Bano *et al*., 2003). Ammonia production was detected by addition of 0.5 mL of Nessler's reagent to the bacterial suspension after incubation and observed for colour change. The intensity of the colour developed from yellow to brown differs based on the ammonia produced by an isolate.

HCN production

All the isolates were screened for the HCN production using King's B medium amended with 0.44 % of L-glycine.100µL bacterial inoculum was plated on the medium (Bakker *et al*., 1987) Whatman No.1 filter paper discs soaked in a mixture of 0.5% picric acid and 2% Na_2CO_3 solution were fixed to the lid of the petridishes inoculated with the test cultures. The plates were sealed with parafilm and incubated at 28±2°C for 48 h. The appearance of orange colour indicates cyanogenic activity of the test isolates.

Estimation of exo-polysaccharides (EPS)

Active bacterial isolates were inoculated in 50 mL of trptone soya broth and incubated in a shaker with 120 rpm atfor 48 hat 28°C. The culture suspension was centrifuged for 10 min at 10000rpm. The pellet was extracted, dissolved in 0.4 percent KCl and centrifuged for 10 minutes at 5000 rpm. The supernatant was collected and diluted with two volumes of cold ethanol and refrigerated overnight. The cold mixture was centrifuged at 15000 rpm for 20 min. The pellet was resuspended in 1mL of demineralized water and further used for EPS estimation using Anthrone reagent method (Celik *et al*., 2008).

Bio-film formation

All the bacterial isolates were inoculated in sterile polypropylene tubes containing King's B broth medium and incubated for 36-48 h at 28°C. After incubation, the medium was decanted and the tubes were rinsed with phosphate buffered saline (pH 7.3) and dried. The tubes were then stained with 0.1% Crystal violet for a minute and decanted. The formation of violet ring at the top is an indication for biofilm formation (Ansari *et al.,* 2018).

Detection of siderophores

Active bacterial cultures were spot inoculated on the CAS Blue-agar medium (Schwyn *et al*., 1987) for detection of siderophores. Plates were incubated for 24 h at 28°C. The positive result is indicated by an orange halo around the bacterial colony.

Detection of extracellular enzymes

(i) Detection of Amylase

Qualitative analysis for amylase activity was conducted by using Nutrient Agar medium amended with Starch (1% w/v). Bacterial culture was spot inoculated and incubated at 28±2°C for 48 h. The plates were then stained with iodine for 10 min. A zone of clearance observed around the isolates with a dark blue or black background indicates a positive result (Luang-In *et al*., 2019).

(ii) Detection of Cellulase and Protease

Cellulase was qualitatively assessed using Nutrient agar amended with 1 percent carboxy methyl cellulose whereas

protease was assessed using 1 percent skimmed milk powder as sole carbon source in mineral salts medium (Booth, 1971). The test cultures were spot-inoculated on respective plates and incubated for 48 hours. Isolates with a clear zone were considered positive for protease. The test plates for cellulase were stained with 0.1 percent Congo red solution for 10 min, washed with 1M NaCl and then flooded with 1M acetic acid for 10 min. The zone of clearance around the colony indicates cellulase activity.

(iii) Detection of Pectinase

Pectinase screening agar was prepared with $NaNO_3$ 2g, KCl 0.5g, $MgSO_4$ 0.5g, K_2HPO_4 1g, tryptone 0.5g, agar 20g, pectin 10g, and distilled water 1L. Active bacterial cultures were spot-inoculated and incubated at 37°C for 24 h and observed for clear zone formation (Tabssum *et al*., 2018).

(iv) Detection of chitin-chitosan modifying enzymes(CCME)

The test cultures were inoculated in 5 mL King's B medium and incubated in a shaker at 28°C for 24 h and were centrifuged at 10,000 rpm for 10 min to remove the bacterial cells. Supernatant was dried in liquid nitrogen and the samples were freeze dried under vacuum in a lyophilizer. Dot blot assay was used to screen for the presence of CCME on 5×5 cm polyacrylamide gels. Substrate was prepared by dissolving 1mg (chitosan 56%) in 1 mL of 100mM glacial acetic acid and incubated overnight by shaking. The protein samples were diluted in 50 mM sodium acetate buffer (pH 5.2) to a final concentration of 10 mg mL^{-1} and spot inoculated (3 L) on to the prepared gels. The gels were incubated overnight at 37°Cin a moist chamber. After incubation, the gels were stained with calcofluor white for 5 min and rinsed twice with water. Lytic zones were revealed by UV illuminationin a gel documentation system (Kumar *et al.,* 2019).

Results

Quantitative estimation for production of IAA and GA_3 by *Pseudomonas* isolates

Of all the isolates of *Pseudomonas* screened for phytohormone production, thirteen isolates produced IAA and twelve isolates produced GA_3. Across the isolates, the range of 15.70 (P105) to 39.73µgmL^{-1}(P84) was observed for IAA production and the range varied from 16.24 (P116) to 38.57 µgmL^{-1} (P118) in case of GA_3 production. Six isolates showed both IAA and GA_3 (Table 1).

Table 1 : Quantitative estimation of IAA and GA_3

S.No.	Isolates	IAA (µg mL^{-1})	S.No.	Isolates	GA_3(µg mL^{-1})
1.	P78	18.90(±2.55)	1.	P76	37.98(±1.18)
2.	P80	32.90(±1.58)	2.	P84	23.91(±1.39)
3.	P84	**39.73(±1.20)**	3.	P87	26.64(±1.18)
4.	P87	24.81(±1.66)	4.	P95	29.16(±0.65)
5.	P92	37.56(±1.99)	5.	P103	33.52(±1.50)
6.	P94	30.68(±0.99)	6.	P105	18.12(±1.51)
7.	P100	32.23(±1.85)	7.	P110	18.88(±0.69)
8.	P105	15.70(±1.74)	8.	P116	16.24(±1.28)
9.	P108	34.54(±2.31)	9.	P118	**38.57(±1.01)**
10.	P110	28.30(±1.76)	10.	P120	34.86(±1.06)
11.	P113	25.60(±1.94)	11.	P123	30.25(±0.89)
12.	P118	29.85(±1.81)	12.	P125	29.16(±0.65)
13.	P120	26.06(±1.37)			

Ammonia Production

All the bacteria were tested for ammonia production and were categorized into three groups viz., weak, moderate and high ammonia producers based on the colour developed (Table 2). Of all the isolates tested, 15isolates (30%) were weak ammonia producers, 18 were moderate (36%) and 10(20%) were strong ammonia producers. However, seven isolates turned-out to be non-ammonia producers (Table 2 and Fig 1).

Table 2 : Ability of the test *Pseudomonas* isolates to produce ammonia *in vitro*

Weak	Moderate	Strong	Non producers
P76, P77, P80, P86, P89, P94, P96, P98, P99, P100, P109, P110, P117, P118, P123 **(15)**	P78,P79,P83,P84, P85, P87, P93, P95, P101, P104, P106, P108, P111, P113, P115, P119, P120, P124**(18)**	P82,P88,P91,P92,P97**,** P105, P116, P121, P122, P125**(10)**	P81,P90,P102,P103, P107, P112, P114**(7)**

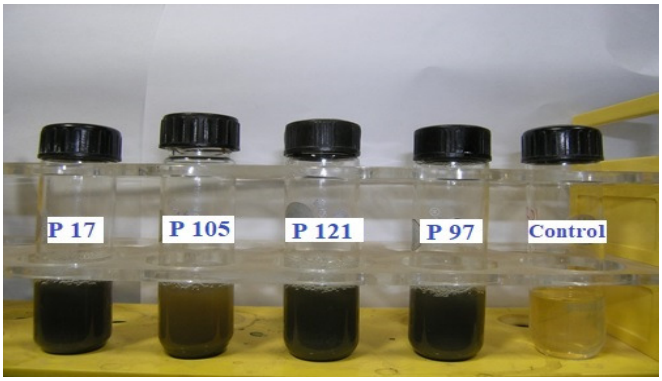


Fig. 1: Ammonia production by *Pseudomonas* isolates

HCN Production

Of all the isolates tested for HCN production, P87 produced maximum amount of HCN. P83 and P125 were moderate producers, whereas P93, P105 and P110were found to be weak producers. Remaining 45 isolates were non-HCN producers (Table 3 and Fig 2).

Table 3 : *Pseudomonas* isolates producing HCN

S.No	Isolates	HCN Production
1.	P83	++
2.	P87	+++
3.	P93	+
4.	P105	+
5.	P110	+
6.	P125	++

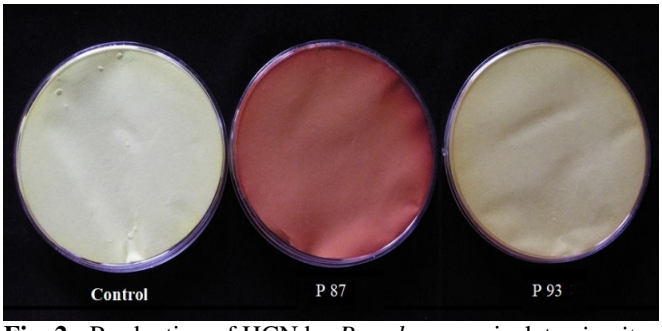


Fig. 2 : Production of HCN by *Pseudomonas* isolates *in vitro*

Quantification of EPS

EPS production was estimated in all the test isolates and the result differed significantly in the range of 2.93 to 60.58 µg mL^{-1}. The highest EPS production was observed in P105 (60.58 µg mL^{-1}) and the lowest production was recorded in P103(2.93 µg mL^{-1}) (Table 4).

Table 4 : Exopoly saccharide production by *Pseudomonas* isolates

S.No.	Isolates	EPS(µg/mL)
1.	P76	49.13(±2.11)
2.	P80	23.41(±1.17)
3.	P83	43.64(±1.03)
4.	P84	13.25(±0.37)
5.	P87	4.59(±0.78)
6.	P90	25.28(±2.23)
7.	P95	34.54(±0.64)
8.	P99	31.82(±2.37)
9.	P103	2.93(±0.21)
10.	P105	**60.58(±1.01)**
11.	P108	40.18(±1.22)
12.	P110	40.10(±1.11)
13.	P111	33.75(±1.61)
14.	P113	53.07(±2.45)
15.	P115	20.21(±0.90)
16.	P118	33.31(±0.77)
17.	P119	14.73(±1.67)
18.	P120	22.55(±1.54)
19.	P121	28.99(±0.74)
20.	P123	17.16(±1.56)
21.	P125	38.08(±1.12)

Biofilm formation

The ability to form biofilm was checked among the *Pseudomonas* isolates. Of all the tested bacterial cultures, 13 could form thin film, 15 isolates formed medium film whereas8 isolates formed thick biofilm. However, 14 isolates did not form biofilm (Table 5 and Fig 3).

Table 5: Qualitative screening for biofilm formation by *Pseudomonas* isolates

Thin	Medium	Thick	Non-producers
P76, P77, P81, P83, P84, P90, P95, P103, P111, P114, P118, P119, P121(**13**)	P79,P80,P86, P87, P91, P92, P98, P99, P100, P106, P110, P112, P115, P120, P125(**18**)	P85,P93,P96, P105, P108, P117, P122, P124 (**8**)	P78, P82,P88, P89, P94,P97, P101,P102, P104, P107, P109, P113, P116, P123 (**14**)

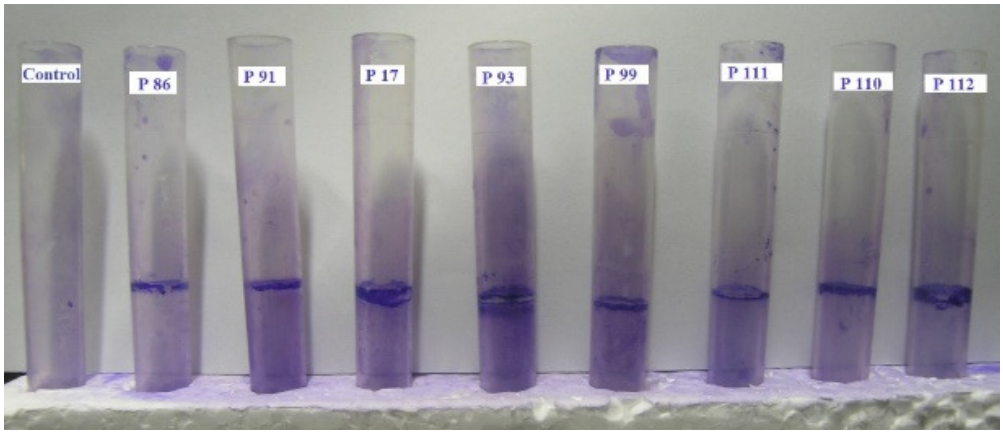


Fig. 3 : Qualitative assay to detect formation of biofilm by *Pseudomonas* isolates

3.6. Production of Siderophores

Out of 50 isolates screened for production of siderophores,P77, P81, P83, P95, P99, P105, P108, P110, P118 (i.e., 22%) isolates were found to produce siderophores (Table 6 &Fig 4).

Table 6: Qualitative screening for Siderophores production by *Pseudomonas* isolates

Producers of Siderophores	Non-Producers of Siderophores
P77, P81, P83, P95, P99, P105, P108, P110, P118 **(7)**	P76, P78, P79, P80, P82, P84, P85, P86, P87, P88, P89, P90, P91, P92, P93, P94, P96, P97, P98, P100, P101, P102, P103, P104, P106, P107, P109, P111, P112, P113, P114, P115, P116, P117, P119, P120, P121, P122, P123, P124, P125 **(43)**

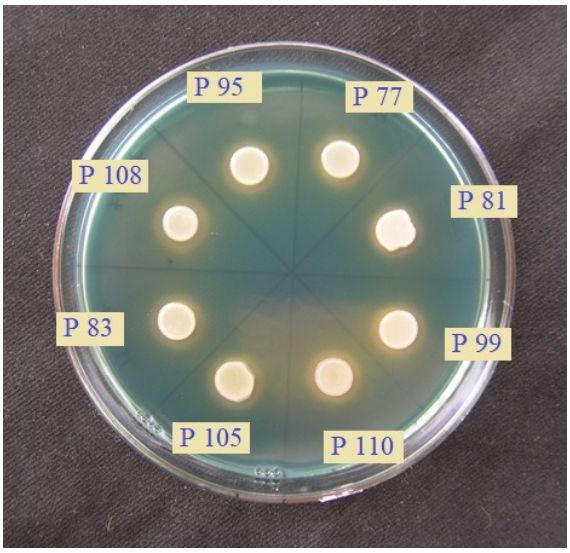
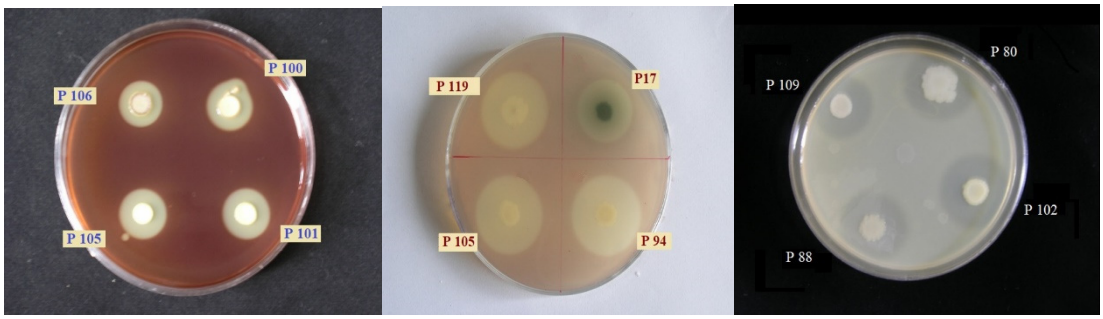


Fig. 4 : Screening for production of siderophores *in vitro* in CAS medium by various isolates of *Pseudomonas*

3.7. Production of hydrolysing enzymes

The *Pseudomonas* isolates were tested for production of hydrolysing enzymes. Across the isolates, 46% (23) isolates for amylase production, 56% (28) for cellulase production, 42% (21) for pectinase and 30%(15) isolates for protease production were positive with clear zones (Table 7 and Fig. 5). The remaining isolates were negative with no zone of clearance.



A B C

Fig. 5 : Detection of cellulase (A), pectinase (B) and protease (C) activity *in vitro* by *seudomonas* isolates

Table 7 : *Pseudomonas* isolates showing hydrolytic enzymes production

Amylases	Cellulases	Pectinases	Proteases
P77, P78, P81, P83, P84, P86, P87, P92, P96, P99, P100, P103, P104, P106, P107, P110, P111, P114, P117, P118, P120, P123, P125**(23)**	P76, P77, P79, P80, P82, P84, P85, P87, P88, P89, P91, P93, P94, P95, P98, P100, P101, P105, P106, P107, P110, P111, P112, P116, P120, P121, P124, P125 **(28)**	P78, P79, P83, P85, P86, P90, P91, P94, P95, P96, P98, P99, P102, P105, P107, P113, P114, P118, P119, P121, P123**(21)**	P80, P83, P88, P93, P95, P87, P104, P102, P108, P109, P111, P120, P115, P122, P125**(15)**

3.8. Chitinolytic Activity

Out of 50 isolates screened for the chitinolytic activity using the substrate chitosan 56% DA, 18% (i.e., 9) were positive with clear halo zones and 82% (i.e., 41) were found to have no activity. (Table 8 and Fig.6)

Table 8 : Production of CCME by *Pseudomonas* isolates

Strong	Medium	Weak	Non-Producers
P83, P87 **(2)**	P93, P110, P116, P120 **(4)**	P77, P82, P121 **(3)**	P76, P78, P79, P80, P81, P84, P85, P86, P88, P89, P90, P91, P92, P94, P95, P96, P97, P98, P99, P100, P101, P102, P103, P104, P105, P106, P107, P108, P109, P111, P112, P113, P114, P115, P117, P118, P119, P122, P123, P124, P125 **(41)**

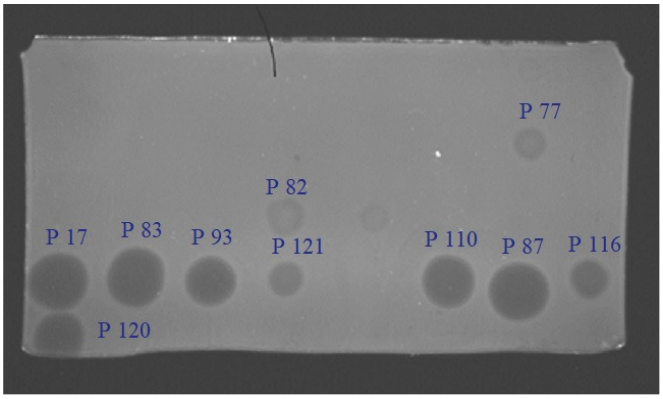


Fig. 6 : Dot blot assay for qualitative detection of chitinase/chitosanase activity

To summarize the results of characterization of isolates for various PGPR traits as mechanisms of plant growth promotion, it was observed that 26% isolates were IAA producers, 24% were GA_3producers, 42% were exopolysaccharide producers, 86% isolates produced ammonia, 20% produced siderophores, 72% formed biofilm, 12% were positive for hydrogen cyanide, 46% exhibited amylase activity, 56% exhibited cellulase activity, 42% produced pectinase, 30% produced protease and 18% showed chitinase activity (Fig7).

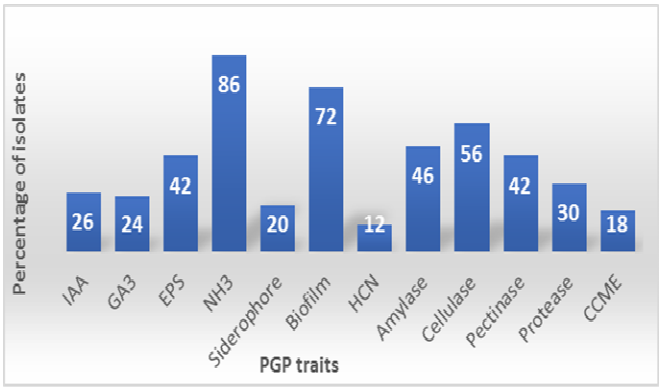


Fig. 7 : Distribution pattern of various PGPR traits

Discussion

In the current investigation, *Pseudomonas* spp. from diverse agro-ecologies of India were screened for plant growth promoting traits. Out of 50 *Pseudomonas* isolates, 13 isolates produced IAA and 12 isolates produced GA_3.The highest amounts of IAA and GA_3were recorded with P 84 and P118respectively.The results are in line with other findings where the Pseudomonas have been reported to produce IAA and GA_3 (Praveen Kumar *et al.,* 2012) and other plant growth regulators. Some of the isolates exhibited a strong ammonia producing ability. HCN production have been reported to play an essential role in the biological control of pathogenic fungi and deleterious rhizobacteria (Kumar *et al.,* 2015). The possible use of siderophores as biopesticides paved special interest to study Pseudomonas (Wilson, 1997). Systemic resistance is also induced by siderophores (Aznar *et al.,* 2015). In the present study, 10 isolates were found to produce siderophores indicating that they may be involved in induction of resistance against stresses in crop plants. Variation in their ability to produce hydrolysing enzymes among the isolates was observed in the present study. Production of hydrolytic enzymes plays a vital mechanism in suppression of phytopathogens. These enzymes are implicated as one of the modes of action of biocontrol agents and also helps in enhancing soil fertility (Bibi *et al.,* 2018). When screened for chitinolytic activity, 50 isolates of *Pseudomonas* responded differentially. Chitosanases with low degree of deacetylation possess anti-microbial action and chitosanases with high degree of deacetylation contribute to plant growth (Glick *et al*., 1999). Evaluation for EPS production and biofilm formation was carried out with all the isolates. EPS producing rhizobacteriahelps in maintaining soil moisture and formation of soil aggregates (Uzma Sultana *et al.,* 2021). In the current study, EPS production among the isolates was significant.

Conclusion

The isolates of *Pseudomonas* used in this study exhibited diversity in their plant growth promoting traits and thus could prove to be effective biostimulants. While majority of the isolates produced ammonia and biofilm, very few isolates showed CCME activity. Other traits were at moderate level for all the isolates. The diversity in the PGP traits demonstrates importance of the agro-ecological variability and emphasizes need for in-depth analysis of the microbial diversity. A particular trait is not responsible for the overall development of the plant. Plant growth promotion is attained by the synergistic action of all the plant growth promoting traits. Further investigations on these *Pseudomonas* for their ability to promote under pot culture studies followed field evaluation could give promising strains to promote growth and productivity of the crops.

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