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Modulation of Rhizobacterial Auxin Production at Different pH and Temperature

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ABSTRACT: *Auxins have been reported to be synthesized by many plant associated bacteria that are involved in enhanced plant growth and root/stem elongation. The aim of the study was to analyze Indole Acetic Acid production by Rhizobacteria at different pH and temperature range. Out of ten isolates, five strains gave a positive primary screening Salkowski test that were proceeded further. Our proposed hypothesis that increasing pH and decreasing temperatures lead to poor production of Auxin was justly supported by the results as all strains showed maximum activity of IAA production at pH 6 among which the most notable strains were AUX-G (36.75 µg/ml) and AUX-M (33.65 µg/ml) with highest IAA production. All strains showed maximum IAA producing activity at 37°C temperature. The least production was associated with decreased temperature i.e. 25° C where AUX-W (2.37 µg/ml) showed the least IAA levels. Hence, maximum IAA yield was obtained at 37°C at 6 pH which also indicated an increased log phase of bacterial growth.*

Key words: *PGPRs, Auxin Estimation, Salkowski reagent , Temperature, pH*

INTRODUCTION

A major phytohormone auxin is produced as a secondary metabolite by almost 80% of bacteria residing the soil rhizosphere (Idris et al., 2007). Auxin aids plants in growth by root and shoots elongation along with inhibition of leaf abscission and promoting flowering in plants (Zhao, 2010). In plants, auxin is produced in young leaves, stem and seeds from transamination and decarboxylation reactions of Tryptophan (Lebuhn and Hartmann, 1997). Biosynthesis of auxin by plant growth promoting bacteria serves as one of a direct mechanisms of providing the plant with essential nutrients whereas the PGPRS also acts as biofertilizers or bioinoculants that uses these microbes to

promote plant growth according to the desire of the farmer (Tittabutr et al., 2007). The biosynthesis of auxin compounds can be done through two common pathways. One is the tryptophan dependent pathway and the other is tryptophan independent pathway. The major pathway is the Indole -3- pyruvate pathway or the IPyA pathway that utilizes indole-3-pyruvic acid (IPyA) as its precursor molecule (Enders and Strader, 2015). The bacterial genera expending this pathway include *Bacillus, Pseudomonas, Bradyrhizobium, Azospirillum, Rhizobium* and*Enterobacter*. Tryptophan as precursor molecule is often supplemented in the culture media to enhance the production of IAA by bacterial cells. Tryptophan is converted into Indole Pyruvic acid IPA which changes to indole-3-acetaldehyde by the process of decarboxylation by indole-3-pyruvate decarboxylase enzyme (Costacurta et al., 1994). Genes encoding these enzymes if inactivated can hinder the production of IAA remarkably. Other pathways for IAA production includes Indole-3-Acetamide pathway (Persello-Cartieaux et al., 2003) exhibited by *Rhizobium fredii*and *B. japonicum* (Sekine*et al.,* 1989), tryptamine pathway shown by *Bacillus cereus* (Perley and Stowe, 1966) and tryptophan side-chain oxidase pathway that has only been demonstrated by *Pseudomonas fluorescens* (Oberhänsli et al., 1991). IAA is regulated by *ipd C*gene which increases its expression in the presence of increased quantity of IAA in the bacterial growth media (Somers et al., 2005). In addition, IAA production has also been found to be associated with quorum sensing regulatory genes (Chalupowicz et al., 2009). Indole Acetic acid production has been reported to stimulate root growth and total root area effectively and faster. Strains of *Pseudomonas* specie were found to produce indole acetic acid with the concentration ranging from 5-10mg/ml which induced root and shoot growth in *Beta vulagris*(Loper et al., 1986). IAA is also a significant signaling molecule in microbes that other than inducing quorum sensing promotes extensive growth, for example *Saccharomyces cerevisiae*, protects against stress along with increased production of EPS, LPS and biofilm formation. This has been demonstrated by *E.coli* (Bianco et al., 2006).In plant-bacteria symbiotic associations, auxin plays a key role in nodule formation, initiation and differentiation. In some cases, the bacterial auxin produced by *Rhizobia* meddles with plants auxin transport and alters plant's auxin homeostasis in return (Mathesius, 2008). Typically, nodulated roots require increased auxin as compared to the non-nodulated ones with increase in bacterial N_2 fixation ability as well (Hunter, 1989). Plant growth promoting bacteria increase root surface, and root hair aiding the plant to efficiently take up minerals

and nutrients. These plant growth promoting bacteria are also involved in phytostimulation (Dobbelaere et al., 2003).

MATERIALS AND METHODS

Sample Collection & Bacterial isolates

Soil sample was obtained from the rhizospheric region of the wheat (*Triticumaestivum var*. Uqab-2000) plant from local fields of Central Park, Lahore local. Ten purified strains were isolated from the wheat soil sample and proceeded for IAA screening.

Screening of Bacterial Isolates for IAA Production

For the initial screening of indole acetic acid producing bacteria, the purified bacterial strains were inoculated in the LB broth medium at pH 6 along with 0.1 µg/ml L-tryptophan supplemented in the broth (Loper and Schroth, 1986).The inoculated broth was incubated at the 37 °C for 24-48 hours. After incubation, the culture was centrifuged at 13,000 rpm for 20 minutes to obtain supernatant and cell pellet. The pellet was discarded and the supernatant was treated with Salkowski reagent in 2:1 ratio. In 2ml supernatant, 1 ml Salkowski reagent was added and stored in a dark place for half an hour for a color change to appear (Holt *et al.*, 1994). The treated supernatant's optical density was determined at 530 nm absorbance for auxin production and the results were noted for all the ten isolates (Fig 2).

Characterization of IAA Producing Isolated Strains

Five IAA producing isolated strains (Fig 1) were studied for its morphological and biochemical characteristics (Table 1 & 2). A series of biochemical tests were performed for each isolate individually including oxidase test, catalase test, citrate utilization test, urease test, DNase agar test, MP-VP test, motility test, H_2S and nitrate reduction tests (Fig 3&4).

Effect of pH & Temperature on IAA Production

LB broth was prepared and was supplemented with 500µg/ml concentration of L-tryptophan. The pH of the media was adjusted to 5, 6, 7 and 8 respectively. Purified bacterial strains were inoculated in the LB broth & the culture was centrifuged at 13,000 rpm for 20 minutes to obtain supernatant. The pellet was discarded and the supernatant was treated with Salkowski reagent in 2:1 ratio. In 2ml supernatant, 1 ml Salkowski reagent was added and stored in a dark place for half an hour for a color change to appear.The treated supernatant's optical density was determined at 530 nm with a spectrophotometer for auxin

production and the results were noted for all the five isolates and two replicates per strain.

For temperature optimization, the purified bacterial strains were inoculated in the LB broth medium adjusted at pH 6 along with 500µg/ml concentration of L-tryptophan supplemented in the broth. The inoculated broth was incubated at different temperature ranges (25°C, 30°C, 37°C and 40°C) for 42-52 hours and Salkowski test was repeated as mentioned for pH optimization.

Statistical Analysis

All the experiments were carried out thrice and the results were statistically analyzed. Results were determined by calculating the mean, standard deviation and ± standard error for the replicates. The results were also presented in tabular form as well as graphically for better visual representation.

RESULTS

Morphological Characterization of IAA Producers

On the basis of size, colonies were classified as small to moderate having circular and irregular shape with entire, lobate and undulate margins on LB agar plates (Fig 1). Apart from colony size, shape, margins other characters such as elevation, color, texture and optical properties were also observed for all the five isolated and purified strains (Table 1).

Biochemical Characterization of IAA Producers

After morphological characterization, a series of biochemical tests were performed for biochemical characterization of the isolated strains from the rhizosphere of *Triticum aestivum*plant. The tests included oxidase test, catalase test, urease test, citrate utilization test, indole test, methyl red, Voges-Proskauer test, H_2S production test and motility test (Table 2). All the strains showed positive results for oxidase, catalase and methyl red test (Fig 3 & 4). Except AUX-G all strains demonstrated positive results for indole test. For urease and citrate utilization test, AUX-Y and AUX-G were negative for urease test and rest were positive whereas for citrate utilization test the strains labeled AUX-W and AUX-M were positive only. AUX-G showed motility when inoculated in SIM Medium where rest of the four bacterial strains appeared to be non-motile. For H_2S gas production test, AUX-M strain showed back coloration in the medium indicating a positive result. AUX-W was positive for DNase agar test after observing the precipitation on the agar plate.

Effect Of pH and Temperature on IAA Production

All strains showed the maximum IAA production at pH 6 with slight variations. AUX-G (36.75 µg/ml) produced the maximum IAA followed by AUX-M (33.6 µg/ml) and W.W (31.6 µg/ml). The least IAA production was shown by AUX-Y (4.94µg/ml) at this pH.At pH 7 and pH 8 the strains showed a decrease in IAA production indicating that basic pH was not suitable for the growth of these isolates. AUX-G (26.8 µg/mol), AUX-M (22.85µg/ml) and AUX-O (20.28 µg/mol) showed maximum IAA production at pH 7, and AUX-Y (2.82 µg/ml) the least. At pH 5 AUX-G (20.4 µg/ml) and AUX-M (20.05 µg/mol) recorded the maximum IAA production and AUX-Y (2.7 µg/ml) the least. At pH 8 all the strains were restrained from producing high IAA levels where AUX-G (26.7 µg/mol) managed to produce the highest IAA and AUX-Y (1.79 µg/mol) with the least IAA levels (Fig 5).

At different temperature (25° C, 30° C, 37° C, and 40° C) all of the strains showed highest IAA production at 37 °C where the most notable strain was AUX-M (33.6 µg/mol) followed by AUX-W (33.5µg/mol) and AUX-G (31.5 µg/mol). Least IAA production was shown at 25°C suggesting that lower temperatures did not favor the bacterial growth at all. At 30° C strains showed similar results to 37° C with slight variations but the IAA levels started to decline with an increase in temperature (Fig 6).

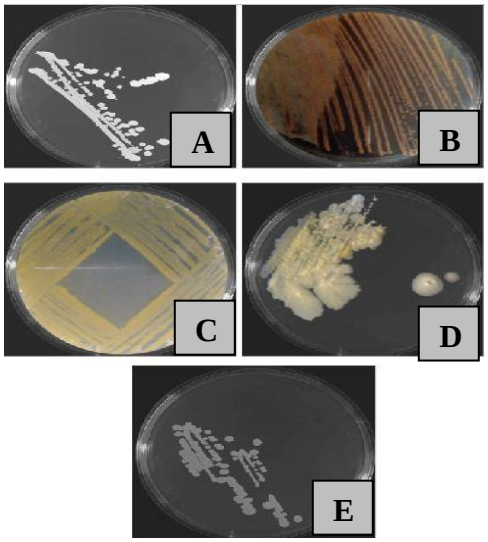

Fig. 1: Purified bacterial colonies isolated from T*riticum aestivum* rhizosphere where image A shows AUX-W, B shows AUX-O, C shows AUX-Y, D shows AUX-M and E shows AUX-G.

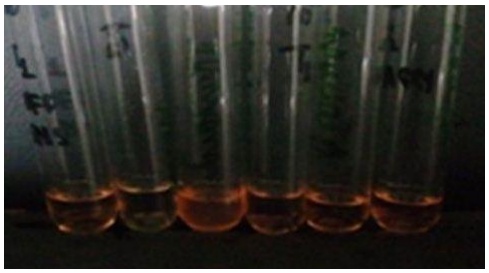

Fig 2: Screening of bacterial strains for IAA production. Development of pink color indicates all isolates as positive for IAA production.

Fig 3: Biochemical Characterization IAA producing bacteria. The image shows a positive catalase test for all the five isolates tested positive for IAA production.

Fig 4: Biochemical characterization of IAA producing bacteria. The image on right shows a positive Citrate utilization test indicated by color change of slants from green to blue. On left is the image of positive Indole Test and H_2S production in SIM Media.

Table1: Morphological Characteristics of IAA producing bacterial strains

Sr. No.	Characteristics	AUX-W	AUX-O	AUX-Y	AUX-G	AUX-M
1.	Colony Texture	Smooth	Smooth	Smooth	Smooth	Smooth
2.	Colony Size	≤4mm	≤6mm	≤5mm	≤4mm	≤8mm
3.	Elevation	Raised	Flat	Flat	Raised	Umbonate
4.	Margin	Lobate	Entire	Entire	Lobate	Undulate
5.	Pigment	White	Orange	Yellow	Grey	White
6.	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
7.	Colony Shape	Irregular	Circular	Circular	Circular	Irregular

Table 2: Biochemical Characteristics of IAA producing bacterial strains

Sr. No.	Biochemical Test	AUX-W	AUX-O	AUX-G	AUX-Y	AUX-M
1.	Oxidase	Positive	Positive	Positive	Positive	Positive
2.	Catalase	Positive	Positive	Positive	Positive	Positive
3.	Citrate utilization	Positive	Negative	Positive	Negative	Positive
4.	Urease	Positive	Positive	Negative	Negative	Positive
5.	DNase Agar Test	Positive	Negative	Negative	Negative	Negative
6.	Methyl Red	Positive	Positive	Positive	Positive	Positive
7.	Voges-Proskauer	Negative	Negative	Negative	Negative	Negative
8.	Indole	Positive	Positive	Negative	Positive	Positive
9.	H_2S Production	Negative	Negative	Negative	Negative	Positive
10.	Motility	Negative	Negative	Positive	Negative	Negative
11.	Nitrate Reduction	Negative	Negative	Negative	Negative	Negative

DISCUSSION

Out of ten isolates that were collected from the soil rhizosphere of *Triticum aestivum*, 5 were screened positive for IAA production and were named as AUX-W, AUX-O, AUX-G, and AUX-Y and AUX-M respectively. The Indole acetic acid production ability of the isolated strains was qualitatively determined by the Salkowski reagent test. Salkowski reagent was used as the primary test in the detection of IAA producing strains isolated from the rhizosphere as it detects the IAA precursor Indole-3-pyruic acid (IPyA) and other indolic compounds (Rahman et al., 2010).

Different pH range was employed for the optimization of Indole acetic acid production by the five isolates that were screened positive for IAA. The strains showed maximum activity of IAA production at pH 6 among which the most notable strains were AUX-G (33.7 µg/ml) and AUX-M (33.65 µg/ml) with highest IAA production. With an increase in pH there was a considerable decrease in the IAA production that indicated the lower pH of the *Triticum aestivum* rhizo spheric soil region from where these bacteria

were isolated. *Paenibacillus* sp. was found to have a higher IAA production activity at acidic pH of 6 as compared to pH 7 which was decreased to 42% (Acuña et al., 2011). It was also found that the acidic pH accelerated the bacterial growth that was responsible for increase in IAA levels. The pH below above 6 showed a low bacterial growth, hence less IAA production (Fig 7); (Table 3).

The effect of temperature was also studied on the production of IAA by bacterial isolates. All strains showed maximum IAA producing activity at 37°C temperature. The least production was associated with a decreased temperature i.e. 25° C where AUX-W (2.37 µg/ml) showed the least IAA levels. *Streptomyces atrovirens* has an optimum temperature for auxin production at 30° C (Abd-Alla et al., 2013). In earlier studies, IAA producing bacteria have been found to show maximum IAA production between 30° C to 37° C temperature range above and below which there was a reduction found in the IAA levels. Such species include *Streptomyces viridis, Pantoea agglomerans, Bacillus megaterium, Lactobacillus case* and *Bacillus licheniformis* which showed maximum IAA production at 37 °C (Ozdal et al., 2017). The increase in bacterial growth and the IAA levels was also noticed to be proportional. Hence, at 37 °C the bacterial growth increased that consequently increased the IAA production. This shows that the temperature above and below 37 °C were not favorable of the growth of the bacteria that were producing IAA (Fig 8) (Table 4).

CONCLUSION

The study showed that IAA production was enhanced at 37° C temperature and the pH of 6. This indicates that plant growth promoting bacteria can yield higher IAA when their growth conditions are optimized favorably. Active bacterial multiplication was also correlated with high IAA production. Such advantageous effects of bacterial secondary metabolites can become a good candidate for bio-fertilizers and bio-control agents in future replacing the increasing use of agrochemicals with agricultural cost-effective products. Such properties of bacteria can be exploited for economical purposes where their inoculations can lead to an improved and enhanced crop yield.

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