Indian J. Applied & Pure Bio. Vol. 39(1), 460-474 (2024). **ISSN: 0970-2091**
A Web of Science Journal

Isolation, molecular characterization and evaluation of efficient *Azotobacter* on *Sorghum* plant

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Abstract

Fly ash is a rich source of essential plant nutrients hence it is suited for crop production and thus facilitates alternate use for fly ash. In the present investigation, 99 *Azotobacter* isolates were isolated from the rhizospheric soil from cereal fields, All the isolates were morphologically, biochemically characterised. Isolates were screened to obtain efficient *Azotobacter* isolates. The *Azotobacter* isolate BUDA2 fixed highest amount of nitrogen 13.3g^{-1} of mannitol. Based on 16s rRna analysis of an isolate BUDA2 was identified as *Azotobacter chroococcum*. A pot culture experiment was conducted to study the effect of BUDA2, fly ash and RDF on the growth parameters of sorghum plant. The inoculation of BUDA2 along with 10% fly ash and 75% RDF showed significant increase in growth parameters of sorghum.

Key words : *Azotobacter*, flyash, nitrogen fixation, molecular characterization, sorghum.

Azotobacter plays a key role in maintaining soil fertility through several beneficial effects in the rhizosphere of cereals and grasses[51]. *Azotobacter* maintain a heavily niche for growing plants.[26] Bacteria are the most abundant microbes in the rhizosphere and hence they are bound to influence the plant in a significant manner up to 15% of the total root surface may be associated with a variety of bacterial strains. *Azotobacter* is an aerobic free living bacterium widely distributed throughout the world and characterized by an added advantage of nitrogen fixation[5] Flyash is an inorganic solid waste mineral product from the combustion of coal during power generation in the thermal power plants. Since more than 70% of the energy today is generated by thermal power plants, enormous amount of fly ash is produced during coal combustion. Physically fly ash occurs as fine particles

having a average diameter or less than 10 mm, low to medium bulk density, high surface area and very light texture. Chemically, the compostion of fly ash from the thermal power station consists oxides of Si, Al, Fe, and Ca and about 0.5 to 3.5 percent of Na, P, K and S and the remainder of the ash is composed of trace elements[12] Flyash consists of partially all the elements present in the soil except of organic carbon and nitrogen. Thus, it was found that this mineral could be used as an additive or amendment material in agriculture applications[46]. Cereal crops are an essential nutritional source for worldwide population, and they are of great economic importance both as food and feed.[7] Application of *Azotobacter chroococcum* inoculants in cultivation of cereals is being recommended as well as commonly practised.[18]

Rhizosphere soil samples were collected from various cereal fields around Raichur Thermal Power Station (RTPS) located in Shaktinagar, Raichur district. Fly ash was collected from the dumped sites of RTPS, shaktinagar. Further Rhizospheric soil was amended with the fly ash and one gram of the fly ash amended soil sample was suspended in 9ml sterilized distilled water and serially diluted up to 10^{-4}. From dilution 10^{-4} 0.1 ml of the soil suspension was spread on Waksman No.77 N-free agar plates[4,57] these plates were incubated at 30p C for 3-6 days.

Morphological characterization :

Colonial morphology of the isolates such as shape, elevation, colour, surface, opacity and consistency was observed after incubation period of 3-7 days at 28p C. Microscopic examination like gram staining and formation of cyst was carried out for the isolates as per Bergey's Manual of Systematic Bacteriology (2001).

Biochemical characterization :

All the biochemical characterization of the isolates was done using standard biochemical methods as given in Bergey's Manual of Systematic Bacteriology (Bergey's et al., 2001).

In vitro nitrogen fixation :

In vitro nitrogen fixation by the *Azotobacter* sp. isolates was determined as per standard protocol by Kjeldahl method[23].

Molecular characterization :

Genomic DNA was extracted by using HiPurA Bacterial DNA purification spincolumn kit (MB505-250PR, Hi Media, India) and checked on 1% agarose gel electrophoresis. PCR amplification of bacterialspecific 16s Rrna gene (1500 bp) was carried outby using primers F27(5'AGAGTTTGAATCMTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGAC TT3') (Jill E. Clarridge,III, 2004). The PCR reaction was performed in 25µL volume containing 12.5 µL EmeraldAmp GT PCR Master Mix, 2x (Takara Bio USA), 1 µL DNA template (50-100 ng), 1.25 µL (10 µL) of each primer (forward and reverse) and 9µL of free- nuclease water. PCR amplification was performed using Applied Biosystems Veriti Thermal Cycler as follows: denaturation at 94 °C for 5 min followed by 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.30 min and a final cycle at 72°C for 7 min.

PCR products were detected by staining with GelRed Nucleic Acid Gel Stain on 1% agarose electrophoresis gel in (IX) TBE buffer and visualised under UV transilluminator (Protien Simple Red Imager SA-1000). PCR product was purified using Exonuclease I and Shrimp Alkaline Phosphatase Purification Kit (New England Biolabs, Inc) and cycle sequenced using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with conditions as follows: denaturation at 96°C for 1 min, 50°C for 05 s, and 60°C for 4 min. Cycle sequenced amplicons were purified using sodium acetate ethanol method (Thermo Fisher Scientific) and sequencing reactions were run on a 3500XL Genetic Analyzer (Applied Biosystems, USA). Sequencing files (.ab1) edited using CHROMASLITE (version 1.5) and further analysed by Basic Local Alingment Search Tool (BLAST) with closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences[3]. The program compares nucleotide or protein sequences to sequence database and calculate the statistical significance of matches[18]. The BLAST algorithms usedto infer functional and evolutionary relationships between sequences as well as help identify members of gene families. (i) Initial search to find potentially closely related **type strain** sequences using the BLASTN program[3] (ii) Pair wise alignment to calculate the sequence similarity values between the query sequence and the sequences identified in step (i) (States *et al*., 1991). Therefore, each isolate is reported with the first five –ten hits observed in the said database. Further multiple sequence alignment and phylogenetic analysis is therefore recommended for accurate species prediction and evolutionary relationship[29].

Pot culture trail :

A pot culture experiment was conducted to study the effect of isolate BUDA2 on growth and yield of *Sorghum* under green house condition. The medium deep black soil obtained from Agriculture Research Station, University of Agricultural Sciences, was sterilized and filled in earthen pots with 5kg soil, FYM (farm yard manure), sand in the ratio 3:2:1 supplemented with different concentration Viz., 5%, 10%, 20% and 30% of fly ash sample. The *Sorghum* seed variety M -35 obtained from seed unit, University of Agriculture Sciences, Raichur were surface sterilized by 3% (v/v) sodium hypochlorite solution for 2-3 minutes and rinsed in sterilized distilled water 2-3 times and dried in shade for 10 -15 minutes and used for sowing in pots, 3-5 seeds were sown in each pots. Inoculation with BUDA2, before sowing seed treatment was done with BUDA2 (containing approximately 10^8 cells /g) prepared from its 24 hours old culture on Walksman No. 77 broth grown for one week in shaker and then $1x10^8$ cells/g cells was used to prepare carrier based inoculum at the ratio of 1:2.5 and lignite as carrier, these seeds were treated and sown in the treatments (table-1). The experimental pots were kept weed free and watered regularly. Plant protection measures were taken as per recommended package of practices.[28] The following growth parameters Viz., plant height, number of leaves, root height, dry weight of shoot, dry weight of leaves and dry weight of root were recorded at 30 days of sowing (DAS), 60DAS and at harvest.

Treatments :

Table-1. There were eleven treatments in the experiment, the details of which are as follows

Sl. No.	Particulars	Details
1	Design	Completely Randomized Block Design
2	Replications	Three
3	Treatments	T_1 – control
		T_2 -Recommended dose of fertilizer(RDF) 100%
		T_3 -RDF 75%
		T_4 -5% flyash + 75% RDF
		T_5 -10% flyash +75% RDF
		T_6 - 20% flyash 75% RDF
		T_7 -30% flyash+75% RDF
		T_8 -5% flyash + +75% RDF
		T_9 -10% flyash+ BUDA2 +75% RDF
		T_{10} -20%flyash+ BUDA2+75% RDF
		T_{11} -30%flyash+ BUDA2+75% RDF
4	Crop	Sorghum
5	Variety	M-35
6	Soil type	Medium black soil

Estimation of soil enzyme activity :

The dehydrogenase activity in soil was determined by the method given by Klein *et al*.,[37] and the phosphatase activity in soil was determined by the method given by Tabatabai[58].

Morphological characterization :

A total of 99 isolates of *Azotobacter* were isolated from the rhizosphere soil collected from the various cereal fields. Colonies of all the isolates were round, raised to convex, white, smooth glistening, translucent to opaque and moist.[59] After one week the colour of the colonies changed from white to dark brown. Isolates were gram negative and capable of forming cyst.[11]

Biochemical characterization :

All the selected *Azotobacter* isolates were subjected for biochemical characterization. Isolate BUDA1, BUDA2, BUDA4, BUDA6, BUDA7, BUDA9 AND BUDA10 were catalase positive, oxidase positive, indole positive, methylred positive, voges proskauer negative, citrate positive and starch positive. Isolate BUDA3 is positive for catalase, oxidase, indole, citrate and starch hydrolysis, whereas methylred and voges proskauer negative. BUDA5 and BUDA10 is positive for catalase and oxidase but negative for indole,

methylred, voges proskauer, citrate and starch hydrolysis.[21,41]

In vitro Nitrogen fixation :

All ten isolates of *Azotobacter* fixed nitrogen. Isolate BUDA2 fixed highest amount of nitrogen at a range of 13.3 mg g^{-1}, followed by BUDA1which fixed about 12.4 mg g^{-1}. (Table-1 and figure 2) The lowest amount of nitrogen fixed[35,57] was by the isolate BUDA4 Viz., 8.14mg g^{-1}.

Molecular characterization :

The selected isolate of *Azotobacter,* BUDA2 was sent to NCIM, CSIR-NCL Pune for 16s rRNA (1500 bp) sequencing and for phylogene. The isolate BUDA2 was identified as *Azotobacter* chroococcum, the strain showed closest homology with *Azotobacter sp.*(Closer to chroococcum). Phylogene also showed closest homology with *Azotobacter* sp. (closer to chroococcum). (Figure 1) Evolutionary analyses were conducted in MEGA6 [59].

16S rRNA gene sequence of BUDA2

>907RC_704F_Seq185_BUDA2

GCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAG

GGGTGCTTGCATCCCGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTG

CCCGATAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTA

CGGGAGAAAGTGGGGGCTCTTCGGACCTCACGCTATCGGATGAGCCTAGGTCGG

ATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCT

GAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA

GGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCG

CGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTCGGGAGGAAGGGT

TGTAGGTTAATACCTTGCAGCCTTGACGTTACCGACAGAATAAGCACCGGCTAA

CTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTAC

TGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGTTGGATGTGAAAGCCCCGGGC

TCAACCTGGGAACTGCATCCAAAACTGCCTGACTAGAGTACGGTAGAGGGTGGT

GGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGG

CGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAG

CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCG

TTGGGCTCCTTGAGAGCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGG
GGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTG
ACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTA
ACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGA
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGC
CCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAA
GCCGCGAGGCGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGCAGTCT
GCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGC
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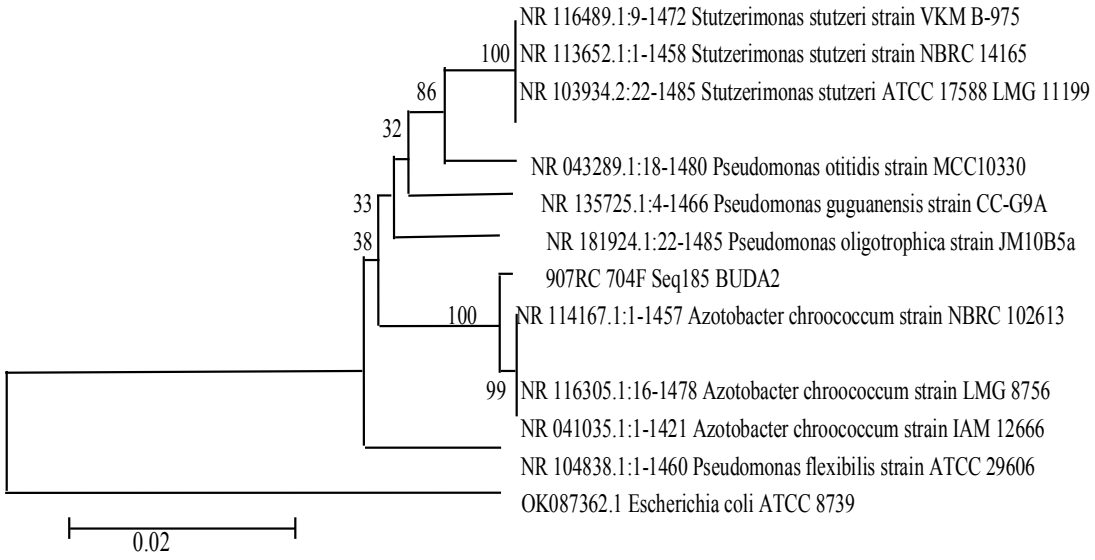

Figure 1: Phylogenetic tree of the 16S rRNA sequence of the strain BUDA2 along with evolutionary relationship of taxa.

The evolutionary history was inferred using the Neighbor-Joining method. (Saitou N. *et al*. 1987) The optimal tree with the sum of branch length = 0.23445573 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstraptest (1000 replicates) are shown next to the branches.[17] The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2- parameter method[34] and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1361 positions in the final dataset. Evolutionary analyses were conducted[59] in MEGA6. Based on the results of screening two isolates Viz., BUDA1 AND BUDA2 were selected as efficient *Azotobacter* isolate. BUDA2 showed the highest PGPR activity hence isolate BUDA2 was selected for pot culture trail.

Pot culture trail :

Pot culture experiment was conducted to study the effect of BUDA2, fly ash and RDF on the growth parameters of sorghum under green house condition. The observations recorded on various plant growth parameters at different period of crop growth are as follows.

Plant height (cm) :

The data pertaining to plant height of sorghum at different stages of crop growth revealed that treatments in the experimentation have significantly influenced the plant height of *Sorghum* at 30 days after sowing (DAS), 60DAS and at harvest (Approximately 90 to 120 days). The plant height ranged from 12.8cm to 75.3cm. (Table-2) Highest plant height of *Sorghum* crop at 30 DAS was observed in treatment T9 (25cm), at 60DAS in T9(39.4cm) and at harvest in T9(75.3cm) (Table-2). Doifode[14] Sultana *et al.*,[56]. Kalaiarasi[28], Inam[24] and Gupta[20] also reported the same and this was observed in sorghum and maize plant. However, plant height at all stages of crop growth was found to be significant and increased with the combined application of BUDA2. 10% fly ash and 75%RDF. Inoculation of beneficial and nitrogen fixing (Abdel- Fattah *et al.*,[1], Chi *et al.*,[9] and Bhardwaj *et al.*,[6] and Sahoo *et al.,*[49]) microbes as biofertilizers can produce beneficial substances such as plant hormones indole acetic acid, gibberellins and cytokinins which helps in plant growth. Chemically fly ash contains elements like Ca, Fe, Mg, and K, essential to plant growth[24].

Number of leaves :

Significant difference due to combined application of fly ash isolate BUDA2 and 75% RDF were observed in number of leaves at all stages. (Table-2) It was observed that number of leaves increased between 30DAS to harvest. At 30DAS, the maximum number of leaves were recorded in T9 (6), at 60DAS in T9 (7) and at harvest in T9 (21) (Table-2), same results were observed in lettuce by (Zahra Razmjooei *et al.*,[48]). Maize plants inoculated with *Azotobacter* sp. gives maximum leaf growth[22] Use of low doses of fly ash increases chlorophyll content in the leaves may be due to higher accumulation of

micronutrients such as Mn, Fe, Cu, and Zn[15].

Root length (cm) :

The data on the length of root of *Sorghum* at different stages of crop growth is presented in (Table-2). Highest root height at 30DAS was recorded in T9(13.7cm), 60DAS T9(38.7cm) and at harvest T9(45.2cm) (Table-2)[30,39,55]. The use of free nitrogen fixing bacteria *i.e.*, *Azotobacter* sp.as a bioinoculant is widely applied for a variety of crops, such as rice, wheat, maize, sorghum and sugarcane due to its property like nitrogen fixation, secretion of plant growth promoting substances, vitamins, antifungal metabolites, phosphate solubilization, soil aggregation and tolerance to pesticides Plant root height at all stages of crop growth was found to be significant and increased with the combined application of BUDA2 with 10% fly ash and 75% RDF.

Shoot dry weight (g plant^{-1}) :

Generally there was increase in shoot dry weight from 30DAS till the harvest with the increase being significant and is presented in (Table-3). At 30DAS, the highest shoot dry weight was recorded in treatment T9 (0.245g plant^{-1}) at 60DAS in treatment T9(0.293)as well as at harvest in treatment T9 (4.821)[2,27,45,55] and was superior over other treatments.

Dry weight of leaves :

The data on dry weight of leaves of sorghum at different stages of crop growth is presented in (Table-3). At 30DAS the treatment T9 (0.993g plant^{-1}) recorded highest leaves dry weight, found superior over other treatments[10]. Similarly highest leaves dry weight at 60DAS and at harvest was recorded in T9 (1.981g plant^{-1} and 4.289g plant^{-1}) it was superior over other treatments.

Root dry weight :

The data pertaining to root dry weight of sorghum at different stages of crop growth revealed that treatment in the experimentation have significantly influenced the root dry weight of sorghum at 30DAS 60DAS and at harvest. The root dry weight ranged from 0.055g plant^{-1} to 0.298g plant^{-1} and is presented in (Table-3). The highest root dry weight at 30DAS, in treatment T9 (0.196g plant^{-1}) and at 60DAS, T9 (0.287g plant^{-1}) as well as at harvest also it was observed in treatment T9 (0.298 g plant^{-1})[19].

Estmation of soil enzyme activity :

In general, enzyme activity is considered as a good index of soil quality because of their intimate relationship to soil biology, ease of measurements, and rapid response to change in soil management practices[13]. The data pertaining to dehydrogenase activity was recorded, the highest dehydrogenase activity was observed in the treatment T9 receiving 10% flyash, BUDA2 and 75% RDF (36.1µg TPF/g soil), the lowest dehydrogenase activity was observed in T7 supplemented with 30% fly ash and 75% RDF (19.9µg TPF/g soil) compared to control (25.2µg TPF/g soil) (Table-4 and figure 3). The result of alkaline phosphatase activity also revealed the highest phosphatase activity in treatment T9 (93.04µg

P- Nitrophenol/g of soil) amended with 10% fly ash, BUDA2 and 75% RDF, while the lowest was recorded in T7(42.00µg P-Nitrophenol /g soil) amended with 30% fly ash and 75% RDF compared to control (73.00 µg P- Nitrophenol /g soil) (Table-4 and figure 4). Supplementation of fly ash at the rate of 10 t ha^{-1} was optimum for bacterial population, soil dehydrogenase activity and microbial biomass[36]. Alkaline phosphatase activity in soil enriched with fly ash gets elevated. Sarangi *et al*., (2001) reported increase in the rate of carbon dioxide evolution as well as activity of soil enzymes increases with increasing application of fly ash up to 10 t ha^{-1}, but decreased with further higher levels. For comparison a reference strain of *Azotobacter* was collected from Department of Microbiology, Agriculture University, Raichur.

Table-1. Nitrogen fixing capacity by *Azotobacter* isolates

Azotobacter isolates	*In vitro* Nitrogen fixed (mg N_2 fixed g^{-1} of mannitol)
BUDA1	12.4
BUDA2	13.3
BUDA3	11.0
BUDA4	8.14
BUDA5	10.4
BUDA6	12.3
BUDA7	9.21
BUDA8	11.2
BUDA9	12.1
BUDA10	10.8
AZTG4 (Ref strain)	8.28
SEm±	0.10
CD at 5%	0.31

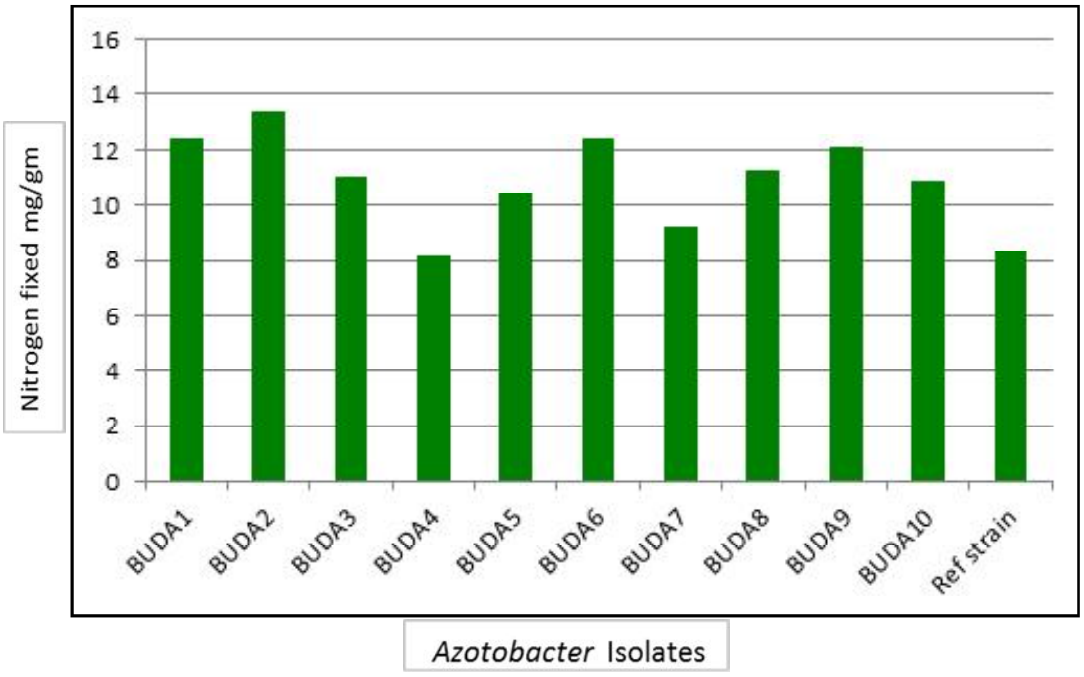

Figure 2. Nitrogen fixed (mg/gm) by *Azotobacter* Isolates

Table-2. Effect of isolate BUDA2 in combination with fly ash and RDF on plant height(cm), number of leaves and root height(cm) at different stages of *Sorghum*

Treat-ment	Plant height(cms)			Number of leaves			Root height (cms)		
	30 DAS	60 DAS	At harvest	30 DAS	60 DAS	At harvest	30 DAS	60 DAS	At Harvest
T1	12.8cm	18.1cm	34.0cm	4.0	4.0	09	6.5cm	19.2cm	24.6cm
T2	15.6cm	20.4cm	56.1cm	5.0	6.0	16	10.8cm	28.7cm	29.4cm
T3	14.5cm	19.0cm	52.0cm	4.0	5.0	14	9.2cm	24.1cm	26.2cm
T4	15.0cm	26.2cm	48.1cm	5.0	6.0	13	9.0cm	26.4cm	30.4cm
T5	20.2cm	28.4cm	64.6cm	6.0	7.0	19	13.0cm	29.2cm	38.6cm
T6	17.6cm	24.3cm	54.6cm	5.0	8.0	12	9.0cm	20.6cm	33.2cm
T7	15.9cm	22.4cm	53.0cm	4.0	5.0	10	8.7cm	21.3cm	31.5cm
T8	18.0cm	32.6cm	58.1cm	5.0	6.0	13.5	9.8cm	25.6cm	39.6cm
T9	25.0cm	39.4cm	75.3cm	6.0	7.0	21	13.7cm	38.7cm	45.2cm
T10	18.8cm	30.2cm	53.7cm	5.0	6.0	14	10cm	22.4cm	37.4cm
T11	15.6cm	29.0cm	50.5cm	4.0	5.0	11	7.5cm	20.3cm	32.5cm
SEm±	0.20	0.33	0.64	0.04	0.05	0.18	0.12	0.32	0.37
CD at (5%)	0.61	0.98	1.90	0.14	0.16	0.54	0.36	0.95	1.09

1)T1-Control, 2)T2-Recommended dose of fertilizer (RDF)100%, 3)T3-RDF 75%, 4)T4-5% Flyash+ 75% RDF 5)T5-10%Flyash + 75% RDF 6)T6-20% Flyash + 75% RDF 7)T7- 30% Flyash +75% RDF, 8)T8-5% Flyash+ BUDA2+ 75%RDF,9) T9-10% Flyash + BUDA2+ 75%RDF, 10)T10- 20% Flyash+ BUDA2+ 75%RDF 11) T11- 30% Flyash + BUDA2+ 75%RDF

Table-3. Effect of isolate BUDA2 in combination with fly ash and RDF on shoot dry weight (g/plant), dry weight of leaves, and dry weight of roots (g/plant) at different stages of *Sorghum*

Treat-ment	Dry weight of shoot(g)			Dry weight of leaves(g)			Dry weight of roots(g)		
	30 DAS	60 DAS	At harvest	30 DAS	60 DAS	At harvest	30 DAS	60 DAS	At harvest
T1	0.060	0.162	1.646	0.089	1.172	2.622	0.055	0.086	0.094
T2	0.093	0.181	1.684	0.324	1.377	2.814	0.083	0.124	0.147
T3	0.072	0.133	1.529	0.236	1.291	3.672	0.061	0.091	0.113
T4	0.124	0.176	2.661	0.214	1.264	2.154	0.171	0.195	0.204
T5	0.231	0.291	3.711	0.847	1.792	4.213	0.180	0.226	0.252
T6	0.103	0.142	2.633	0.498	1.527	3.141	0.111	0.201	0.220
T7	0.091	0.122	2.602	0.392	1.615	2.118	0.093	0.192	0.211
T8	0.136	0.206	2.754	0.611	1.688	3.222	0.124	0.233	0.246
T9	0.245	0.293	4.821	0.993	1.981	4.289	0.196	0.287	0.298
T10	0.193	0.222	2.705	0.572	1.642	3.186	0.131	0.242	0.265
T11	0.175	0.191	2.693	0.461	1.609	3.165	0.101	0.201	0.243
SEm±	0.002	0.005	0.047	0.011	0.017	0.043	0.002	0.002	0.003
CD at (5%)	0.008	0.015	0.138	0.034	0.052	0.128	0.006	0.008	0.009

1)T1-Control, 2)T2-Recommended dose of fertilizer (RDF)100%, 3)T3-RDF 75%, 4)T4-5% 'Flyash+ 75% RDF 5)T5-10%Flyash + 75% RDF 6)T6-20% Flyash + 75% RDF 7)T7- 30% Flyash +75% RDF, 8)T8-5% Flyash+ BUDA2+ 75%RDF,9) T9-10% Flyash + BUDA2+ 75%RDF, 10)T10- 20% Flyash+ BUDA2+ 75%RDF 11) T11- 30% Flyash + BUDA2+ 75%RDF

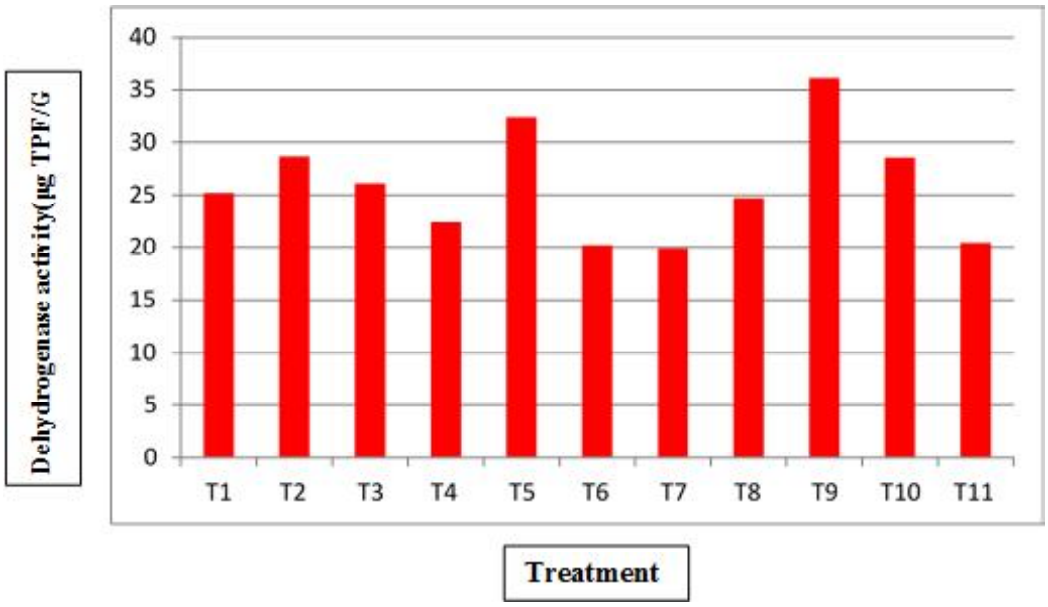

Fig. 3. Effect of BUDA2 in combination with fly ash and RDF
On Dehydrogenase activity of soil

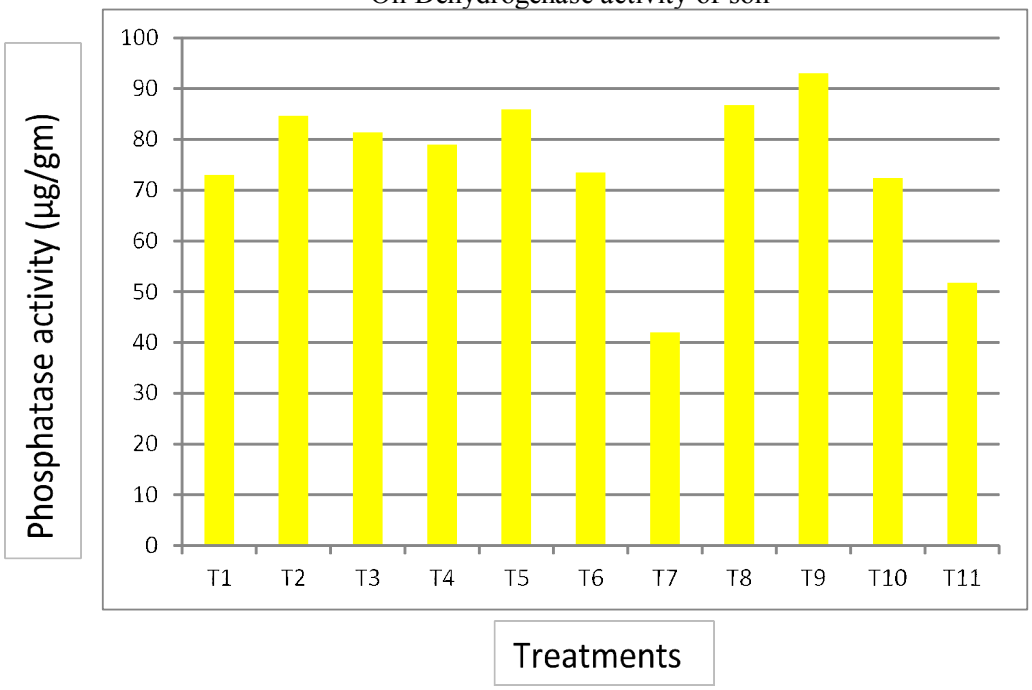

Fig. 4. Effect of BUDA2 in combination with fly ash and RDF
On Phosphatase activity of soil

Table-4. Effect of BUDA2 in combination with fly ash and RDF on dehydrogenase activity and phosphatase activity of soil

Treat-ment	Dehydro-genase Activity (Mg Tpf/G Soil)	Phosphatase Activity/hour (µg of P- nitro phenol/gm of soil)
T1	25.2	73.00
T2	28.6	84.66
T3	26.1	81.42
T4	22.4	79.01
T5	32.3	85.90
T6	20.2	73.51
T7	19.9	42.00
T8	24.7	86.75
T9	36.1	93.04
T10	28.5	72.38
T11	20.4	51.78
SEm±	0.32	0.811
CD at (5%)	0.95	2.393

1)T1-Control, 2)T2-Recommended dose of fertilizer (RDF)100%, 3)T3-RDF 75%, 4)T4-5% Flyash+ 75% RDF 5)T5-10%Flyash + 75% RDF 6)T6-20% Flyash + 75% RDF 7)T7-30% Flyash +75% RDF, 8)T8-5% Flyash+ BUDA2+ 75%RDF,9) T9-10% Flyash+ BUDA2+75%RDF,10)T10-20% Flyash+ BUDA2+ 75%RDF 11) T11- 30% Flyash + BUDA2+ 75%RDF

Agrochemical are increasingly used to meet the feed needs of a growing population. The continuous use of chemical fertilizers over the years has been detrirmental to soil health as well as quality. A total ninty nine rhizosphere bacterial isolates were isolated from rhizosphere soils of cereals. Based on screening results, two isolates BUDA1 and BUDA2 were selected as efficient *Azotobacter* isolate the BUDA2 showed the highest PGPR activity. PCR amplification of bacterial specific 16s Rrna gene (1500 bp) was carried out by using the following primers F27 (5'AGAGTTTG-AATCMTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3') and confirms and identified as *Azotobacter chroo-coccum*, the strain showed closest homology with *Azotobacter* sp (closer to chroococcum). Phylogene also showed closest homology with *Azotobacter* sp. (closer to chroococcum). Evolutionary analyses were conducted[60] in MEGA6. The inoculation of BUDA2 along with 10% fly ash and 75% RDF led to significant increased growth parameters of sorghum. In addition the isolate showed significant increase in mg N_2 fixed g^{-1} of mannitol was superiorly 13.3 g^{-1} of mannitol which is superior over reference strain and over control plants which is not received by inoculums of any Bacteria.

References :

1. Abd El-Fattah D. A, W. E Ewedab, M. S Zayed, and M. K Hassaneina (2013) Effect of carrier material, sterilization method, and storage temperature on survival and biological activities of *Azotobacter chroococcum* inoculants. The prokaryotes, vol. 2. Berlin, Heidelberg: Springer Verlag. P. 795-817. 2.Ann Agric Sci. *58:* 111-118.
2. Ali A. Abdel Salam, H. Elhosainy Omar, R. Wissam, A. Zahra Mohamed, Salam Abdel and A. Hashem Inas (2015) *Egypt. J. of Appl. Sci., 30*(7):
3. Altschul S. F, W Gish, W Miller, E. W Myers, and D. J. Lipman (1990) *J. Mol. Biol*. Oct 5, *215*(3): 403-10. Pubmed

PMID: 223171.

4. Aneja, K. R (2002) Experiments in microbiology plant pathology, tissue culture and mushroom production technology. New age International Publishers, 3rd edn: pp. 165-167.
5. Becking, J.H. (1981) The family Azotobacteraceae In: Starr MP, Stolp H, Triiper HG, Balows A, Schlegel HG, editors.
6. Bhardwaj Deepak, Ansari Mohammad Wahid, and Tuteja Narendra. (2014) Microbial Cell Factories 13, Article number: 66.
7. Bottalica A. (1998). *J. Plant pathol. 80:* 85-103.
8. Bjelic Dragana D.,B. Marinkovic Jelena, B. Branislava Tintor, Lj. Tancic Sonja, M. Nastasic Aleksandra, and B. Mrkovacki Nastasija (2015) *Matica Srpska J. Nat. Sci. Novi Sad, 129:* 65-72.
9. Chi F, P Yang, F Han, Y Jing, and S. Shen (2010) *Proteomics. 10:* 1861-1874
10. Chih Li Yu, Deng Qi, Jian Siyang, E. Jianwei Li, Kudjo Dzantor and Hui Dafeng (2019). *Environmental pollution, 250:* 137-142.
11. Dadook Mohammad, Mehrabian Sedigheh, Salehi Mitra and Irian Saeed. (2014) *Jundishapur J Microbiol, 7*(4): e 9415.
12. Davison R. L., D. F. S. Natusch, J. R. Wallace, J.R. Evans, and C.A.(1974) *Environmental Sciencesand Technology 8:* 1107-1113.
13. Dick R. P., D. P. Breakwell, R. F. Turco (1996) Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In Doran J.W., Jones. A. J. (Eds). Methods of assessing soil quality. SSSA special publication 49. Soil science society of of America Madison. WI. USA. PP. 247-271.
14. Doifode V.D. (2021) Effect of biofertilizer on the growth and yield of sorghum crop. *Science Progress and Research. 1*(2): 19-23.
15. Dwivedi S, RD Tripathi, S Srivastava, S. Mishra, M.K. Shukla, K. K. Tiwari, R Singh and U.N. Rai (2007) *Chemosphere 67:* 140-151.
16. Elseewi A.A., A.C. Chang and I. Straugham (1980) *J. Environ. Qual, 9:* 333-344.
17. Felsenstein J. (1985). *Evolution 39:* 783-791.
18. Gertz E.M. (2005) Blast Scoring Parameters, 16 March 2005.
19. Gupta Dharmendra K., N. Rai Upendra, Inouhe Masahiro (2002). *Journal of Pant Research 115:* 401-409.
20. Gupta Avinash and Mayuri Deshmukh (2022) Varietal response to graded levels of nitrogen and bio-fertilizers on forage yield of Sorghum (*Sorghum bicolor* L. Moench) during the summer season. *International Journal of Current Microbiology and applied sciences. 11*(07):
21. Hamid Abdel, S. Marva; A. F. Elbaz; A.A. Ragab; H.A. Hamza and K.A. EI Halafawy (2010) *J. Of Agricultural Chemistry and Biotechnology, 1*(2): 93–104.
22. Hamid Mukhtar, Hina Bashir, Ali Nawaz, and Ikramul Haq. (2018) *Journal of Bacteriology and Mycology. 6*(5):
23. Humpries E. C. (1965) Mineral components and ash analysis, In:Modern Methods of plant Analysis. Ed. Peach, K. and M. V. Tracey, Springer, Verlag, Berlin, pp. 468-502.
24. Inam A.(2007) *Pollution res; 26*(1): 34-42.
25. Inamdar S., R. U. Kantikar, and M. G. Watve (2000). *Current sciences. 25:* 234-240.

26. Islam M.Z., D.I. Sharif and M. A. Hossain (2008). *J. Soil. Nature 2*(3): 16-19.
27. Kakkad Ajeet and Debbarma Victor (2013) *International Journal of plant and soil science. 35*(8): 90-97.
28. Kalaiarasi, R. and S. Dinakar (2015) *International Journal of Current Microbiology and applied sciences 4*(10) *:* 190-196.
29. Karlin. S. and S. F. Altschul (1990) *Proc. Natl. Acad. Sci. USA 87:* 2264-2268.
30. Karthick A. and V. S. Jayashree (2008); *Nature Environment and Pollution Technology, 7:* 123-126.
31. Kasa Parameshwari, Modugapalem Hemalatha and Battini Kishori (2015) *The Journal of Natural Science, Biology and Medicine 6*(2): 360-363.
32. Khan M. R. and K. Singh (1997) *Plant Pathol, 46:* 33-43.
33. Khan R. K. and Khan. (1996) *Environ. Pollut, 92:* 105-111.
34. Kimura M. (1980). *Journal of Molecular Evolution 16:* 111-120.
35. Kizilkaya Ridvan (2009) *Journal Environ Biol. 30*(1): 73-82
36. Kohli. S. J., and D. Goyal (2010) *Acta Agrophys 16:* 327-335.
37. Klein D. A., T. C. Loh, and R. L. Goulding (1971). *Soil Biology and Biochemistry. 3*(4): 385-387.
38. Kumar Vipin and Pramila Gupta (2010) *Research Journal of Agriculture and Biological Sciences, 6*(3): 280-282.
39. Lee H, HS Ha, and CH Lee, *et al.* (2006) *Bioresour. Technol. 97:* 1490-1497.
40. Mishra L.C. and K. N. Shukla (1986). *Environ Pollut, 42:* 13.
41. Nag Navin Kumar, Biplab Dash, Gupta Shyam Bihari, Khokher Dharmendra and Ravindra Soni. (2018) Evaluation of stress tolerance of *Azotobacter* isolates. Article in *Biologija. 64*(1):
42. Nayak A. K., R. Raja, K. S. Rao, A. K. Shukla, Sangita Mohanty, Shahid Mohammed, Tripathy R., B.B. Panda, P. Bhattacharyya, B. Anjani Kumar, Lal, S.K. Sethi, C. Puri, D. Nayak and C.K Swain (2014) *Ecotoxicology and Safety.* http://dx.doi.org/10.1016/j.ecoenv2014.03.033.
43. Pati. S.S. and S.K. Sahu (2004) *Geoderma 118:* 289-301.
44. Raghav D. and A. A. Khan (2009) *Thai Journal of Agriculture Sciences 35:* 187-194.
45. Rai Pallavi and Kehri Harbans Kaur. (2017) *J. Indian bot. Sci. 96*(1&2): 1-17.
46. Rautaray S. K., B. C. Ghosh, and B. N. Mittra (2003). *Bioresource technol. 90:* 275-283.
47. Rizvi R. and A. A. Khan (2009). *Biology and Medicine, 1:* 20-24.
48. Razmjooei, Zahra, Mohammad Etemadi, Asghar Ramezanian, Abarghuei Faezeh Mirazimi and Alizargar Javad. (2022) *MDPI. Journal plants. 11*(3): 406; https://doi.org/ 10.3390/ plants 11030406.
49. Sahoo R. K., M. W Ansari, M Pradhan TK Dangar, S Mohanty, and N. Tuteja (2014) *Plant signalling & Behaviour 9:* e29377; PMID:24874118.
50. Saitou N. and M. Nei (1987). *Molecular biology and evolution 4:* 406-425.
51. Sandeep Upadhyay, Narendra Kumar, V.K. Singh and Anshuman
52. Singh. (2015) *Journal of Applied and Natural Sciences 7*(2): 984-990.
53. Singh D, K Raghuvanshi, S. K Pandey, and PJ. George (2016) *Research Environmental Life and Sciences. 9*(3): 385-386.
54. Singh K., A.A. Khan and Safiuddin (2011)

Nematol medit. 39: 127-131.

55. Suliasih and Sri Widawati, (2017) Effect of plant growth promoting rhizobacteria and molasses on seed germination and seedling growth of Sorghum bicolour L. Moench The 1st satreps conference, Bogor. Nov 2016
56. Sultana, Uzma, Suseelendra Desai and Gopal Reddy (2016) *World Journal of Microbiology. 3*(1), 043-049.
57. Swapna K. Vendan Tamil, Mahadevaswamy, D.S. Aswathanarayana and R.C. Gundappagol. (2017) *International Journal of Current Microbiology and Applied Ssciences, 6*(11):
58. Tabatabai M. A. (1994) Soil enzymes in: weaver. RW., Angle. J.S., Bottomley. P. S. (Eds). Methods of soil analysis. Part 2: Microbiological and biochemical properties. Soil Sciences Society of America. Madison. Wl. Pp. 775-833.
59. Talabani, SH. K., O.A. Fattah, and A. K. Khider, (2019) *Applied Ecology and Environmental Research 17*(5): 12491-12506.
60. Tamura K., G. Stecher, D. Peterson, A. Filipski and S. Kumar (2013). *Molecualar Biology and Evolution 30 :* 2725-2729s
61. Upadhyay Sandeep, Narendra Kumar, V.K. Singh and Anshuman Singh (2015) *Journal of Applied and Natural Sciences 7*(2) : 984-990.
62. Wankar S. S. and V. S. Wadhai. (2017) *International Journal Current Research in Biology and Medicine. 2*(4):