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Isolation and Characterization of Cellulolytic Nitrogen Fixing *Azotobacter species* from Wheat Rhizosphere of Khyber Pakhtunkhwa

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Abstract: Thirty two isolates of cellulolytic Nitrogen fixing *Azotobacter* species were isolated from seventy soil samples collected from Charsadda, Mardan, Peshawar, Swabi, Nowshera and Dir districts of Khyber Pakhtunkhwa. Among them, twelve isolates were chosen by using Congo red assay for further analysis and were considered as efficient with their cellulolytic zone ratios 1.23-2.71 (low to moderate activity) in Congo red assay. Cellulase activity of each selected isolates was quantitatively measured through Dinitrosalicyclic (DNS) calorimetric method. The results of both these methods were comparable. In contrast only one isolate showed maximum zone ratio of 2.71 by Congo red assay and minimum enzyme activity of 0.003 IU/mL, Nitrogen fixing activity of efficient *Azotobacter* isolates was detected by color changes in Glucose Nitrogen Free Mineral Medium (GNFMM) containing Bromothymol Blue and Phenate method. Blue color change of colonies and significant amount of NH_4-N of 11-94 mg/L released by isolates, indicated Nitrogen fixation. Phosphate solubilizing potential was checked by using Agar spot and pH drop methods. All these isolates can solubilize tricalcium phosphate in Pikovskaya's agar medium with solubility index 1.14-1.61 and can drop the pH of Pikovskaya's broth medium in the range 2.99-5.83. Indole Acetic Acid (IAA) producing and antifungal activities of these isolates were also studied. All these *Azotobacter* isolates were found positive for IAA production by the development of pink color in GNFM medium. Among twelve isolates, seven displayed antifungal activities against *Fusarium oxysporium*. These isolates of cellulolytic *Azotobacter* showed low to moderate cellulolytic activities and good Nitrogen fixing, Phosphate solubilizing, IAA producing and antifungal activities. Therefore the results of the present study may provide the basis for the development of bioinoculants on cellulosic wastes and production of biofertilizers for Wheat plants in Khyber Pakhtunkhwa.

Key words: *Azotobacter* · Cellulolytic Nitrogen Fixing *Azotobacter Species* · Carboxymethyl Cellulose · Cellulose · Ammonium Nitrogen · Glucose and Pikovskaya's Medium

INTRODUCTION

Owing to expansion of urbanization, agricultural land squeezed to a greater extent. Once Pakistan was reckoned in the ranks of agricultural countries, but now the situation has been rapidly changing. Pakistan is becoming day by day a dependent state rather than an independent in the field of agriculture. Barren lands are also being converted into attractive housing schemes. This dangerous trend is continuing globally, but the scientists in some foreign countries are taking the trend very seriously and pursuing different mechanisms to balance their agricultural needs such as adopting drum technology or even they adopted methods of growing vegetables etc. at their roof tops. Population growth globally is another factor affecting the existing agricultural

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production. In some countries they somehow managed to control the population growth, but in Pakistan it seems impossible to control due to various traditional and religious factors. Pakistanis scientists must practically cogitate upon this very serious issue, how to enhance the agricultural production in the prevailing circumstances. Research oriented, purposeful and practical steps are the only solution to the grave problem [1].

Farmers are nowadays totally dependent on using the chemical fertilizers which includes mainly Nitrogen and Phosphorus. Excess use of chemical fertilizers cause water pollution problems, effect the soil properties such as pH, water holding capacity, osmotic pressure etc, harm soil biota and are also hazardous to human health. Shift to bio-fertilizer will be more beneficial and progressive [2].

Large number of soil microorganisms consuming mineral compounds and degrading organic matter, affects the turnover of most of the elements in soil, regulating elemental cycles and plant nutrition [3]. These microorganisms include those capable of carrying out Nitrogen fixation, phosphate solubilization, cellulolytic degradation and killing the pathogenic microbes [1]. Various bacteria, fungi and algae have the potential to produce physiological active quantities of auxins which may exert great effects on plant growth [4].

Azotobacter is a genus of aerobic free-living Nitrogen fixing bacteria in soil, water and sediments. They have beneficial effects on plant yields because of having nitrogen fixing ability. They also can produce growth promoting hormones, like gibberellins, auxins and cytokinins [5] therefore are efficiently utilized as biofertilizers as their effects were observed on maize growth and yield [6] and on Wheat [7]. Nowadays there is an increase interest on different species of *Azotobacter* to be used as biofertilizers because of their high potential for Nitrogen fixation and Phosphate solubilization [5].

These soil microorganisms as biofertilizers can maintain good soil health by increasing soil fertility and can enhance the production to a greater level. They can also avoid harmful impacts of chemical fertilizers. However development of biofertilizers requires the growth of these microbial cultures in pure form and for that purpose a food source is the main requirement [8].

Cellulose is the abundant organic compound present as agricultural, industrial and residential wastes, posing environmental pollution as a major problem in some countries [9, 10]. They are commonly disposed by burning and composting. Scientists have found new means of disposal as the bioconversion of such wastes into useful products such as cheap energy source, animal feeds and biofeuls etc [11]. This can be possible only by the cellulolytic enzymes produced by microorganisms, which have significant applications in industries and agriculture [12].

The chosen research topic is a quest to contribute in the pursuit to minimize the use of chemical fertilizers thereby enhancing the productivity in the lesser portion of agricultural land. The main objective of this research study was to isolate *Azotobacter* species from rhizospheric soil samples, having the ability to degrade cellulose and fix atmospheric nitrogen. The desired isolates should also be able to solubilize phosphates, produce growth hormones and kill pathogenic fungal species. Utilization of such efficient species will help in the development of potent biofertilizers by the use of waste materials.

MATERIALS AND METHODS

Sample Size and Location: A Total of 70 soil samples from Wheat rhizosphere were collected from Wheat growing districts of Khyber Pakhtunkhwa including Charssada (11sample), Mardan (13 sample), Peshawar (12 sample), Swabi(10 sample), Nowshera (12 sample) and Dir (12 sample).

Sample Collection Procedure: For each sample, soil was first dug out with sterile shovel to expose the lateral roots. The soil associated with roots was collected from approx 15 cm depth by using disposable and sterile wooden plough. Soil was then mixed thoroughly and foreign materials like roots and stones etc were removed. Approx 10 g was transferred to sterile zip lock bag. All the soil samples after collection were properly sealed, labeled and sent to laboratory where they were kept at 4°C.

Screening, isolation and purification of cellulolytic Nitrogen fixing *Azotobacter species*: Screening and isolation of cellulolytic *Azotobacter species* was done on Nitrogen Free Carboxy-Methyl Cellulose (NF-CMC) medium (Table 2) by enrichment method. 1 g of soil sample was added in 100 mL NF-CMC medium in flask, thoroughly mixed and incubated at 28°C for 7 days in shaking incubator. After incubation, 1 mL of inoculum from each flask was taken, spread on NF-CMC agar media plates and were incubated at 28°C for 7 days. After incubation, media plates were flooded with 0.2% (w/v) Congo red dye for 20 minutes (Congo red assay). Dye was discarded and plates were flooded with 1 M NaCl for 20

Table 1: Identification Tests of *Azotobacter* species

S#	Isolate code	Gram staining	Catalase	Oxidase	Starch hydrolysis	Gelatin hydrolysis	Citrate	Indole test	Motility
1	2M	G^{-ive}	+	+	+	+	+	+	+
2	3M	G^{-ive}	+	+	+	-ive	+	+	+
3	4M	G^{-ive}	+	+	+	+	+	+	+
4	12 M	G^{-ive}	+	+	+	+	+	+	+
5	6M	G^{-ive}	+	+	+	+	+	+	+
6	10S	G^{-ive}	+	+	+	+	+	+	+
7	9 S	G^{-ive}	+	+	+	+	+	+	+
8	3D	G^{-ive}	+	+	+	+	+	+	+
9	7D	G^{-ive}	+	+	+	+	+	+	+
10	11D	G^{-ive}	+	+	+	+	+	+	+
11	7S	G^{-ive}	+	+	+	+	+	+	+
12	1S	G^{-ive}	+	+	+	+	+	+	+
13	1P	G^{-ive}	+	+	+	+	+	+	+
14	2P	G^{-ive}	+	+	+	+	+	+	+
15	6P	G^{-ive}	+	+	+	+	+	+	+
16	11P	G^{-ive}	+	+	+	+	+	+	+
17	8P	G^{-ive}	+	+	+	+	+	+	+
18	9P	G^{-ive}	+	+	+	-ive	+	+	+
19	6S	G^{-ive}	+	+	+	+	+	+	+
20	3C	G^{-ive}	+	+	+	+	+	+	+
21	1D	G^{-ive}	+	+	+	+	+	+	+
22	4D	G^{-ive}	+		+	+	+	+	+
23	2S	G^{-ive}	+	+	+	+	+	+	+
24	7M	G^{-ive}	+	+	+	-ive	+	+	+
25	5C	G^{-ive}	+	+	+	+	+	+	+
26	6C	G^{-ive}	+	+	+	+	+	+	+
27	4S	G^{-ive}	+	+	+	+	+	+	+
28	2C	G^{-ive}	+	+	+	+	+	+	+
29	9C	G^{-ive}	+	+	+	+	+	+	+
30	1N	G^{-ive}	+	+	+	+	+	+	+
31	7N	G^{-ive}	+	+	+	+	+	+	+
32	10N	G^{-ive}	+	+	+	+	+	+	+

Table 2: Composition of different media used

S #	Media/reagents/solutions	Composition in Gram/ Litre (g/L)
1	Nitrogen Free Carboxymethyl cellulose broth	K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.2), $MnSO_4.H_2O$ (0.02), $FeSO_4.7H_2O$ (0.02), $CaCl_2.2H_2O$ (0.02), CMC (5), pH (10.5)
2	*Azotobacter* Glucose Agar	K_2HPO_4 (1), $MgSO_4$ (0.2), NaCl (0.2), $FeSO_4$ (0.005), Glucose (10), Agar (15), pH (7.6+0.2)
3	Glucose Nitrogen Free Mineral agar	Glucose (10), K_2HPO_4 (1), $MgSO_4$ (0.2), NaCl (0.2), $CaCO_3$(1), $NaMoO_4$(0.005), $FeSO_4$(0.1), Agar (20), pH (7.0)
4	Bromothymol blue (BTB) solution	Bromothymol blue (0.5), Ethanol (100 mL)
5	Pikovskaya's agar medium	Glucose (10). $(NH_4)SO_4$ (0.5), $MgSO_4.7H_2O$ (0.1), KCl (0.2), NaCl (0.2), $FeSO_4$(0.002), Yeast extract (0.5), $MnSO_4$(0.002), Agar (15),50 mL TCP solution: Tri calcium phosphate (TCP) (2.5), Gum Arabic (0.025)
6	Potato Dextrose Agar	Potato infusion (200), Dextrose (20), Agar (20),

minutes. NaCl was discarded. Colonies were marked and were subcultured on *Azotobacter* Glucose agar (AGA) medium (Table 2) for purification.

Storage and Maintenance of Isolates: Purified isolates were cultured in Glucose Nitrogen free mineral medium with 15% glycerol and were stored at 4°C.

Identification of *Azotobacter species*: *Azotobacter species* were identified by using standard identification tests for *Azotobacter* according to Bergey's Manual of Determinative Bacteriology [13] including Gram staining, Catalase, Oxidase, Starch hydrolysis, Gelatin hydrolysis, Citrate, Indole and Motility tests (Table 1).

Detection of Cellulase Producing Activity of *Azotobacter species*

Congo Red Assay for Cellulase Producing Activity of *Azotobacter species*: Cultures were spotted on NF-CMC agar media plates and were incubated at 28°C for 7 days. After incubation, media plates were flooded with 0.2% (w/v) Congo red dye for 20 minutes (Congo red assay). Dye was discarded and plates were flooded with 1 M NaCl for 20 minutes. NaCl was discarded and zones were measured [14].

CMCase assay of *Azotobacter species*: Quantitative analysis of cellulase production was done by CMCase assay for Endo-beta-1, 4-glucanase. For this purpose, Dinitrosalicyclic acid (DNS) method was used for reducing sugar estimation [15]. Before starting DNS, different solutions were made including Citrate buffer (0.005 M), Glucose stock solution (0.01 M or 2 mg/mL), DNS reagent, CMC substrate (2% w/v) and Glucose standard solutions (0.1, 0.2, 0.3, 0.5, 0.6 and 1 mg/0.5 mL).

Fresh culture of each cellulolytic *Azotobacter species* was inoculated in NF-CMC broth medium and incubated for 7 days at 28°C in shaking incubator. After incubation the broth was centrifuged at 8000 rpm for 20 min at 4°C. Supernatant was taken and used for processing. Enzyme dilutions were made, at least two for each sample.

Standard glucose solutions were made daily in five test tubes. 0.5 mL CMC substrate was taken in all the tubes including Enzyme (E), Enzyme blanks (Eb), Standard (S) and Spectro blank. 0.5 mL solution from enzyme dilution tubes was added in enzyme (E) tubes. All the tubes were incubated at 50°C for 30 min in water bath. 3 mL DNS reagent was added in the tubes to stop the reaction. 0.5 mL enzyme dilution from each dilution was added in enzyme blanks one by one. 0.5 mL Citrate buffer was added in Spectro blank tube and 0.5 mL glucose standards were added in standard tubes one by one. Final volume of each tube was 4 mL. All the tubes were boiled at 100°C in water bath for 5 minutes and then cooled in ice bath. 10 mL distilled water was added in each tube for further dilution. Absorbance was checked on UV spectrophotometer at 540 nm. (Model no UV-1800ENG240V).

Detection of Nitrogen Fixing Activity of Cellulolytic *Azotobacter species*

Qualitative Analysis of Nitrogen Fixing Activity: Nitrogen fixation of *Azotobacter species* was qualitatively checked on Glucose Nitrogen Free Mineral (GNFM) agar medium with BTB [9] (Table 2). *Azotobacter* species were inoculated on prepared media plates and were incubated at 28°C for 7 days. Color change of colonies was determined.

Phenate Method for Detection of Nitrogen Fixing Activity: Quantitative analysis of Nitrogen fixation was done by Phenate method to detect for the production of Ammonium Nitrogen (NH_3-N) [16]. Before starting the method, different solutions were prepared including Ammonium stock solution (1000 mg/L), Phenol, Sodium Nitroprusside solution (0.5% w/v), alkaline citrate, 5% Sodium hypochlorite, Oxidizing solution and Ammonium standard solutions (0.1, 0.2, 0.5 and 1 mg N/L).

Fresh culture of each cellulolytic *Azotobacter* species was inoculated in NF-CMC broth medium and was incubated for 7 days at 28°C in shaking incubator. After incubation the broth was centrifuged at 8000 rpm for 20 min at 4°C. Supernatant was taken and used for processing. Sample dilutions (10^{-1} to 10^{-3}) were made. Ammonium standard solutions (S1 to S4) were made in separate flasks. Corning tubes were labeled as Sample tubes (N), Standards (S), Reagent blank (RB) and DDI blank. 25 mL solution from each sample dilution was added in sample tubes. 25 mL DDI water was taken in DDI blank and reagent blank. 25 mL standard solutions were taken in their respective standard tubes. 2.5 mL Oxidizing solution, 1 mL Phenol solution and 1 mL Sodium Nitroprusside solution was added in all the tubes except DDI water blank. All these tubes were kept for at least 1 hour to develop color and then absorbance was checked at 640 nm on UV spectrophotometer (Model no UV-1800ENG240V).

Detection of Phosphate solubilizing activity of cellulolytic *Azotobacter species*: Phosphate solubilizing activity of cellulolytic *Azotobacter species* was qualitatively checked on Pikovskaya's agar (PKV) and broth medium (Table 2) by Agar Spot and pH drop methods. PKV agar plates were inoculated with efficient *Azotobacter species* by using Agar Spot technique and were incubated for 7 days on 28°C. Zones of clearance around colonies were measured. For pH drop, PKV broth medium was inoculated with these *Azotobacter species* and were incubated for 7 days at 28°C. The pH of broth was checked daily for 7 days [17].

Detection of Indole Acetic Acid (IAA) Producing Activity: IAA production was checked on GNFM broth medium (Table 2) supplemented with tryptophan (0.5mg/mL tryptophan) [19]. *Azotobacter species* were inoculated

and incubated at 28°C for a week. Each culture was than centrifuged at 10,000 rpm for 5 min. 1 mL of supernatant was taken and was mixed with one drop of Orthophosphoric acid. 2 mL Solkoski's reagent was added in all the tubes and was kept for some time to develop color. Color intensity was noted.

Detection of Antifungal Activity: Production of antifungal metabolites by *Azotobacter species* was determined on Potato Dextrose Agar (PDA) plates (Table 2) [9]. *Fusarium oxysporium* (indicator fungi) and *Azotobacter species* were cultured separately for fresh culture to develop. *Fusarium oxysporium* was spread on each of the four plates and was kept for some time. *Azotobacter* culture spots were placed on these plates along with a positive control of *Bacillus specie* over fungal lawn and were incubated at 28°C for 7 days. Zones of inhibition were measured in mm around bacterial spots.

RESULTS AND DISCUSSION

Samples Collected: Total of 70 soil samples from rhizospheric region of Wheat crops from different districts of Khyber Pakhtunkhwa were collected as given in the Table 3.

Screening, isolation and purification of cellulolytic and Nitrogen fixing *Azotobacter species*: Screening and isolation procedure resulted in 118 Cellulolytic Nitrogen fixing isolates from different soil samples which on further sub culturing on *Azotobacter* Glucose Agar (AGA) medium gave 32 pure isolates. These were suspected to be *Azotobacter species* based on colony morphology (Fig 1) and Gram staining**.**

Identification of *Azotobacter species*: These 32 isolates were further subjected to microscopic studies and different biochemical tests for the identification of *Azotobacter species*. Microscopy showed Gram negative rods. Biochemical tests revealed that all these species are positive for Catalase, Oxidase and Starch hydrolysis, Indole, Motility tests and Citrate utilization.

Results of Gelatin hydrolysis showed that 29 isolates were positive while 3 were negative for gelatin hydrolysis. Biochemical tests are given in Table 1. These results confirmed that these isolates belong to the Genus *Azotobacter.*

Detection of Cellulase Producing Activity

Congo Red Assay for Cellulase Production: All the 32 *Azotobacter species* showed clear zones around colonies.

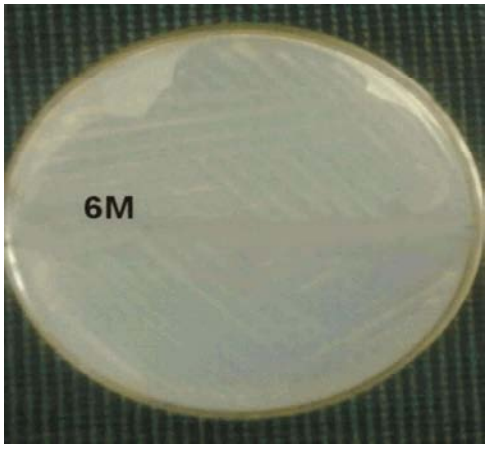

Fig 1: *Azotobacter* pure culture on Nitrogen free Carboxymethyl cellulose agar medium.

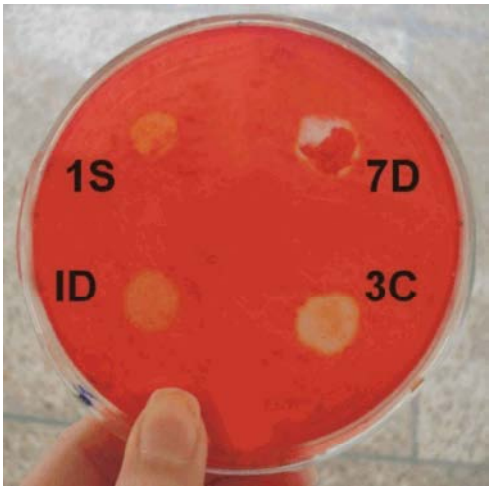

Fig 2: Cellulolytic zones of different *Azotobacter* isolates in Congo red assay.

Table 3: Soil samples from different districts

District	No. of samples collected
Charsada	11
Mardan	13
Peshawar	12
Swabi	10
Nowshehra	12
Dir	12

Out of these, only 12 *Azotobacter species* were selected for further quantitative analysis based on their relatively higher zone ratio as given in Table 5. The selected isolates were considered as efficient. Out of these 12, 6 isolates showed low cellulolytic activity in the range of 1-2 ratios while 6 showed moderate cellulolytic activity in the range of 2.1-3 ratios as given in the Table 4. Cellulolytic zones can be seen in Fig 2. Among the efficient isolates, maximum cellulolytic zone measured was 2.714

Table 4: Activity ranges of Cellulolytic *Azotobacter species.*

32 isolates of cellulolytic *Azotobacter species* Range (Ratio 1-2.7 mm)			
Activity range	=1	1- 2	2.1- 3
No. of isolates	20	6	6
Activity level	Very low activity	Low activity	Moderate activity

Table 5: Absorbance, Glucose concentration and Enzyme units of 12 test samples by spectroscopy

S #	Isolate code	Zone ratio* of efficient Cellulolytic *Azotobacter*	Glucose concentration mg/0.5 mL*	Enzyme units(IU ml^{-1})*
1	2M	2.4	0.121	0.044
2	12M	2.25	0.085	0.031
3	6M	2.714	0.010	0.003
4	7D	1.71	0.036	0.013
5	1P	1.3	0.021	0.007
6	11P	1.266	0.014	0.005
7	8P	1.454	0.021	0.007
8	6S	2.42	0.107	0.039
9	3C	2.22	0.077	0.028
10	1D	2.333	0.100	0.037
11	4D	1.5	0.022	0.008
12	2S	1.23	0.011	0.004

Note: *=given values are mean of duplicate value, IU/mL=International units per milliliter, mg=Milligrams

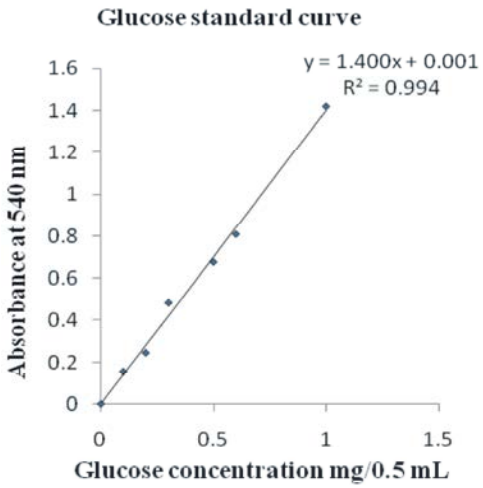

Fig. 3: Glucose Standard Curve.

while minimum cellulolytic zone was 1.23. These results indicated that the isolated *Azotobacter species* have low to moderate ability to produce cellulases.

CMCase assay of *Azotobacter species*: These 12 *Azotobacter species* were processed by DNS method. The absorbance of glucose standards were recorded and linear standard curve was made.

Efficient *Azotobacter species* processed through DNS method produced different levels of glucose concentration in mg/0.5 mL which were determined from glucose standard curve as shown in the Fig 3. These glucose concentrations were translated into enzyme concentration in International units (IU) [15]. Maximum amount of CMCase enzyme was released by isolate labeled as 2M and was determined to be 0.044 IU ml^{-1} while minimum amount was released as 0.003 IU ml^{-1} by isolate labeled 6M (Table 5). The remaining efficient isolates also displayed very low to moderate cellulolytic activities in this method on CMC substrate. By comparing the qualitative and quantitative results of cellulolytic activity of all the efficient isolates, it was found that 2M isolate had displayed significant zone ratio in Congo red assay and maximum amount of enzyme in DNS method.

In contrast to this peak result of 2M isolate, isolate 6M had displayed maximum zone ratio in Congo red assay while showed minimum amount of enzyme in DNS method. As it was reported by Immanuel *et al*, in 2006 that different physical and chemical parameters such as carbon source, cellulose quality, temperature, pH value and incubation time are responsible in the optimization of cellulase production by bacteria, therefore this contradictory result can be justified through their findings. They worked on cellulolytic endoglucanases of different bacterial species isolated from coir fibre and found that enzyme production by bacteria was optimal at pH 7 and 40°C temperature [18]. Our results can be supported by other research finding [19]. He isolated different cellulolytic bacteria from different samples including soil, rumen fluid and cow dung and checked their cellulolytic potential by DNS at different temperatures of 30, 37, 40 and 50°C and pH 3, 4, 5 and 6. He found that different bacteria gave different sugar

Table 6: Results of qualitative and quantitative methods for Nitrogen fixation

S #	Isolate code	Blue color change on GNFMM with BTB	$[NH_4\text{- }N]_{Tube}$* mg N/L	$NH4\text{-}N_{sample}$= NH4-N x DF mg N/L*	$[NH_4\text{-}N]$ /1 g carbon source
1	2M	+	0.798	80	8.0
2	12M	+	0.207	21	2.1
3	6M	+	0.188	19	1.9
4	7D	+	0.202	20	2.0
5	1P	+	0.939	94	9.4
6	11P	+	0.703	70	7.0
7	8P	+	0.170	17	1.7
8	6S	+	0.154	15	1.5
9	3C	+	0.107	11	1.1
10	1D	+	0.199	20	2.0
11	4D	+	0.195	20	2.0
12	2S	+	0.162	16	1.6

*=Given values are mean of duplicate values, g=Grams, mg N/L=Milligrams Nitrogen per Litres, NH_4-N=Ammonium Nitrogen, DF=Dilition facter

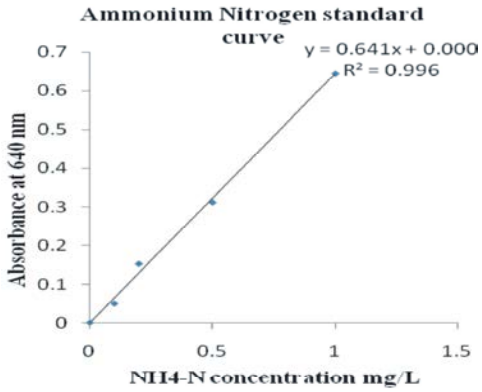

Fig. 4: Ammonium Nitrogen Standard Curve.

yields. Highest yield was recorded at 40°C and pH 6 while minimum yield was recorded at 30°C and pH 3 [19]. Therefore 6M isolate may require different temperature and pH for enzyme production. Thus optimization for cellulase enzyme production in DNS method by the each *Azotobacter* isolate is required.

There may be another possible reason for the low activity of isolate 6M in DNS method. It was reported by Fan, L. T. in 1980 that enzymatic hydrolysis of celluloses depends on important factor of accessible surface area to enzyme action [20]. Cellulose hydrolysis may be slowed down by the inability of enzymes access to the additional substrate because the hydrolytic products and some enzyme complexes cover some surface chains due to which enzyme molecules cannot bind to the substrate. Walker *et al*. worked on fragmentation of cellulose by the action of *Thermomonospora fusca* cellulases, which showed that fragmentation takes place before the release of reducing sugar [21]. Thus there is a possibility of slow release of glucose due to reduced fragmentation.

Qualitative and quantitative results of the remaining efficient isolates were analogous on comparison indicated capability to release enzymes in broth properly. However our results of DNS method are low in contrast to the cellulolytic activities of bacteria determined in 2008 by Ei Phyu Kyaw [9]. Their DNS findings were in the range of 0.39-0.45 mg/0.5 mL on CMC substrate and our results are comparatively low meaning thereby *Azotobacter species* of our locality have low ability to degrade cellulose.

Detection of Nitrogen Fixing Activity

Qualitative Analysis of Nitrogen Fixing Activity: Growth of all the 12 *Azotobacter species* on NFGM agar medium showed significant color change of colonies to dark blue indicated nitrogen fixation as given in the Table 6.

Phenate Method: Absorbance of Ammonium standards by Phenate method was recorded at 640 nm and standard linear curve was made. Absorbance of 12 test samples of *Azotobacter species* was recorded and concentration of NH_3-N mg/L was determined (Table 6) from the standard curve shown in Fig 4 by using the formula given below [16].

$[NH_4\text{-}N]_{Tube}$ mg/L = (y–b) /m (as y = mx + b)

The resultant values of the conducted activity were in the range of 11-94 mg N/L. Maximum concentration of NH_4-N was 0.94 shown by 1P isolate in 10^{-2} dilution indicated that it can produce 94 mg NH_4-N/L. This means that it can produce 9.4 mg NH_4-N per 1 g of carbon used (as 1L broth contains 10 g glucose). This is the peak value which nearly approximates the known value of 10 mg N/ 1g of carbon source which is the maximum concentration

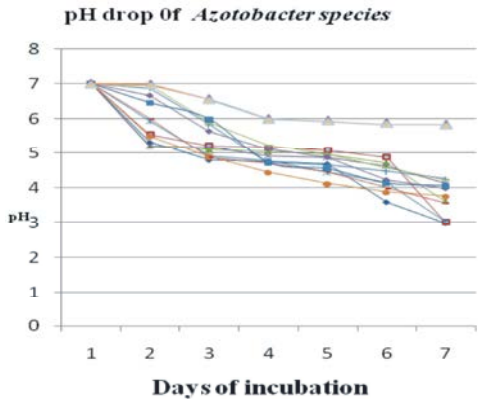

Fig 5: Drop of efficient *Azotobacter species* in Pikovskaya's broth medium.

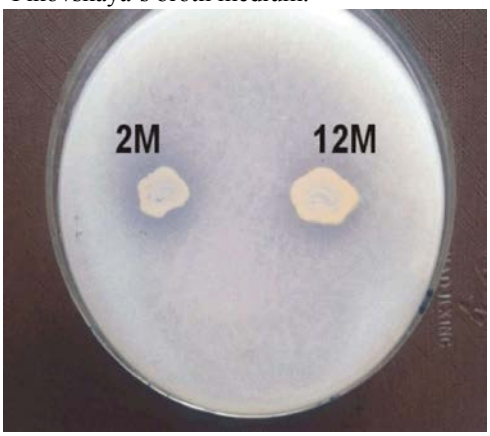

Fig 5: Phosphate Solubilization activity indicated by clear zones around colonies.

of Nitrogen released by *Azotobacter species in vitro* conditions stated by Arun [22] and S Sheraz Mehdi and his colleagues [23].

Minimum concentration of NH_4-N was 0.11 shown by 3C isolate in 10^{-2} dilution indicated that it can produce 11 mg NH_4-N/L. This means that it can produce 1.1 mg NH_4-N per 1 g of carbon used. The remaining efficient isolates also produced different concentrations of NH_4-N mg/L.

The results of both the qualitative and quantitative methods for the detection of Nitrogen fixing activity are comparable indicating Nitrogen fixation.

Detection of Phosphate Solubilization of Cellulolytic *Azotobacter species*: All the *Azotobacter species* are able to solubilize Phosphates to a significant level indicated by both methods of agar spot and pH drop. Phosphate Solubilizing activity was shown in the range of 1.142 - 1.611 zone ratio in Agar Spot technique, while in pH drop method; pH fall was recorded from pH 7 to 2.99 – 5.83 (Table 7).

Table 7: Phospahte solubilizing activity of 12 *Azotobacter species*

S #	Isolate code	Total zone/ Colony ratio*	pH fall in PKV broth from pH 7*
1	2M	1.611	2.99
2	12M	1.591	3.01
3	6M	1.346	3.61
4	7D	1.25	4.12
5	1P	1.308	3.02
6	11P	1.333	3.76
7	8P	1.214	4.25
8	6S	1.458	3.56
9	3C	1.230	4.22
10	1D	1.292	3.98
11	4D	1.273	4.06
12	2S	1.142	5.83

*=Given values are mean of duplicate value

Table 8: Color development in GNFMM

S #	Isolate code	Development of pink color
1	2M	++
2	12M	++
3	6M	++
4	7D	++
5	1P	++
6	11P	++
7	8P	+
8	6S	+
9	3C	+
10	1D	+
11	4D	+++
12	2S	+

Note: + slight pink, ++ pink, +++ dark pink

The results of Agar spot and pH drop method on comparison revealed that 2M *Azotobacter* isolate showed maximum zone ratio in agar spot while in pH drop method, it showed significant drop of pH, indicated that 2M has maximum Phosphate solubilizing potential.

Azotobacter isolate 2S showed minimum zone ratio in Agar spot method while it decreased the pH to low level, indicated that it has low ability to solubilize Phosphates. The remaining isolates also showed corresponding results in both these methods indicated different Phosphate solubilizing potential (Fig 5). Our results are analogous to the results of agar spot and pH drop methods by Rashid, M *et al*. 2004 in which bacterial isolates showed significant amount of Solubility index value and drop of pH [17]. *Azotobacter species* with significant Phosphate Solubilizing ability can be taken into account for utilization as biofertilizers.

Detection of IAA production of *Azotobacter species*: All of the 12 *Azotobacter species* were able to produce IAA on GNFMM with 0.5 mg/L tryptophan and developed pink color with different intensities when reagents were applied. *Bacillus specie* was used as a positive control as shown in the Fig 6. The results are shown in the Table 8.

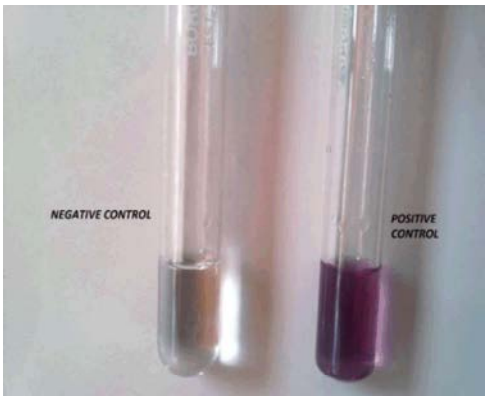

Fig 6: Positive and Negative Control Samples for IAA activity.

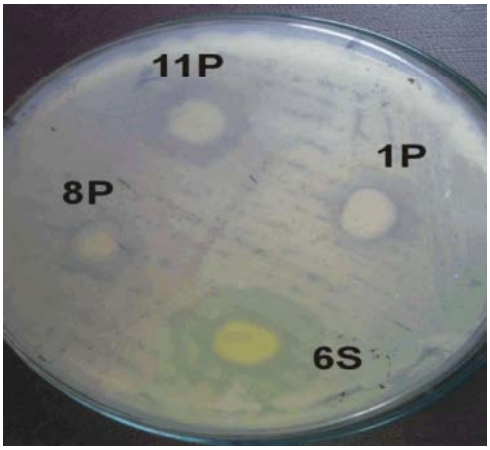

Fig 7: Clear Zones around Colonies indicating Production of Antifungal Substances.

Table 9: Zones of inhibition of 12 *Azotobacter species* against *Fusarium oxysporium*

S#	Isolate code	Total zone(Zone/Colony)
1	2M	2.22
2	12M	0
3	6M	0
4	7D	0
5	1P	1.75
6	11P	2.11
7	8P	1.5
8	6S	2.6
9	3C	1.55
10	1D	1.33
11	4D	0
12	2S	0

Our results are similar to the research findings in which all their *Azotobacter species* had shown Indole acetic acid production conducted by Vikram Patil [24], Muhammad Arsalan Ashraf [25] and Ei Phyu Kyaw [9]. These results also indicated that each *Azotobacter species* have IAA producing ability in different levels thus IAA levels should be properly evaluated to be used further for biofertilizer development and application *in vitro*.

Detection of antifungal potential of *Azotobacter species*: Clear zones appeared around some of the *Azotobacter species* indicating that they have antifungal activity. Zones of inhibition were measured (Table 9). Minimum zone is 1.33 while maximum zone is 2.6.

Out of 12 *Azotobacter* isolates, 7 showed antifungal activities against *Fusarium oxysporium* by forming clear zones around colonies while 5 formed no zones around colonies thus showed no antifungal activity. Our results are similar to the findings of Ei Phyu Kyaw and his colleagues who worked in 2008 on antifungal activities of *Azotobacter* and their results also showed that some species have antifungal activity against *Fusarium oxysporium* [9]. *Azotobacter species* with antifungal activity can be considered for further use as biofertilizer.

CONCLUSION

This research work concluded that *Azotobacter species* isolated from different districts of Khyber Pakhtunkhwa showed the low to moderate ability to degrade Carboxymethyl Cellulose in the media. They also significantly showed their abilities to fix atmospheric Nitrogen, solubilize tricalcium phosphates, Indole acetic acid and antifungal metabolites production against indicator fungal species. However the cellulase production indicated that these species have low to moderate ability to degrade CMC meaning thereby these *Azotobacter species* can be used for the development of bioinoculants on cellulosic wastes so as to generate potent biofertilizers.

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