CHARACTERIZATION OF MICRORGANISMS FROM PROBIOTIC PRODUCTS

In the present study, the main focus was on the isolation and genotypic characterization of microorganism isolated from probiotic products of agricultural importance. Diversity of probiotic microorganisms was studied using 16S rDNA gene sequence analysis for species identification. Furthermore, plant growth promotion of probiotic microorganisms was investigated and also effect of these microorganisms on growth sorghum seedlings was studied.

ISOLATION OF PROBIOTIC MICROORGANISMS

Three different types of samples of varying fermentation time were used for the isolation of probiotic microorganisms and each of these three samples was designated as S1 (T1, T2, T3), S2 (T1, T2, T3) and S3 (T1, T2, T3). All the samples (except S3) were enumerated for microbial count on second day of preparation, maturity (8^{th} day for S1 and S2; 16^{th} day for S3) and expiry 22^{nd} day for S1 and S2. The sample S3 could not be processed at expiry date as it got totally dried up. For the isolation of different type of microorganisms, tenfold serial dilution of the samples were made and plated using different medias such as King's B media for fluorescent pseudomonas, Soil Plate Count Agar (SPCA) for broad range of bacteria, Pikovskaya's media for phosphate-solubilising bacteria and Kenknight's media for actinomycetes. Based on colony morphology, four to five different types of colonies were selected from each of the sample making a total of 89 isolates. Selected isolates were streaked on the respective media to obtain pure cultures and photographed.

Screening on King's B

Further pure cultures were screened for variable morphological types using King's B media, which showed dirty and milky white round mucoid colonies, dry white irregular colonies, mucoid colonies with irregular dry margins and white shiny raised, yellow smooth, white flat colonies. Based upon the variable colony morphology, different types of colonies were selected for further studies. The selected isolates were preserved in slant culture.

Molecular Characterization of the Isolates

All the isolates were grown overnight in Trypticase soya broth and DNA was isolated using standard protocol.

PCR for amplification of 16SrDNA: PCR amplification of 16S rRNA gene of the selected isolates was done under standard conditions using the Universal primers (5'AGAGTTTGATCCTGGCTCAG3') and reverse primer (5'AAGGAGGTGATCCAGCCGCA 3'). The amplified product was visualized on agarose gel.

ARDRA (Amplified Ribisomal DNA Restriction Analysis)

To study the diversity in the isolated microorganisms, three restriction enzymes *Bam* HI, *Eco* RI, *Hind* III were used together for restriction of amplified 16rDNA. After the restriction product was run on 1.5% agarose and the banding pattern for each sample was recorded.

Sequencing: Amplified 16rDNA samples of selected isolates representing all the groups based on ARDRA results were sent for sequencing (XCELRIS Labs)

Plant Growth Promoting Traits

Selected ten isolates were tested for PGP traits like P and Zn solubilization, IAA, Siderophores, HCN production, Antagonism against fungal plant pathogens (*Macrophomina phaseolina* and *Sclerotium rolfsii*) etc using standard protocols.

Plant Growth studies

The ten selected isolates with PGP traits were tested as seed inoculants on sorghum to evaluate their effect on plant growth under sterile soil in plastic cups.

Results

Isolation and purification of Microorganisms

After incubation 0.5mm to 3mm sized different colony morphologies were observed, such as dirty and milky white mucoid round colonies with regular margins, transparent and opaque dry white colonies with irregular margins, Irregular and regular dry colonies, orange round colonies, yellow smooth regular colonies, dirty white shiny round colony, dry flat colonies on Kings B and milky white shiny and mucoid regular colonies and dry irregular and round dirty white colonies and dry raised colonies on SPCA media and Pinpoint round white shiny colonies with entire margin were observed on Pikovskaya's (Fig. 1-4) and plate count was performed for

different set of samples for enumeration studies. In general it was observed that there was increase in population of bacteria from 2nd day to maturity day and further decrease up to expiry day (Table 1), indicating that the reduction in the number of microorganisms can be related with expiry of the sample. Interestingly good population of efficient P-solubilizers was observed in all the samples, indicating potential of the product for plant growth promotion. Further morphologically not much difference was observed among different treatments. Therefore 3-4 morphotype from each sample type were selected for molecular studies.

When streaking on King's B media, the presence of 7-8 morphotypes with dominance of dirty and milky white round mucoid colonies, pseudomonas type colonies, mucoid colonies with irregular margins and white shiny raised, yellow smooth, white flat colonies respectively was observed (Fig. 6). Based upon the variable colony morphology, different types of colonies were selected for further studies. The selected isolates were preserved in King'B slants under refrigerated conditions.

Table 1.1 Total count of bacteria on SPCA medium

TOTAL COUNT (cfu/ml)			
Sample	**2nd Day**	**After 7 Day**	**14 days (Maturity)**
S1T1	**76×10^7**	**25×10^7**	**21×10^7**
S1T2	**20×10^7**	**16×10^7**	**3×10^7**
S1T3	**1×10^7**	**37×10^7**	**25×10^7**
S2T1	**16×10^7**	**16×10^7**	**3×10^7**
S2T2	**16×10^7**	**10×10^8**	**6×10^7**
S2T3	**80×10^7**	**10×10^7**	**6×10^7**
	2nd Day	**After 21 days**	
S3T1	**37×10^7**	**38×10^7**	**-**
S3T2	**38×10^7**	**3×10^8**	**-**
S3T3	**12×10^8**	**5×10^8**	**-**

Table 1.2 Total count of bacteria on King's B medium

Sample	**TOTAL COUNT (cfu/ml)**		
	2nd Day	After 7 Day	14 days (Maturity)
S1T1	**50×10^7**	**10×10^7**	**28×10^7**
S1T2	**33×10^7**	**35×10^7**	14×10^7
S1T3	7×10^6	**38×10^7**	**25×10^7**
S2T1	26×10^6	**15×10^7**	**36×10^8**
S2T2	**5×10^7**	**10×10^8**	**18×10^7**
S2T3	**8×10^7**	**5×10^7**	**90×10^7**
	2nd Day	**After 21 days**	
S3T1	**23×10^7**	**76×10^7**	-
S3T2	**10×10^7**	**23×10^8**	-
S3T3	**65×10^7**	**16×10^8**	-

Table 1.3 Total count of bacteria on Pikovskaya medium

Sample	**TOTAL COUNT (cfu/ml)**		
	2nd Day	After 7 Day	14 days (Maturity)
S1T1	**8×10^7**	**26×10^7**	**1×10^6**
S1T2	**40×10^7**	22×10^7	2×10^6
S1T3	**4×10^7**	**28×10^7**	**8×10^6**
S2T1	**6×10^7**	19×10^7	**1×10^6**
S2T2	**3×10^7**	51×10^7	**2×10^6**
S2T3	**8×10^7**	**1×10^7**	**1×10^6**
	2nd Day	**After 21 days**	
S3T1	**25×10^7**	**12×10^7**	--
S3T2	**13×10^7**	**15×10^7**	--
S3T3	**72×10^7**	**15×10^8**	--

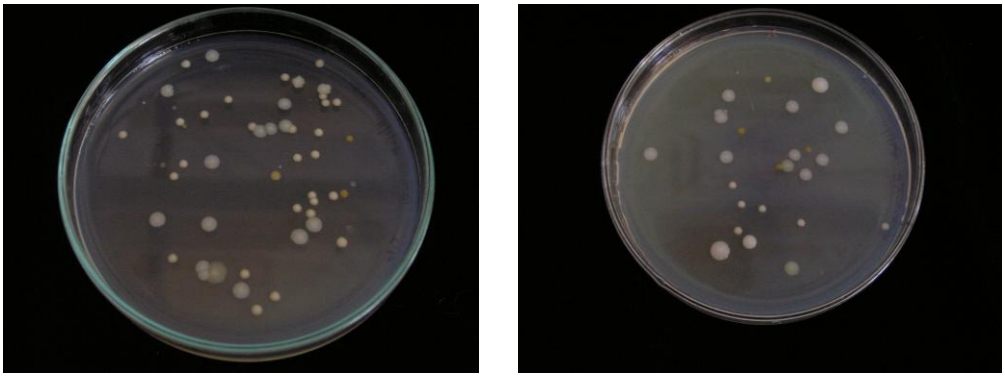

Fig. 1 Colony morphology in King's B Media

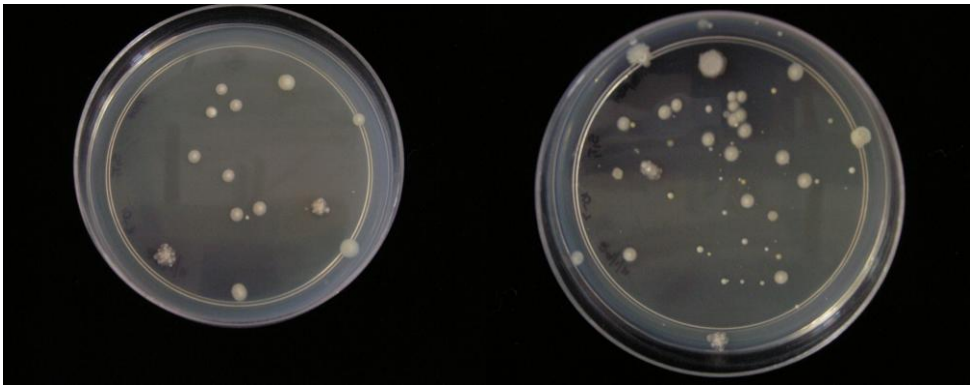

Fig. 2 Colony morphology on SPCA Media

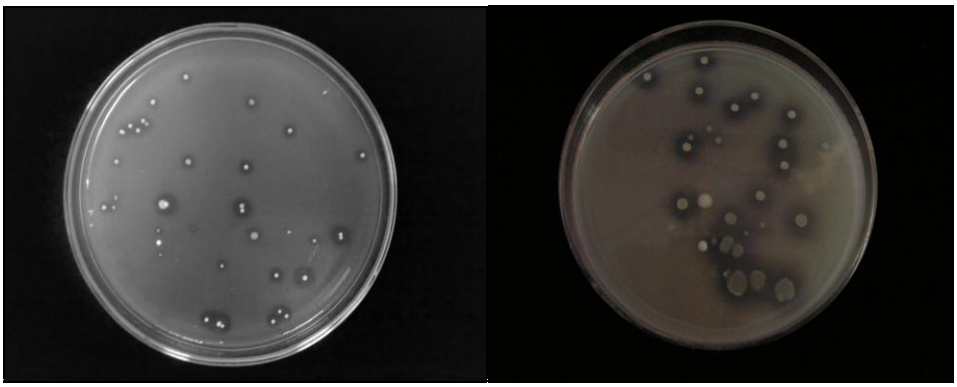

Fig. 3 Colony morphology in Pikovskaya's Media

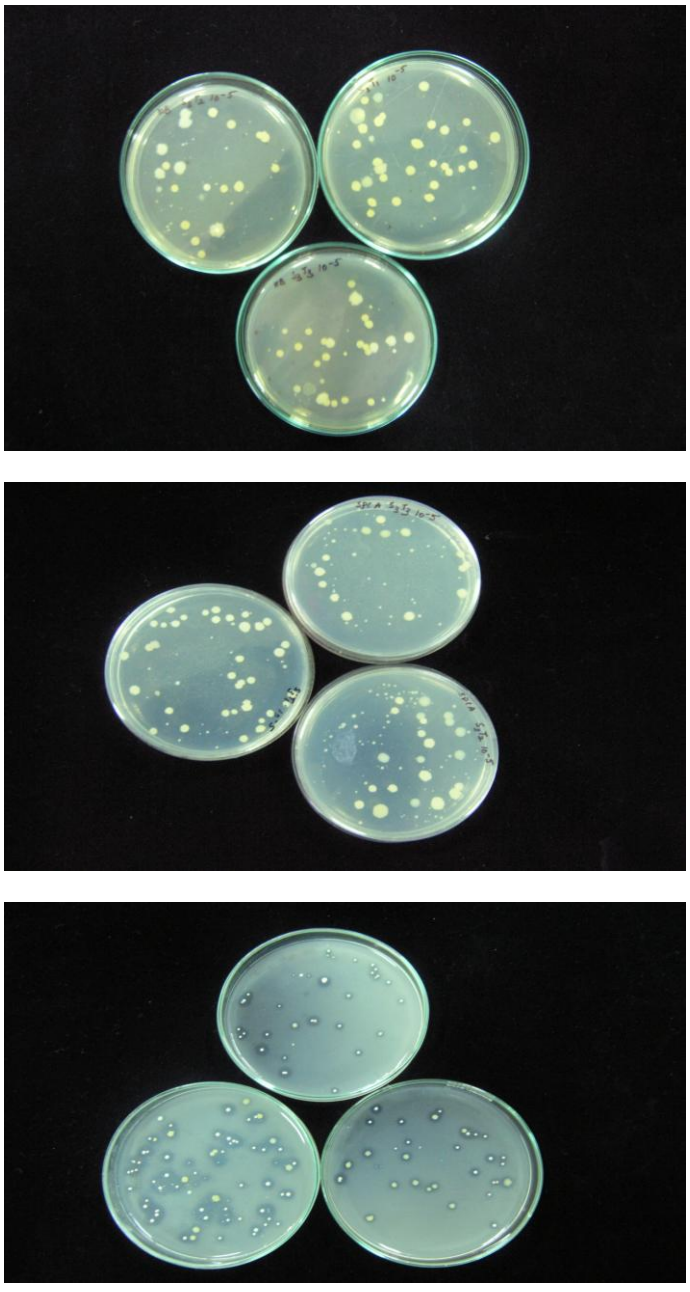

Fig : 4 Variants of S3 (S3T1, S3T2, S3T3) sample showing similar types of Morphotypes on King's B, SPCA and Pikovskaya's Media

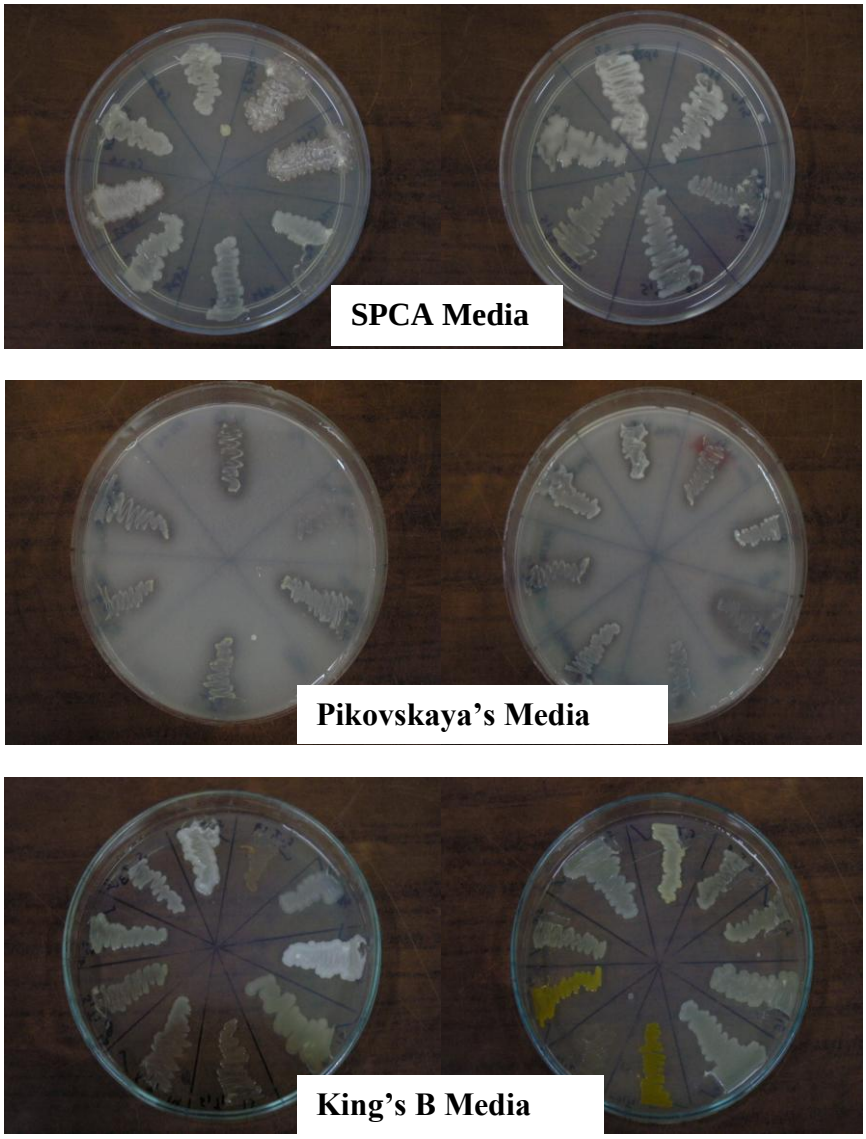

Fig. 5 Purified Isolates on respective media

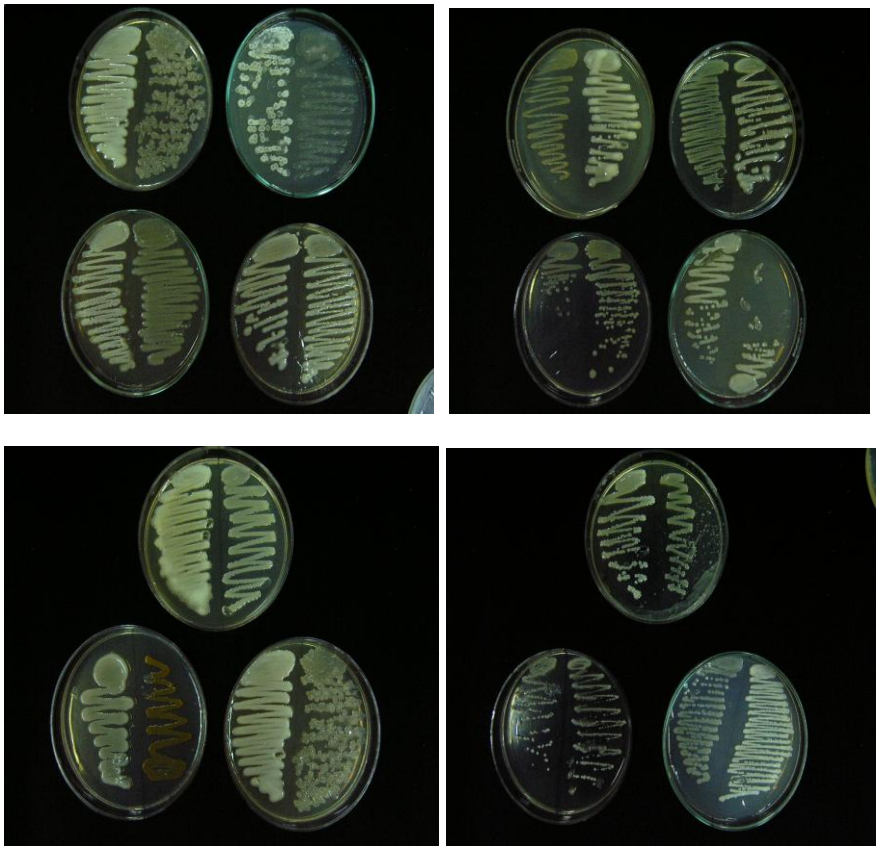

Fig. 6 Different Morphotypes on king's B Media

GENOTYPIC CHARACTERIZATION OF PROBIOTIC MICROORGANISMS

DNA Isolation and PCR

Molecular identification of the selected isolates was performed by the isolation of Genomic DNA (Fig.7) followed by 16S r DNA gene analysis using universal primers (Fig. 8). A total of 89 DNA samples were isolated and PCR was also performed. Amplified product (1500 bp) for samples was observed, which was used for ARDRA.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Phylogenetic analysis was done using RFLP analysis and 16S rDNA sequencing method. RFLP was performed using different restriction enzymes. Seventy three isolates with good PCR product showed Eight different types of banding pattern named as BP1-BP8 (Fig. 9, Table 1). The major group was formed by 56 isolates giving banding pattern BP-2, indicating similarity in these organisms. Further based

on the restriction band pattern, the samples were selected for identification to species level by sequencing.

Sequencing of Amplified 16SrDNA product

The 16SrDNA product of selected isolates representing each of the ARDRA banding patterns were sent for sequencing for identification of isolates at Genus and Species level. (The results are awaited soon)

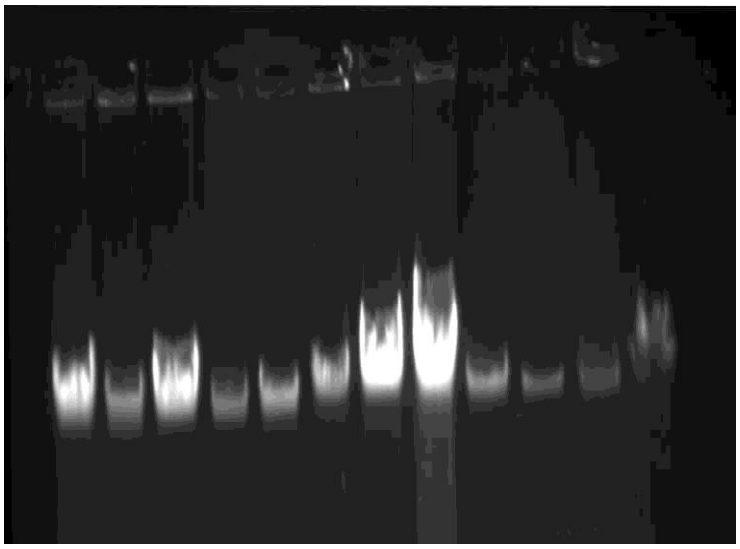

Fig. 7 Genomic DNA run in agarose Gel Electrophoresis

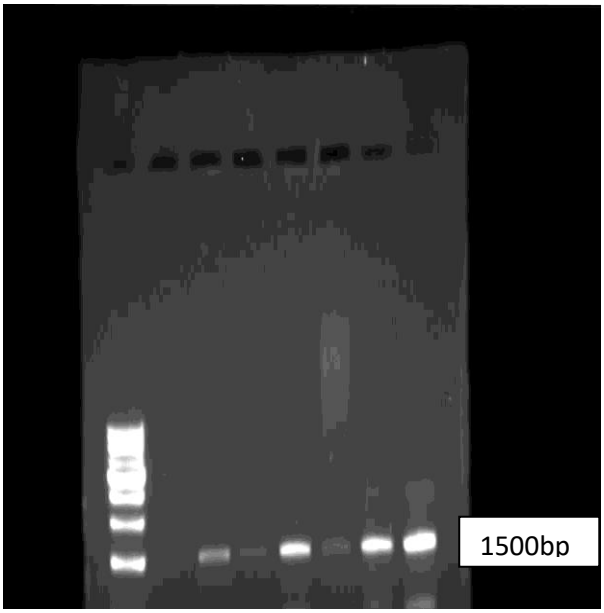

Fig. 8 Amplified 16S rDNA (1500 bp) in agarose gel

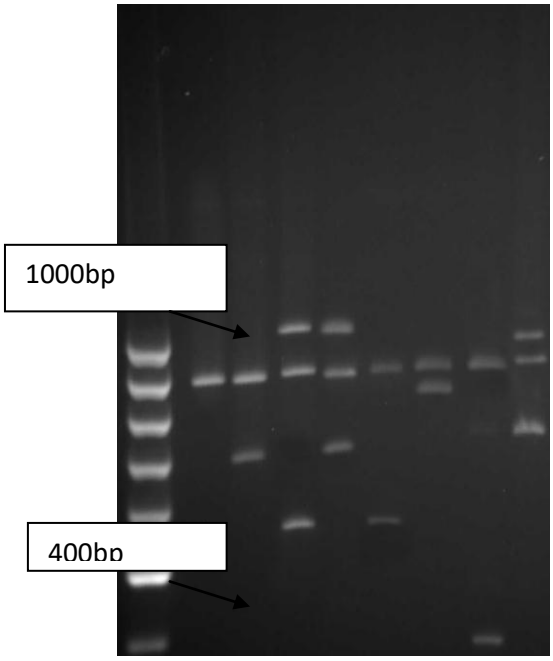

Fig. 9 ARDRA of PCR product (16SrDNA) using three restriction enzymes

Table 2: Grouping of isolates according to ARDRA banding pattern

Banding Patern	No. of Isolates
BP-1	4
BP-2	56
BP-3	1
BP-4	1
BP-5	3
BP-6	2
BP-7	1
BP-8	5

PLANT GROWTH PROMOTING (PGP) PROPERTIES OF SELECTED ISOLATES

Each of the selected isolates exhibited multiple PGP properties, indicating their potential as plant growth promoting bacteria (Table 3). All were positive for Indole Acetic Acid (IAA a plant growth hormone) production, P-solubilization, an important nutrient for plant, ammonia production. Antifungal activity against fungal plant pathogens, *M. Phaseolina* and *S. Rolfsii* was exhibited by 8 and 1 isolate respectively. Eight out of ten isolates tested showed antagonisms, indicating strong antifungal properties of probiotic product. This may be the reason for not getting fungal isolates from probiotic products at the time of isolation.

Table 3: Plant Growth Promoting Properties of Selected Bacteria

Isolates	IAA µg mg^{-1} Protein	P-Solubilization (ppm)	NH_3 Prod.	Antifungal activity		HCN	Sidero-phore
				M. phaseolina	*S. rolfsii*		
SP3 S3T2	46.8±0.12	59.23±1.531	+++	++	-	-	-
SP1 S3T3	90.8±0.41	97.24±1.47	++	+++	-	-	-
SP11 S2T2	49.2±0.26	64.41±1.24	++	+++	+++	-	-
SP12 S3T3	58.4±0.67	70.45±1.84	+	+	-	-	-
SP14 S3T1	39.0±0.13	42.41±1.31	+++	++	-	-	-
PK14 S1T3	36.6±0.92	136.4±1.08	+++	-	-	-	-
KB4 S3T2	48.4±0.89	50.08±1.57	++	++	-	-	-
KB3 S1T3	58.4±0.59	60.01±1.42	++	++	-	-	-
KB27 S1T1	43.4±0.81	61.41±1.22	+	+	-	-	-
KB18 S2T2	56.8±0.47	83.96±1.34	+++	-	-	-	-

GROWTH STUDIES

All the ten isolates tested as seed inoculants in sorghum showed remarkable difference in plant growth and shoot height. Observation on biomass and other plant growth parameters are in the progress.

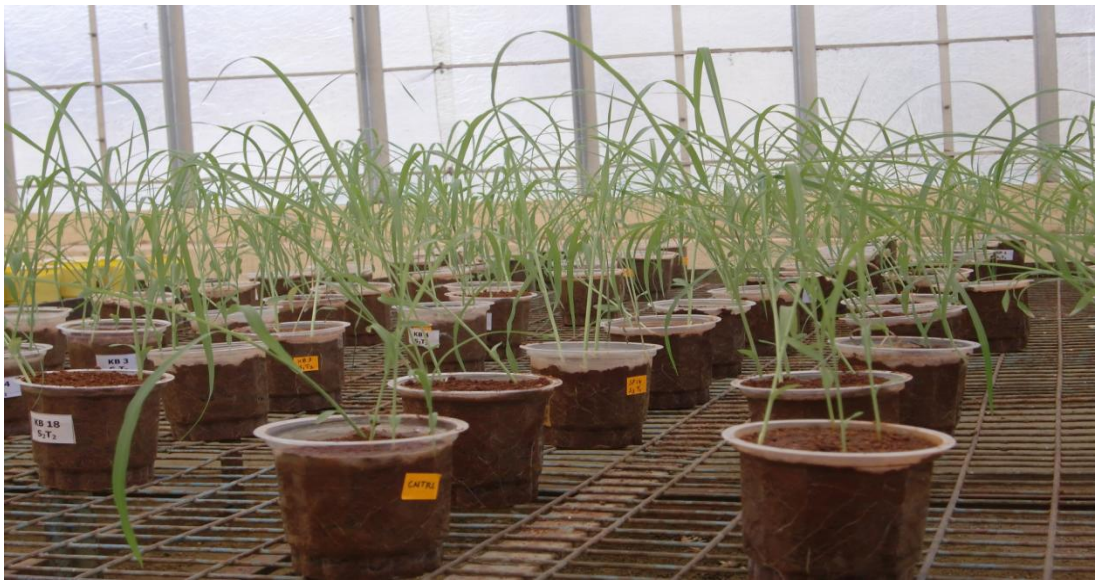

Fig.10. Plant Growth Experiment using Sorghum as a test crop and ten bacterial isolates as seed inoculants (Overview)

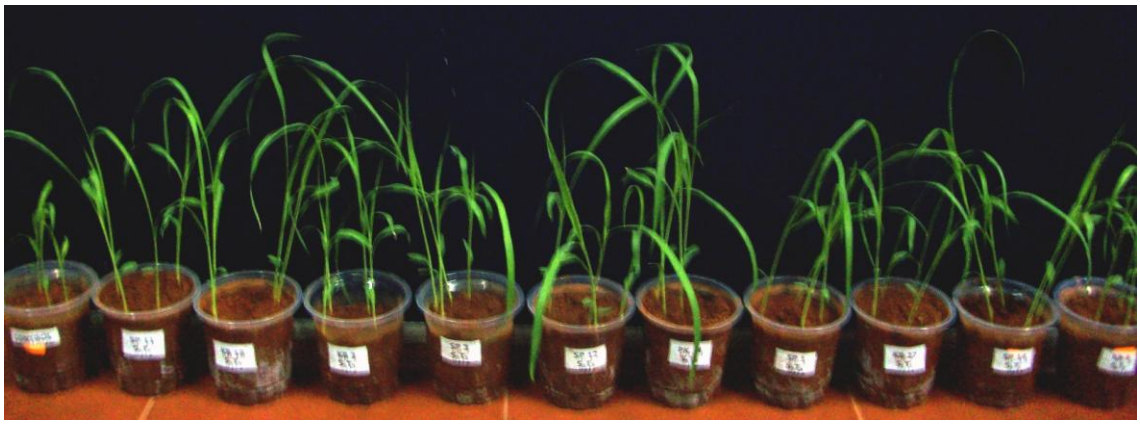

Fig. 11. Effect of bacterial isolates on plant growth of sorghum compared with uninoculated control (extreme left)

Conclusion: The probiotic products contain bacteria with multiple plant growth promoting traits and can be exploited as potential biofertilizers