ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 2 Issue 3 March 2019

Research Article

Detection of Plant Growth Promoting Activities among *Azotobacter* sp. Isolated from the Soil Receiving Pulp and Paper Mill Effluents

Mohd Musheer Altaf* and Abdul Malik

Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India

***Corresponding Author:** Mohd Musheer Altaf, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India.

Received: January 04, 2019; **Published:** February 15, 2019

Abstract

Plant growth promoting rhizobacteria (PGPR) are recognized to improve plant development through different methods (direct and indirect). In this study, 15 isolates of *Azotobacter* sp., were withdrawn through different rhizospheric soil, which had continuously been irrigated with wastewater from pulp and paper mill industries. These isolates were subjected to *In vitro* screening for their plant growth promoting characters such as secretion of indole-3 acetic acid (IAA), siderophore, phosphate solubilization, ammonia (NH_3), hydrogen cyanide (HCN), seed germination, antifungal activity as well as nitrogenase activity. IAA and ammonia production was reported in all the isolates. 26.6% isolates solubilized tricalcium phosphate, whereas 80% isolates showed siderophore production. HCN secretion along with antifungal activity was demonstrated by 33.3 and 60% isolates respectively. All the isolates were also examined for quantitative production of IAA, siderophore, phosphate solubilization, antifungal and nitrogenase activity. IAA production was highest (10-25.6 µg mL^{-1}) at the concentration of 50-500 µg mL^{-1} tryptophan. Twelve *Azotobacter* isolates were found to solubilize phosphate. Isolates Az-11 and Az-12 could solubilize maximum phosphate (26.9 µg mL^{-1}). The level of siderophore production in *Azotobacter* isolates is found to be 14.6µg mL^{-1}. All the *Azotobacter* isolates were positive for seed germination test. Seven *Azotobacter* isolates showed nitrogenase activity in which isolate Az-15 showed highest (274.12 ± 6.50 nmoles of $C_2 H_4$ hr^{-1} mg^{-1} of protein). Nine isolates of *Azotobacter* showed broad-spectrum antifungal activity against *Fusarium oxysporium*, one or more species of *Aspergillus* and *Alternaria*. Moreover, assessment of the isolates depicting different plant growth promoting (PGP) characters on soil-plant system under stress conditions (pulp and paper mill effluents) and molecular mechanism need to be studied.

Keywords: PGPR; IAA; Pulp and Paper Mill Effluents; Gas Chromatography; Nitrogenase Activity

Introduction

Currently, substantial interest has been shown towards industrial wastes that are regularly released on earth or into different water bodies. It is anticipated that the industrial activities will accelerate with the pace of development. This would have adverse impact on agriculture and would cause environmental degradation. The application of industrial effluents for watering agricultural fields is a worldwide phenomenon. This system is inexpensive way for wastewater treatment and disposal along with providing nutrient rich water to farmers [1-3]. India, (a tropical country) shortage of water supply and exhaustion of ground water resources forced to search other irrigation sources. The insufficient irrigation water supply can be increased by using wastewater from pulp and paper industry [4,5].

Pulp and paper manufacturing business is well-known contaminant of the environment. It has been labeled as one of the 6th most polluting industry in the world due to the release of large amount of vastly coloured and contaminated wastewater in the surroundings [6]. It positioned 3rd on the planet in terms of ground water extraction following primary metal and chemical industries. It is predicted that approximately 100-250m^3 of water is needed to produce one tonne paper which ultimately produces 75-225 m^3 of wastewater [6,7]. The pollutants associated with pulp and paper mill effluents are organic matter, inorganic dissolved solids, chlorinated compounds (measured as absorbable organic halides, AOX), fatty acids, tannins, resin acids, lignin, sulphur and sulphur compounds, thermal components as heat, floating solids and microbes [8].

Plant growth promoting rhizobacteria (PGPR) are a diverse assembly of bacteria that are found in association with roots, which enhances plant growth and development directly and indirectly. In previous decade a huge number of bacteria together with species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Burkholderia, Bacillus, Rhizobium and Serratia* accounted to augment plant growth [9,10]. The direct encouragement by PGPR involves through facilitating the plant with a plant growth promoting compound produced by the bacterium itself or assisting the absorption of different plant nutrients from the surroundings. The indirect growth of plant occurs through decreasing the harmful effects produced by plant pathogens.

Moreover, the plant growth promoting beneficial rhizospheric bacteria should have the capacity to live on and inhabit in the rhizosphere. Sadly, the communication among associative PGPR and plants can be unbalanced. The superior results achieved *In vitro* cannot always be repeated in field conditions [11-13]. The inconsistency within the performance of PGPR might be due to a variety of non-living reasons that influence their growth and applied their effect on the plant. The ecological causes comprise a range of abiotic issues such as type of weather, quality of irrigation water, soil characteristics as well as activity of the indigenous microbes.

Although impact of the use of industrial wastewaters on soil, soil microflora, plants and water bodies has been evaluated, database is not adequate to elucidate the influence of long-term use of industrial effluents to agriculture as well as environment as a whole. Therefore, as apparent the above limitation, the present research was planned to evaluate *Azotobacter* sp. for their multiple plant growth promoting activities and to initiate preliminary work on paper mill wastewater pollution.

Materials and Methods

Study area

In India, Saharanpur district (Uttar Pradesh) lies in Indo-Gangetic region between the parallels of 29.97°N latitude and 77.55°E. Famous for agro based industries and one of the main industries is of pulp and paper. The discharge from these industries is being used for the irrigation purposes. As a result of long term irrigation from paper mill polluted wastewater, the region was chosen for the research.

Sample collection

Pulp and paper mill effluent portions were gathered arising out of Star Paper mills at Saharanpur (India) in a 5-l sterile plastic container. Soil specimens were obtained from the 15 cm depth in the agricultural fields irrigated with the Pulp and paper mill wastewater. The composite sample was made for analysis by combining five different field specimens. The specimens of soil were transferred to sterilized polythene bags using sterilized spatula. A total of four samplings were carried out.

Physico-chemical and biological analysis of soil and pulp and paper mill effluent samples

Several physicochemical tests on soil and pulp and paper mill effluent samples were performed for determination of pH, Total solids, Soil texture, Organic C, Organic matter, Water content, Phosphorus, Potassium, Sulphate, Sodium, Chloride, Calcium, Magnesium, Nickel, Copper, Cadmium, Zinc, Chromium, Total Aerobic Heterotrophs, Actinomycetes, Fungi. All these tests were performed according to standard methods [14].

Isolation and characterization

Soil samples were serially diluted in sterile normal saline solution and 0.1 mL sample was plated on Ashby's medium plates and were incubated at 28 ± 2°C for 24-72 h. After incubation on Ashby's medium, colonies showing brownish pigmentation were considered *Azotobacter* sp. The selected isolates were further subjected to biochemical characterization using standard methods [15]. Arbitrarily chosen isolates were preserved in glycerol at –20°C and tentatively named as (Az) in this study.

In vitro screening of bacterial isolates for their plant growth promoting (PGP) activities

Assay for indole-3 acetic acid (IAA) production

IAA secretion by selected isolates was identified by the method of Bric., *et al.* [16]. A (10^7 CFU mL^{-1}) of culture was inoculated in 25 mL Luria Bertani broth containing 0, 50, 100, 200 and 500 μg mL^{-1} of tryptophan. The flasks were incubated for 72-96 h at 28 ± 20^{0}C on rotatory shaker (100 rpm). The Cultures were agitated at 10,000 rpm for 15 min and 2-3 drops of O-phosphoric acid and 2 mL of Salkowsky reagent was added to the supernatant and incubated at room temperature for 25 minutes. Using pure IAA (Hi-media) as standard, absorbance of pink colour was read at 530 nm and IAA was quantitated. Each individual experiment was repeated three times.

Ammonia production

Test isolates were evaluated for the generation of ammonia within peptone water. Newly grown cultures were inoculated in test tube containing 10 mL peptone water and incubated at 28 ± 2° C for 48-72 h. Nessler's reagent (0.5 mL) was added in each tube

after incubation. Production of brown to yellow colour was a positive test for ammonia generation [15].

Hydrogen cyanide production

The test isolates were evaluated for hydrogen cyanide (HCN) production [17]. Bacteria were streaked on modified nutrient agar plates containing 4.4 g glycine l^{-1}. A Whatman filter paper no. 1 dipped in 2% sodium carbonate in 0.5% picric acid solution was kept on the top of the plate. Plates were sealed with parafilm and incubated at 28 ± 2° C for 4 days. Evolvements of orange to red colour specify HCN production.

Phosphate solubilization by test bacteria

The phosphate solubilization was quantified by determining the soluble phosphate secreted in the culture medium, by the method of King [18] as explained by Gaur [19].100 mL of sterile Pikovskaya's broth amended with 0.5% tricalcium phosphate was separately inoculated with test culture (10^7 cells mL^{-1}). The uninoculated medium used as control. At specific time period throughout growth, 10 mL of culture was taken from each flask and centrifuged at 10,000 rpm for 30 minutes. Ten mL of supernatant was poured to tubes. To each sample, 10 mL of ammonium molybdate and 0.25 mL of chlorostannous acid was added and volume was adjusted to 50 mL with distilled water. The developed blue colour was examined at 600 nm. Amount of phosphate solubilized was calculated using the calibration curve of KH_2PO_4.

Siderophore production

Selected bacterial isolates were further assayed for the quantification of catechole-type siderophores using the method described by Alexander and Zuberer [20]. One mL (10^7 CFU mL^{-1}) of each culture was inoculated to 30 mL of Modi medium broth and incubated at 28 ± 2°C for three days. The completely developed cultures were centrifuged at 10,000 rpm for 20 minutes. The supernatant was adjusted at pH 2.0 with diluted HCl. Equal quantity of ethyl acetate was added twice in a separating funnel and separated, the resultant 60 mL ethyl acetate fraction was taken. Five mL ethyl acetate fraction was mixed with 5 mL of Hathways's reagent. The absorbance was read at 700nm for benzoate and 560 nm for salicylates. The concentrations of benzoates and salicylates were calculated with the help of standard 2-3 Dihydroxy benzoic acid (1-30 µg mL^{-1}) and Salicylic acid (1-35 µg mL^{-1}) respectively.

Petri dish bioassay

In vitro seed germination test was performed on the seeds of *Vigna radiata* var. PDM-139 by applying the method as described by Shende and Apte [21] and Elliot and Lynch [22] with slight alterations. The seeds of *vigna* sp. were surface sterilized by 0.1% $HgCl_2$ solution for 3 min. and washed with sterile distilled water. The seeds were kept in 70% ethanol for 1 minute. Seeds were again washed at several instances with sterile distilled water. Surface sterilized seeds were inoculated with 10^7 cells mL^{-1}. The seeds were kept for 30 minutes in the culture medium. These seeds were placed on petri plates containing soft agar. Each plate contained 5-7 seeds and each bacterial inoculation was repeated three times. These plates were incubated at 28 ± 2°C for three days or more. A control plate of soft agar without bacterially treated seeds was also run. Seedling growth (root and shoot length) was determined after five days of incubation.

Fungal inhibition growth assay

The agar well diffusion method as described by Mehmood., *et al*. [23] was used. The test filamentous fungus used were *Fusarium oxysporium, Aspergillus* sp. and *Altenaria* sp. procured from division of Plant Pathology, IARI, Pusa New Delhi. The test fungus was fully grown on Sabouraud dextrose agar (SDA, Hi-media, India) slants. The spores were scraped and suspended in 10 mL of sterile normal saline solution. 0.1mL of diluted spore suspension (≈10^5 CFU mL^{-1}) of the fungi was spread on Sabouraud dextrose agar plates. Wells of 8 mm diameter were punched into the agar medium and filled with 200 µl (10^7 CFU mL^{-1}) of bacterial culture. Uninoculated control was kept for comparison of results. The plates were incubated for 5-6 days at 28 ± 2°C. The antifungal activity was assessed by measuring the zone of inhibition against test fungi.

Nitrogenase activity (Acetylene Reduction Assay) in bacterial cultures

The Nitrogenase activity was determined in the bacterial samples of (*Azotobacter*) as described by Hardy., *et al*. [24,25]. The slant of appropriate medium (*Azotobacter*-Jensen's N_2 free medium) was prepared. Loop full of culture were streaked on the slants and incubated at 28 ± 2°C for 2-7 days. The cotton plugs were changed by air tight subaseal. After incubation 10% of the total volume of the atmosphere of each tube was replaced with acetylene. Tubes are then incubated at 28 ± 2°C for 24 h.1 mL of gas sample was collected and ethylene (C_2H_4) concentration was calculated on a gas chromatograph (GC 5765, NUCON, New Delhi). Ethylene detection was based on the retention time and was quantified by comparing with the standard curve drawn with pure ethylene gas. All cultures were assayed in triplicate. The Nitrogenase activity was expressed as nM (nanomoles of C_2H_4 produced hr^{-1} mg^{-1} protein).

Protein estimation

The protein volume of bacterial isolates (*Azotobacter*) was predicted by the process given by lowery., *et al.* [26]. Bacterial cells were collected in 2mL 2N NaOH (Sodium Hydroxide) solution. Cell suspension was kept in boiling water bath for 10 minute. After that it was cooled and neutralized with 2 mL 2N HCl. Stock Bovine Serum Albumin (BSA, Hi-media, India) solution and dilutions were freshly prepared for the standard curve. Five mL of copper reagent (Sodium Carbonate 4% w/v, Sodium Potassium Tartrate 4%w/v, and Copper Sulphate 2% w/v, mix all in the ratio of 100:1:1) was added to each sample. The test tubes were mixed well and allowed to stand for 10 minutes at room temperature. 0.5 mL Folin's reagent mixed in the tubes and incubated at room temperature for 30 minutes. At the development of blue colour O. D. was read at 660 nm. The protein concentration was calculated with help of 500 μg mL^{-1} BSA (Bovine Serum Albumin) solution. The amount of protein was expressed in mg gm^{-1}. The experiment was done in triplicate.

Result and Discussion

The physicochemical and microbiological analysis of the agricultural soils and treated pulp and paper mill effluents employed for irrigation are presented in Table 1. The mean pH of treated pulp and paper effluent was found to be 7.8 ± 0.5. The value of TS (1980 mg l^{-1}) exceeded the ISI tolerance limit, which might affect the water quality of receiving wetland and thus also found unfit for irrigation purposes. The concentration of phosphorous, potassium, sulphate, sodium, chloride, calcium and magnesium are in conformity with other authors [27,28]. Baruah., *et al*. [29] accounted that most of the parameters and components of the pulp and paper mill effluent were 4-50 times higher than the WHO permissible limit, specifically in respect to pH, alkalinity, chlorides, sulphates, calcium and residual chlorine which ultimately lead to the pollution of wetland receiving effluent. The concentration of the phosphorous, potassium, sulphate, sodium, calcium and magnesium in soil is found to be high. The higher value was due to addition of nutrients through effluent irrigation over a long period of time [5].

The soil texture investigation demonstrated it to be clay soil having pH 8.4 ± 0.25. The pH of the soil was similar to those of Santosh [27] who documented the pH of agricultural soil ranging from 7.5 – 8.6. Moreover there is no significant change in pH over long time due to application of paper mill effluent, as it does not have any tough salt which on dissociation could alter the pH significantly [1]. The organic carbon and organic matter of soil is found to be higher than the normal soil. The increase in soil organic carbon and organic matter might be attributed to the continuous accumulation of organic matter in the course of effluent irrigation.

The entire metals (Ni, Cu, Cd, Zn and Cr) were estimated in the pulp and paper mill effluent and soil sample by atomic absorption spectrophotometer (Table 1). The analysis of these test samples revealed the presence of Ni, Cu, Cd, Zn and Cr. The levels of Ni, Cu, Cd, Zn and Cr were detected in high amount in soil compared to paper mill effluent. Most of the detected metals are believed to be poisonous to the biological system. Bansal [30] accounted mean level of heavy metals in soil irrigated with sewage water to be 34-36 μg g^{-1} for Cu, 96-100 μg g^{-1} for Zn and 770-8300 μg g^{-1} for Fe. Aleem., *et al*. [31] reported that agricultural soil watered by wastewater has an elevated level of metals in contrast with ground water or non polluted agricultural soil. They reported metal levels in the range of 862-1051μg g^{-1} for Fe, 36.4-48.5 μg g^{-1} for Ni, 32.5-42.3 μg g^{-1} for Cr, 25.4-29.6μg g^{-1} for Cu and 0.58 1.2 μg g^{-1} for Cd. Similar results were also reported by Kumar., *et al*. [5].

A variation in the population(s) of different groups of microflora in the soil following irrigation with pulp and paper mill effluent is summarized in table 1. The total aerobic heterotrophs in soil is 192 × 10^6 CFU g^{-1}, actinomycetes (81×10^3CFU g^{-1}) and fungi (16×10^3 CFU g^{-1}). Our results for the microbiological properties of paper mill effluent are comparable with those of Kannan and Oblisami [32], Chandra [33] and Thawale., *et al.* [34]. The high level of population of bacteria, actinomycetes and fungi in the soil might be due to the continuous addition of microflora from the effluent as these soils were irrigated with paper mill effluent since last 30-40 years. The increase in population might be due to enhancement in the accessible nutrient. Also, a good relationship among microbial biomass and soil C and N contents have been reported by Ross., *et al*. [35].

Isolation and biochemical characterization

On the basis of cultural, morphological and biochemical characteristics a total of 15 rhizospheric isolates were grouped into *Azotobacter* sp., as described in Bergey's Manual of Determinative Bacteriology [36]. General features of the test isolates are illustrated in table 2.

Plant growth promoting traits of test isolates

Evaluating results of PGP traits are depicted in figure 1. 100% *Azotobacter* isolates were found positive for IAA and ammonia production. 80% *Azotobacter* isolates produces siderophore whereas 26.6%, 33.3% solubilize phosphate and produce HCN respectively. However, only 60% displayed antifungal activity. In the present

Variable	Pulp and paper mill effluent (mg l^{-1})	Pulp and paper mill effluent irrigated Soil (mg kg^{-1})
pH	7.8 ± 0.5	8.4 ± 0.25
Total solids	1980 ± 1.84	ND
Soil texture	ND	Clay
Organic C (%)	1.68 ± 0.11	1.36 ± 0.03
Organic matter (%)	N. D	2.34 ± 0.07
Water content (%)	N. D	43 ± 6.13
Phosphorus	3.92 ± 0.34	41 ± 5.11
Potassium	139.1 ± 4.56	39 ± 5.0
Sulphate	192 ±7.08	47 ± 7.12
Sodium	94.4 ± 2.1	310 ± 1.14
Chloride	147 ± 3.45	ND
Calcium	275.2 ± 6.54	33.1 ± 3.98
Magnesium	43 ± 3.47	29.8 ±1.56
Nickel	0.83 ± 0.02	26 ± 1.93
Copper	3.5 ± 0.41	32.1 ± 2.63
Cadmium	0.12 ± 0.05	1.3 ± 0.07
Zinc	1.42 ± 0.25	79.6 ± 1.70
Chromium	3.02 ± 0.12	15.2 ± 0.49
Total Aerobic Heterotrophs	$125\times10^6 \pm 21\times10^6$ *	$192\times10^6 \pm 8.0\times10^6$*
Actinomycetes	$49\times10^3 \pm 4.1\times10^3$	$81\times10^3 \pm 4.5\times10^3$
Fungi	$19\times10^2 \pm 3.0\times10^2$	$16\times10^3 \pm 2.0\times10^3$

Table 1: Physicochemical analysis and biological characteristics of wastewater and soil.

*Each value is the mean of three independent experiments carried out in duplicate

ND: Not Done, * Values are in CFU ml^{-1} and CFU g^{-1}, ± = Standard Deviation.

Morphology/Biochemical reactions	Azotobacter spp.
Gram reaction	Negative
Cell Shape	Rods
Polysaccharide Production	++
Nitrate reduction	93.33
Catalase test	100
Starch hydrolysis	53.33
Gelatin hydrolysis	26.66
Utilization of:	
Citrate	100
Glucose	53.33
Fructose	60.0
Lactose	53.33
Sucrose	66.66
Mannitol	46.66
Indole production	20.0
Methyl red	40.0
Voges proskauer	73.33
Oxidase	100

Table 2: Morphological and biochemical properties of the test isolates.

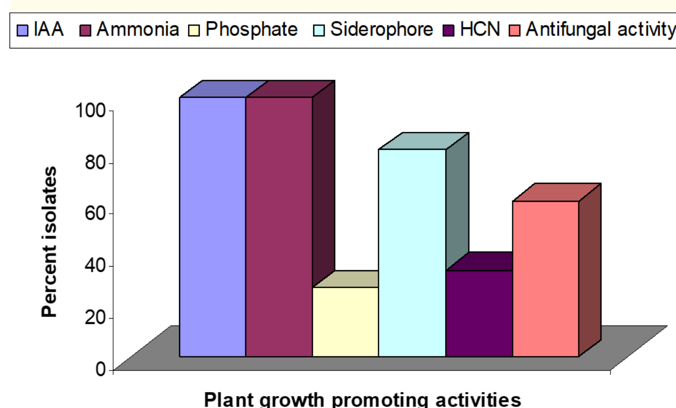


Figure 1: Plant growth promoting activities of *Azotobacter* isolates

analysis IAA secretion in the rhizobacterial isolates are in conformity with results of Ahmad., *et al*. [37].

In the present investigation, potential PGPR (*Azotobacter*) was able to solubilize phosphorous is shown in (Figure 2). Level of phosphate solubilization by *Azotobacter* isolates vary from 3.2-26.9 µg mL^{-1} and Az-11 and 12 solubilize maximum phosphate (26.9 µg mL^{-1}). High salt concentration, pH and temperature hamper phosphate solubilization capacity of rhizobacteria [38]. Mechanisms of phosphate solubilization include organic acid and enzymes production which help in phosphate solubilization [39].

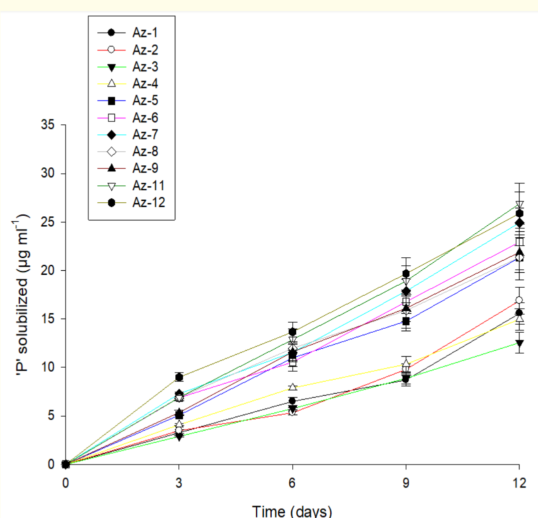
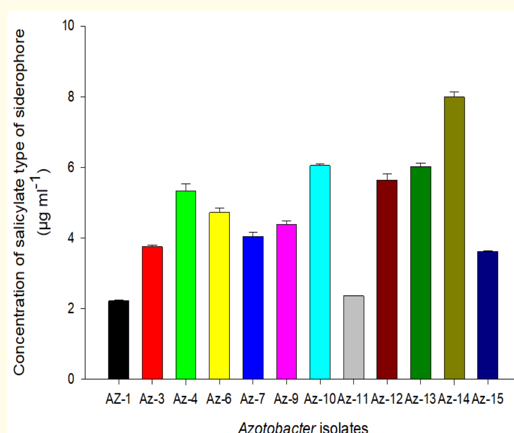


Figure 2: Phosphate Solubilization by *Azotobacter* isolates.

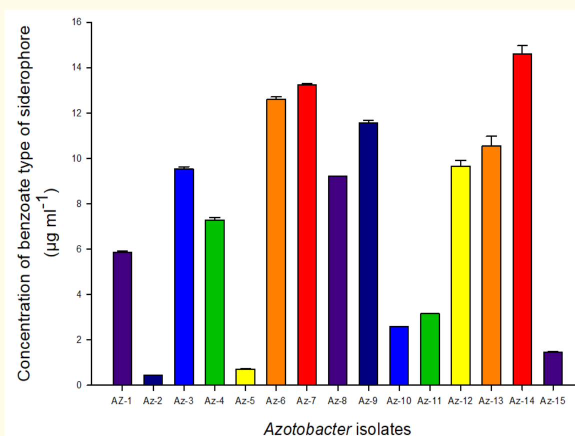
Siderophores openly encourages the biosynthesis of other antimicrobial components through augmenting the availability of these minerals to the bacteria. Antibiotic and siderophores additionally work as stress factor or singles as well as local and systematic host resistance [39]. Therefore, we screened all the *Azotobacter* isolates for siderophore production (Figure 3). All the isolates are found to produce both benzoate and salicylate type of siderophore. Our findings on siderophore production are corroborated with Chennappa., *et al.* [40].

Quantitative estimation of production of IAA in *Azotobacter* sp. is depicted in figure 4. IAA produced by the bacterial isolates was detected in the medium with 0, 50, 100, 200 and 500 µg mL^{-1} tryptophan concentration after 24-30 h of incubation. The amount of IAA produced differed among isolates and were concentration dependent. All the *Azotobacter* isolates produced good amount (6.7-24.6µg mL^{-1}) of IAA. Ahmad., *et al.* [37] has reported increased level of IAA production in the presence of precursors in *Azotobacter*.

In vitro inhibition of fungal growth by *Azotobacter* isolates against *Fusarium oxysporum*, *Aspergillus* and *Alternaria* spp. was investigated by agar well diffusion method using whole cell broth (Figure 5-7). Among the phytopathogens, *Fusarium oxysporum* was found to be the most sensitive strain as compared to other test fungi. In contrast *Aspergillus* sp. was most resistant to test PGPR isolates. All nine isolates of *Azotobacter* inhibit the growth of *Fusarium oxysporum* and *Alternaria* sp., whereas eight isolates inhibit the growth of *Aspergillus* spp., while Az-1, 15 inhibited the growth of all three test fungal isolates. Similar findings of antifungal activity in rhizobacterial isolates have been reported by Chennappa., *et al.* [40].



(a)



(b)

Figure 3: Quantification of Catechol types of siderophores by Azotobacter isolates.
(a) Salicylate type (b) Benzoate type.

Influence of isolated rhizobacterial treatment on seedling growth of *Vigna radiata* var. PDM 139 was examined for 3-5 days on soft agar in petriplates (Table 3). All the *Azotobacter* isolates gave positive results for seed germination. Interestingly none of the test isolates adversely affect the seedling growth. Similar results were also reported for seed germination under the influence of rhizobacteria by Cakmakci., *et al.* [41].

We screened most of the *Azotobacter* isolates which were having potential of plant growth promotion for the presence of nitrogenase enzyme by acetylene reduction assay and data is given in table 4. Our observations revealed that the isolates were able to reduce acetylene under stress conditions and showed varying levels of acetylene reduction. One of the *Azotobacter* isolate (Az-15) produced 274.12 nmoles of C_2H_4 hr^{-1} per mg of protein. Our results are in tune with Gothwal., *et al.* [42]. The *Azotobacter* protects their

S. No.	Bacterial isolates	Ethylene produced (nmoles of C_2H_4 hr^{-1} per mg of protein)
1	Az-1	14.84 ± 1.23
2	Az-2	33.35 ± 2.15
3	Az-6	48.83 ± 2.89
4	Az-7	33.41 ± 2.56
5	Az-13	17.66 ± 1.49
6	Az-14	176.08 ± 4.50
7	Az-15	274.12 ± 6.50
*Values are mean of three replicates with ± SD.		

Table 4: Acetylene reduction assay of *Azotobacter* isolates from agricultural soil receiving pulp and paper mill effluents.

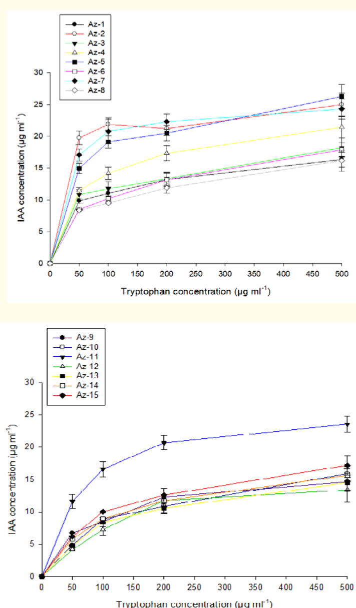


Figure 4: Concentration dependent IAA production by Azotobacter isolates.

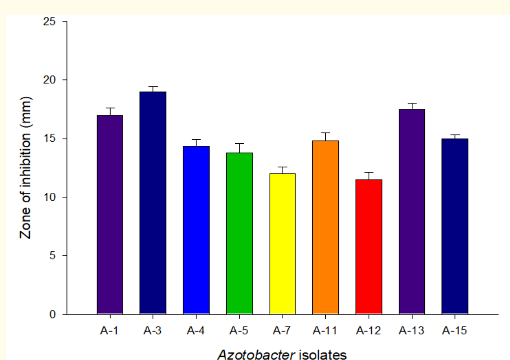


Figure 5: Antifungal activity of *Azotobacter isolates* against *Fusarium oxysporum*.

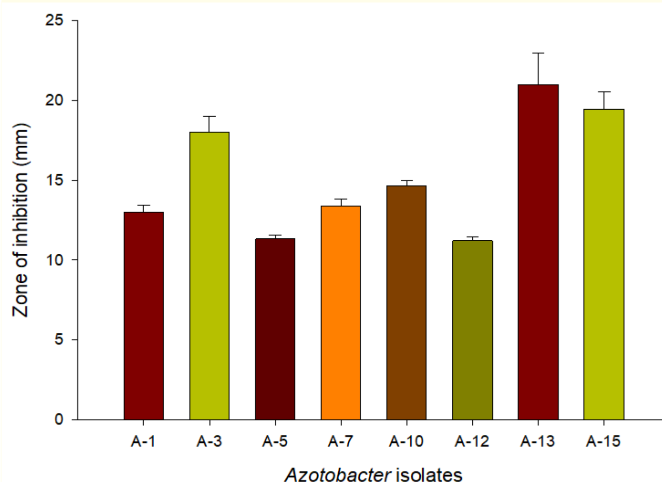


Figure 6: Antifungal activity of *Azotobacter isolates* against *Aspergillus* sp.

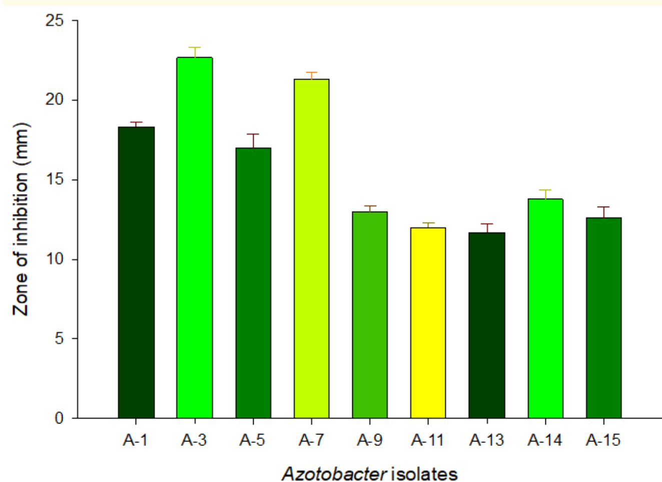


Figure 7: Antifungal activity of *Azotobacter* isolates *against Alternaria* spp.

nitrogenase from environmental stress by slime production [43].

The characteristic PGP features of rhizobacteria (*Azotobacter* sp.) were observed in the present study indicated that most of these isolate showed three or more PGP activities such as production of IAA, Ammonia, Siderophore, HCN, Solubilization of phosphate, seed germination and antifungal activity.

Bacterial isolates	Radicle Length (cm)	Plumule length (cm)
Control	5.60±0.85	6.66±0.98
Az-1	9.33 ± 0.57	12.86 ± 1.15
Az-2	8.60 ± 2.30	5.60 ± 1.70
Az-3	9.03 ± 2.55	13.00 ± 2.00
Az-4	11.06 ± 0.20	14.00 ± 0.00
Az-5	8.33 ± 2.08	11.00 ± 2.64
Az-6	8.56 ± 1.06	11.90 ± 2.97
Az-7	8.13 ±2.82	7.00 ± 2.00
Az-8	9.00 ± 1.00	12.66 ± 1.52
Az-9	10.23 ± 1.59	14.66 ± 1.52
Az-10	6.33 ± 1.52	9.76 ± 1.30
Az-11	8.73 ± 0.64	13.66 ± 0.57
Az-12	10.73 ± 1.41	11.66 ± 0.57
Az-13	10.80 ± 1.25	13.43 ± 0.60
Az-14	9.16 ± 1.52	12.83 ± 0.80
Az-15	8.86 ±1.15	13.86 ± 1.20

Table 3: Effect of *Azotobacter* inoculation on seed germination after 5 days of Growth.

*Values are mean of three replicates with ± SD.

Conclusion

Present finding indicates that the pulp and paper mill effluent contains various kinds of pollutants (organic matter, inorganic dissolved solids, chloride, sulphate, suspended solids and microorganisms). This finding demonstrates that the bacterial population of the contaminated soil can be better used for the development of bioinoculant/biofertilizers because the bacterial population especially *Azotobacter* sp. has exhibited higher levels of plant growth promoting (PGP) activities under stress conditions (soil receiving pulp and paper mill effluents).

Bibliography

1. Chhonkar PK., *et al*. "Impact of Industrial Effluents on Soil and Agriculture-Indian Experience: Part I-Distillery and Paper Mill Effluents". *Indian Journal of Environmental Health* 59 (2000): 350-361.
2. Phukan S and Bhattacharyya KG. "Modification of soil quality near a pulp and paper mill". *Water, Air, and Soil Pollution* 146 (2003): 319-333.
3. Jaramillo MF and Restrepo I. "Wastewater reuse in agriculture: a review about its limitations and benefits". *Sustainability* 9 (2017): 1734.
4. Oblisamy G and Palanisami A. "Studies on the effect of paper and sugars factory effluenton soil microflora and agricultural cropping system - scheme report". Seshasayee Paper Mill, Tamil Nadu Agricultural University, Coimbatore (1991): 297-305.
5. Kumar V and Chopra AK. "Ferti-irrigation effect of paper mill effluent on agronomical practices of Phaseolus vulgaris (L.) in two different seasons". *Communications in Soil Science and Plant Analysis* 45 (2014): 2151-217.
6. Kumar V., *et al*. "Assessment of phytokinetic removal of pollutants of paper mill effluent using water hyacinth (Eichhornia crassipes [Mart.] Solms)". *Environmental Technology* 39 (2017): 2781-2791.
7. Jayabalakrishnan RM., *et al*. "Treating Paper and Pulp Mill Effluent through Vermiculite Clay Mineral Barrier (VCMB) System". In: Proceedings of International Conference on Energy and Environment. (2009).
8. Ranganathan K., *et al*. "Assessment of water pollution in different bleaching based paper manufacturing and textile dyeing industries in India". *Environmental Monitoring and Assessment* 134 (2007): 363-372.
9. Kloepper JW., *et al*. "Free-living bacterial inocula for enhancing crop productivity". *Trends in Biotechnology* 7 (1989): 39-43.
10. Glick BR, *et al*. "Biochemical and genetic mechanisms used by plant growth promoting bacteria". Imperial College Press, London (1999): 267.
11. Chanway CP and Holl FB. "First year yield performance of spruce seedlings inoculated with plant growth promoting rhizobacteria". *Canadian Journal of Microbiology* 39 (1993): 1084-1088.
12. Zhender GW., *et al*. "Microbe-induced resistance against pathogens and herbivores: evidence of effectiveness in agriculture". In: Agarwal, A.A., Tuzun, S., Bent, E. (Eds.), Induced Plant Defenses Against Pathogens and Herbivores: Biochemistry, Ecology and Agriculture. APS Press, St Paul, MN (1999): 33.
13. Gouda S., *et al*. "Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture". *Microbiological Research* 206 (2018): 131-140.
14. APHA. "Standard methods for the examination of water and wastewaters, 19th edn, APHA AWWA WPCF, Washington DC (1998).

15. Cappuccino JC and Sherman N. In: Microbiology: A Laboratory Manual, New York (1993): 125-179.

16. Bric JM., *et al*. "Rapid in situ assay for indole acetic acid production by bacterial immobilization on a nitrocellulose membrane". *Applied and Environmental Microbiology* 57 (1991): 535-538.

17. Lorck H. "Production of hydrocyanic acid by bacteria". *Physiologia plantarum* 1 (1948): 142-146.

18. King JE. "The colorimetric determination of phosphorus". *Biochemical Journal* 26 (1932): 292- 295.

19. Gaur AC. "Physiological functions of phosphate solubilizing micro-organisms". In: Gaur, A.C. (Ed.): , Phosphate Solubilizing Micro-organisms as Biofertilizers. Omega Scientific Publishers, New Delhi 16-72.

20. Alexander DB and Zuberer DA. "Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria". *Biology and Fertility of Soils* 12 (1991): 39-45.

21. Shende ST., *et al*. "Influence of Azotobacter on germination of rice and cotton seeds". *Current Science* 46 (1977): 675-676.

22. Elloit LF and Lynch JM. "Pseudomonas as a factor in the growth of winter wheat (Triticum aestivum L.)". *Soil Biology and Biochemistry* 16 (1984): 69-71.

23. Mehmood Z., *et al*. "Indian medicinal plants: a potential source of anticandidal drug". *Pharmaceutical Biology* 37 (1999): 237-242.

24. Hardy RWF., *et al*. "The acetylene -ethylene assay for N2 fixation: laboratory and field evaluation". *Plant Physiology* 43 (1968): 1185-1207.

25. Hardy RWF., *et al*. "Applications of the acetylene-ethylene assay for measurement of nitrogen fixation". *Soil Biology and Biochemistry* 5 (1973): 47-81.

26. Lowry OH., *et al*. "Protein measurement with the Folin Phenol Reagent". *Journal of Biological Chemistry* 193 (1951): 265-275.

27. Santosh KS. "Effect of irrigation with paper mill effluent on the nutrient status of soil". *International Journal of Soil Science* 2 (2007): 74-77.

28. Mehdi UJ., *et al*. "Effect of pulp and paper mill effluent on seed germination and seedling growth of mustard (Brassica compestris), pea (Pisum sativum) and rice(Oryza sativa) seeds". Pollution Research 27 (2008): 437-442.

29. Baruah BK., *et al*. "Study on the effect of paper mill effluent on the water quality of receiving wetland". *Pollution Research* 4 (1996): 389-393.

30. Bansal OP. "Heavy metal pollution of soils and plants due to sewage irrigation". *Indian Journal of Environmental Health* 40 (1998): 51-52.

31. Aleem A., *et al*. "Impact of long term application of industrial wastewater on the emergence of resistance traits in Azotobacter chroococcum isolated from rhizospheric soil". *Bioresource Technology* 86 (2003): 7-13.

32. Kannan K and Oblisami G. "Impact of pulp and paper mill effluent on sugarcane soil". Paper presented at the fifth national convention of Environmental Engineers Madras, India (1989): 23-24.

33. Chandra R, *et al*. "Seasonal Bacteriological analysis of Gola river water contaminated with pulp and paper mill waste in Uttranchal, India". *Environmental Monitoring and Assessment* 118 (2006): 393-406.

34. Thawale PR., *et al*. "Characterisation of soil irrigated with pulp and paper mill wastewater for variation in physico-chemical, biological and enzymatic properties". I*nternational Journal of Environmental Technology and Management* 18 (2015): 207-217.

35. Ross DJ., *et al*. "Microbial biomass estimation in soils from tussock grasslands by three biochemical procedures". *Soil Biology and Biochemistry* 12 (1980): 375-383.

36. Holt JG., *et al*. In: Bergy's Manual of Determinative Bacteriology, ninth ed. Williams and Wilkins Pub., MD, USA (1994): .

37. Ahmad F., *et al*. "Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities". *Microbiological Research* 36 (2006): 1-9.

38. Rajankar PN., *et al*. "Study of phosphate solubilization efficiencies of fungi and bacteria isolated from saline belt of Purna river basin". *Research Journal of Agriculture and Biological Sciences* 3(2007): 701-703.

39. Ahemad M and Kibret M. "Mechanisms and applications of plant growth promoting rhizobacteria: current perspective". *Journal of King Saud University - Science* 26 (2014): 1-20.

40. Chennappa G., *et al*. "PGP potential, abiotic stress tolerance and antifungal activity of Azotobacter strains isolated from paddy soils". *Indian Journal of Experimental Biology* 54 (2016): 322-331

41. Cakmakci R., *et al*. "Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions". *Soil Biology and Biochemistry* 38 (2006): 1482-1487.

42. Gothwal RK., *et al*. "Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants". *Applied Ecology and Environmental Research* 6 (2007): 101-109.

43. Madigan MT and Martinko JM. "Brock Biology of Microorganisms". 11th edition, Pearson Prentice Hall Publishing Company (USA) (2006).

Volume 2 Issue 3 March 2019
© All rights are reserved by Mohd Musheer Altaf and Abdul Malik.