Asian J. Adv. Basic Sci.: **2017, 5***(2),* 127-135
ISSN (Print): 2454 – 7492
ISSN (Online): 2347 – 4114
www.ajabs.org

Simple and Effective Method for Development of Longer Shelf Life Liquid Formulation with *Azospirillum brasilense* strain Asp-7

Mounika N.[1*], Muralidhara Rao D.[2], Uma A.[3], Sandhya V.[1] and Ali SkZ[1]

[1*]*Department of Microbiology, Agri Biotech Foundation, PJTSAU Campus, Rajendranagar, Hyderabad, Telangana state, INDIA*
[2]*Department of Biotechnology, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, INDIA*
[3]*Department of Biotechnology, Jawaharlal Nehru Technological University, Hyderabad, Telangana State, INDIA*

[*] *Correspondence: E-mail: mounika.nagabhairava@yahoo.com*

(Received 07 Dec, 2017; Accepted 20 Dec, 2017; Published 31 Dec, 2017)

ABSTRACT: Liquid formulation is an upcoming technology to overcome the limitations associated with carrier based formulation. In our study a total of nine *Azospirillum* spp. were isolated, screened for plant growth promoting traits (PGP) like indole acetic acid (IAA), ammonia, phosphate solubilisation and siderophore production. The selected strain Asp-7 was showed tolerance to drought and temperature stress. Biochemical and molecular characterization of the strain Asp-7 was done and identified as *Azospirillum brasilense* by 16S rDNA sequence analysis and the gene sequence was submitted to Genbank under the accession number KT374217. The *Azospirillum brasilense* strain Asp-7 was further screened with different polymeric additives to support growth and shelf life of liquid formulation. Among the additives tested PVP at 4% concentration showed the highest cell population. Liquid formulation of *Azospirillum brasilense* strain Asp-7 formulated with PVP was evaluated for its shelf life for 16 months. The results of the present study confirmed that addition of PVP has enhanced the shelf life of liquid formulation of *Azospirillum brasilense* strain Asp-7.

Keywords: *Azospirillum brasilense*; Liquid formulation; Plant growth promoting traits; Polymeric additives; Shelf life and Temperature stress.

INTRODUCTION: In most of the agricultural systems nitrogen is one of the major limiting nutrient that dictates crop productivity. Despite its presence in larger quantities in the atmosphere, plants cannot utilize nitrogen since it is inert.[7] Nitrogen is made available in form of chemical fertilizers, and extensive use of these chemical fertilizers causes environmental pollution and kills different beneficial microorganism in soil. Bioinoculants are environmental friendly and these are one of the best modern tools for agriculture.[50] Bioinoculants upon inoculation enhance the plant growth by nutrient mobilization, nitrogen fixation, phytohormone production and induction of defence mechanisms against biotic and abiotic stresses.[27,21,6,38,22&2] In India most of the bioinoculants are solid carriers, with certain constraints such as lower shelf life, high degree of contamination, unavailability and quality inconsistency of carriers, poor survival under adverse environmental conditions and unpredictable field performance.[5 & 28]

Liquid formulations/Liquid bioinoculants is one of the solution to defeat the problems associated with solid carriers.[26] Liquid bioinoculants containing not only the desired microorganisms and their nutrients but also special cell protectants or additives that promote for longer shelf life and tolerance to adverse conditions.[20 & 28] Many kinds of additives have been used for liquid bioinoculant production because of their ability to limit heat transfer, their good rheological properties, lower dosage use and high water activities.[33 & 34] Recent study also revealed that shelf life of *Azospirillum* spp. enhanced up to 9 months by using trehalose.[45] Viability of liquid bioinoculants will be done using different additives or amendments such as sodium alginate, gum Arabic, polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA) and polyethylene glycol (PEG). Therefore, in the present study, an attempt was made to isolate drought and temperature tolerant *Azospirillum* spp. and to evaluate the optimum concentration of additives that could enhance the shelf life of liquid bioinoculants.

MATERIALS AND METHODS:

Isolation of *Azospirillum spp.*: *Azospirillum* spp. were isolated from rhizosphere soil sample collected from different crop production systems covering arid and semi-arid regions in India. The plants were uprooted and the bulk soil removed by gently shaking and root adhering soil (RAS) was carefully collected and used for isolation of *Azospirillum* spp. by serial dilution[18] and inoculated into nitrogen free bromothymol blue (NFB) semi solid medium and kept

for incubation for three days at 30 ± 2ºC. The presence of *Azospirillum* was indicated by the formation of white pellicle few millimeters below the surface of the medium.[13 & 32] For pure culture**,** a loopful of the pellicle was streaked on to congo red agar medium and incubated at 30 ± 2°C for 48–72 h. Typical pink, often wrinkled colonies formation confirmed successful isolation[1]. The pure cultures were maintained on nitrogen free bromothymol blue (NFB) agar media slants under refrigerated conditions. Fresh broth cultures of each strain were prepared and stored for further experiments.

Plant Growth Promoting (PGP) traits: All the *Azospirillum* strains were tested *in vitro* for plant growth promoting traits. For testing ammonia production by bacterial strains, overnight raised cultures were inoculated in 10 ml of peptone water and incubated at 30°C for 2 days, and 1ml of Nessler's reagent was added. Development of yellow to brown colour indicated ammonia production.[11] For hydrogen cyanide (HCN) production, the overnight bacterial culture was streaked on nitrogen free bromothymol blue (NFB) agar and Whatman no. 1 filter paper disc soaked in 0.5% picric acid (in 2% sodium carbonate) was placed inside the lid of Petri plate. The plates were sealed with para film and incubated at 30°C for 4 days for the development of deep orange color.[4] For siderophore production, 10 µl of overnight raised culture in Luria Bertain broth (LB) was spotted on Chrome Azurol S (CAS) agar plates and incubated at 30°C for 48 – 72 h. Plates were observed for the appearance of orange halo around the bacterial colony. [42] The method of[17] was followed for the estimation of IAA. One ml of the broth culture, raised in LB (amended with 5 mM tryptophan), was centrifuged and supernatant was carefully decanted in a separate test tube; 4 ml of Salkowsky reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) was added to 1 ml of supernatant and then the mixture was incubated for 1h at room temperature for the development of pink colour under dark condition. After incubation, the absorbance was read at 530 nm. For studying phosphate solubilisation[29] 5 µl of overnight raised culture was spotted on Pikovskaya's agar plates containing 2% tricalcium phosphate. The plates were incubated at 30°C for 48–72 h and observed for the appearance of the solubilisation zone around the bacterial colonies.

Pot experiment: The protective effect of inoculated *Azospirillum* strains on maize seedlings was studied under sterile soil under greenhouse conditions. Among the nine strains tested for PGP traits, three strains with highest PGP traits were selected for plant studies. Seeds of maize var. DHM117 were surface sterilized with 0.1% $HgCl_2$ and 70% ethanol and bacterized with overnight (10^8 cfu ml^{-1}) grown bacterial cultures, shade dried and sown in 250 ml plastic cups filled with 300 g of soil. Plastic cups (inoculated and uninoculated) were replicated six times in randomized block method. Soil moisture was maintained constant during the experiment by daily sprinkling with sterile distilled water. After 15 days of germination, inoculated and uninoculated seedlings were harvested for further analysis. Plant experiments were conducted in six replicates to study the variation between inoculated and uninoculated seedlings. Shoot and root length were measured according to the manual method[41] and dry biomass was recorded after drying the samples at 105ºC.

Screening for drought stress: Best strains from plant studies were selected and screened for drought tolerance. LB broth with different water potentials (−0.05, −0.15, −0.30, and −0.49 MPa) was prepared by adding appropriate concentrations of Polyethylene glycol 6000 (PEG 6000)[30 & 40] and inoculated with 1% overnight raised bacterial culture. Six replicates of each strain at each concentration were prepared. After incubation at 30°C under shaking conditions (120 rpm) for 24 h, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer.

Screening for temperature stress: LB broth was inoculated with overnight raised broth culture (1%) and incubated at different temperatures ranging from 30-40°C. Six replicates of each strain at each temperature were maintained. After incubation at respective temperatures under shaking conditions (120 rpm) for 24 h, growth was estimated by measuring the optical density at 600 nm using a spectrophotometer.

Biochemical and molecular characterization: The selected strain Asp-7, 24 h culture was used for different biochemical tests (ONPG, Lysine utilization, Ornithine utilization, Urease, Phenylalanine Deamination, Nitrate reduction, H_2S production, Citrate utilization, Voges Proskauer's, Methyl red, Indole, Malonate utilization, Esculin hydrolysis, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose, Lactose, Oxidase and Catalase) using Enterobacteriaceae identification kit (KB003 Hi25™ Hi media, India).

For molecular characterization, bacterial genomic DNA was isolated[8] and subjected to Polymerase Chain Reaction (PCR) for amplification of 16S rDNA gene using universal forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse (5′CGGTTACCTTGTTACGACTT-3′) primers under standard conditions (initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 40 s, extension at 72°C for 90 s,

and final extension at 72°C for 7 min). The PCR (approximately 1.5 kb) product was purified and sequenced (SciGenom Labs, India). The 16S rDNA gene sequence obtained were compared with the existing database of 16S rDNA gene and submitted to Genbank.

Phylogenetic analysis: Sequence analyses of selected strain were performed using Basic Local Alignment Search Tool (BLAST) available on the NCBI homepage (http://www.ncbi.nlm.nih.gov). Homologous gene sequences were collected from the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence data were aligned with ClustalW 1.6 and evolutionary tree construction was done by using Molecular Evolutionary Genetics Analysis 6.0 (MEGA6) software.[48 & 36] Tree branches were evaluated using the bootstrap method.[14 & 23]

Effect of additives on isolates viability: Nutrient broth was amended with different concentrations of additives such as polyvinyl pyrrolidone (PVP) 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v); gum arabica 0.1, 0.3, 0.5, 0.7 and 0.9% (w/v) and sodium alginate 0.1, 0.2, 0.3, 0.4 and 0.5% (w/v). The experiments were carried out in 250 ml flasks containing 100 ml of amended medium. Late log phase culture of the *Azospirillum* strain Asp-7 was inoculated into test media (1% v/v), and incubated in an incubator shaker at 30°C at 140 rpm. After 10 days of inoculation, the viable cell population were determined by spread plate method on nutrient agar medium.

Shelf life studies of liquid bioinoculants: Liquid bioinoculants of *Azospirillum* strain Asp-7 was prepared in 100 ml of nutrient broth amended with 4% PVP with the cell population adjusted to 7 x 10^{11} cfu ml^{-1} using sterile nutrient broth and made up to 250 ml with sterile nutrient broth containing 4% PVP in sterilized high density polyethylene (HDPE) bottles. Same cell population and quantity was maintained in controls (without PVP 4%). The formulated products was stored at room temperature and assessed for their shelf life at monthly interval up to 16 months.

Statistical analysis: A total of six replicates were used for each parameter and the data obtained was analyzed using analysis of variance (ANOVA) and expressed in mean ± SD of six replicates.

RESULTS AND DISCUSSION:

Results: ***Isolation and screening for PGP traits:*** A total of nine *Azospirillum* spp. were isolated from ten rhizosphere soil samples collected from different crop production systems grown under arid and semi-arid regions of India. *In vitro* screening of the strains revealed variations in the production of PGP traits (Table 1). Among the nine strains, Asp-7 solubilized maximum amount of phosphate (15.37 ppm) followed by Asp-6 (12.29 ppm) and Asp-4 (10.76 ppm). Similarly, strain Asp-7 was the best producer of IAA (7.91 µg/mg protein), followed by Asp-6 (6.93 µg/mg protein), Asp-4 (6.06 µg/mg protein) and Asp-5 (3.84 µg/mg protein). Siderophore production was observed only in strain Asp-7. Furthermore, all the nine strains were negative for HCN production but, positive for ammonia production (Table 1). Based on plant growth promoting traits Asp-4, Asp-6 and Asp-7 were selected for plant studies.

Pot experiment: Effect of *Azospirillum* strain on plant growth promotion of maize seedlings was demonstrated using maize (var. DHM117) as a test crop. Bacterial inoculation significantly enhanced the seedling growth in terms of root, shoot length and dry biomass (Figure 1), compared to uninoculated control. Among three different treatments, maize seed treated with *Azospirillum* strain Asp-7 showed significantly higher shoot (48.2 cm), root length (58.2 cm) and dry biomass (0.733 mg) compared to strains Asp-4, Asp-6 and uninoculated control (Figure 1).

Screening for drought and temperature tolerance: Based on PGP traits and plant studies *Azospirillum* strain Asp-7 was selected for further screening for drought and temperature stress. The strain Asp-7 was evaluated for drought tolerance and could grow at a minimum water potential − 0.49 MPa. The strain Asp-7 was also screened for temperature tolerance on LB broth at different temperatures. Strain Asp-7 was able to grow even up to 40°C.

Biochemical and molecular characterization: Microscopic studies revealed *Azospirillum* strain Asp-7 as Gram negative, motile, rod shape bacteria. The strain Asp-7 was positive for ONPG, oxidase, catalase activity, utilized malonate, citrate showed nitrate reduction, esculin hydrolysis and produced indole (Table 2).

A BLASTN search was performed for nucleotide sequence of partial length of strain Asp-7; the sequence showed a 100% homology with the 16S rDNA sequence of *Azospirillum brasilense* strain AZ 74 (KC920689.1). The sequence was submitted to Genbank under the accession no. KT374217.1. Phylogenetic analysis of 16S rDNA sequence was carried out using neighbor joining method which showed significant bootstrap value ranged from 51% to 80% with *Trichoderma turrialbense* strain CBS 112445 as the out group (Figure 2).

Effect of additives and Shelf life of liquid bioinoculant formulation: Among the three different additives, PVP showed excellent viable count at 4% (9.00 cfu ml^{-1} at 10^9) followed by 3% (7.90 cfu ml^{-1} at

10^9), 5% (6.65 at 10^9 cfu ml^{-1}), 2% (5.94 cfu ml^{-1} at 10^9) and 1% (3.21 cfu ml^{-1} at 10^9). After 4%PVP, viable count dropped. Gum arabic showed highest viable count at 0.5% (7.60 cfu ml^{-1} at 10^9) followed by 0.7% (5.92 cfu ml^{-1} at 10^9), 0.3% (3.92 cfu ml^{-1} at 10^9) and 0.1% (2.90 cfu ml^{-1} at 10^9). After 0.5% gum arabic, viable count dropped. In case of sodium alginate viable count was dropped after 0.2% concentration. Sodium alginate showed very low viable count at 0.2% (5.97 cfu ml^{-1} at 10^9). The obtained results showed excellent viable count at 4% PVP compared to gum arabica and sodium alginate (Figure 3).

In both the cases (liquid formulation with 4% PVP and without PVP), the initial set population 7 x 10^{10} cfu ml^{-1} was maintained. Liquid formulation with 4% PVP, 1st month cell population was 9.30 x 10^{10} cfu ml^{-1}, then dropped with lapse of time. In case of liquid formulation without PVP, the cell population was decreased from 1st month onwards. The 1st month cell population was 9.00 x 10^9 cfu ml^{-1} only noticed. The degree of declination was very fast in liquid formulation without PVP than 4%PVP liquid formulation. The liquid formulation without PVP recorded 3.21 x 10^5 cfu ml^{-1} cell population at the end of 6th month (Table 3). After 6th month growth was not observed. But in the liquid formulation with 4% PVP recorded 6.50 x 10^{10} cfu ml^{-1} cell population at the end of 6th month. In case of 4%PVP liquid formulation, the cell population declination was very slow. The study was continued up to 16 months. At the end of the 16th month 2.00 x 10^9 cfu ml^{-1} cell population was recorded (Table 3).

Table 1: Plant growth promoting traits produced *Azospirillum* spp.

Strains	Phosphate solubilisation (ppm)	IAA (µg/mg protein)	Siderophore	HCN	Ammonia
Asp-1	No zone	-	-	-	+ +
Asp-2	No zone	-	-	-	+ +
Asp-3	No zone	-	-	-	+
Asp-4	10.76 ± 0.12^{a}	6.06 ± 1.28^{a}	-	-	+ +
Asp-5	No zone	3.84 ± 0.57^{b}	-	-	+ ++
Asp-6	12.29 ± 0.67^{b}	6.93 ± 0.23^{ca}	-	-	+ ++
Asp-7	15.37 ± 0.15^{c}	7.91 ± 0.82^{d}	+	-	+ ++
Asp-8	No zone	2.48 ± 0.39^{e}	-	-	+
Asp-9	No zone	-	-	-	+ +

Note: *-, absent; +, presence; +, fair; ++, good; +++, excellent. Values are the means of six replicates with ± SD value. Values with different letters are significantly different at P<0.05*

Table 2: Biochemical characterization of *Azospirillum* strain Asp-7.

Biochemical tests	Strain Asp-7
ONPG	+
Lysine utilization	-
Ornithine utilization	-
Urease	-
Phenylalanine Deamination	-
Nitrate reduction	+
H_2S production	-
Citrate utilization	+
Voges Proskauer's	-
Methyl red	-
Indole	+
Malonate utilization	+
Esculin hydrolysis	+
Arabinose	-
Xylose	-
Adonitol	-
Rhamnose	-
Cellobiose	-
Melibiose	-
Saccharose	-
Raffinose	-
Trehalose	-
Glucose	-
Lactose	-
Oxidase	+
Catalase	+

Table 3: Survival of *Azospirillum brasilense* Asp-7 (cfu ml^{-1}) liquid formulation amended with PVP and without PVP.

Months	Control	PVP	Other bacterial
0	7.00 x 10^{10}	7.00 x 10^{10}	-
1	9.00 x 10^{9}	9.30 x 10^{10}	-
2	5.29 x 10^{9}	9.10 x 10^{10}	-
3	4.00 x 10^{8}	8.72 x 10^{10}	-
4	3.32 x 10^{7}	8.50 x 10^{10}	-
5	2.69 x 10^{6}	7.29 x 10^{10}	-
6	3.21 x 10^{5}	6.50 x 10^{10}	-
7	No growth	5.23 x 10^{10}	-
8	No growth	4.00 x 10^{10}	-
9	No growth	9.21 x 10^{9}	-
10	No growth	9.08 x 10^{9}	-
11	No growth	8.02 x 10^{9}	-
12	No growth	6.59 x 10^{9}	-
13	No growth	6.32 x 10^{9}	-
14	No growth	4.20 x 10^{9}	-
15	No growth	3.50 x 10^{9}	-
16	No growth	2.00 x 10^{9}	-

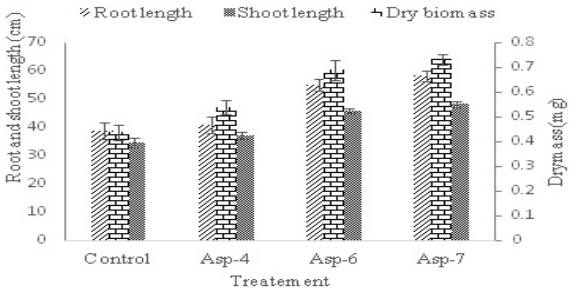

Figure 1: Root, shoot length and dry mass of maize seedlings inoculated with *Azospirillum* spp. strains Asp-4, Asp-6 and Asp-7 along with uninoculated control. Error bars are mean of ± standard deviation with n=6.

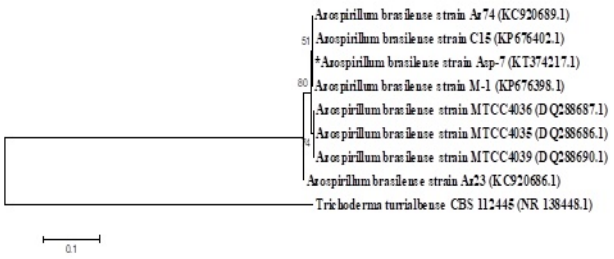

Figure 2: Phylogenetic analysis of *Azospirillum* strain Asp-7 based on 16S rRNA gene sequences available from the NCBI Genbank database. Distances and clustering analysis with the neighbour joining method was performed by using the software packages Mega ver. 6.0. Bootstrap values (n=500) are listed as percentages at the branching points.

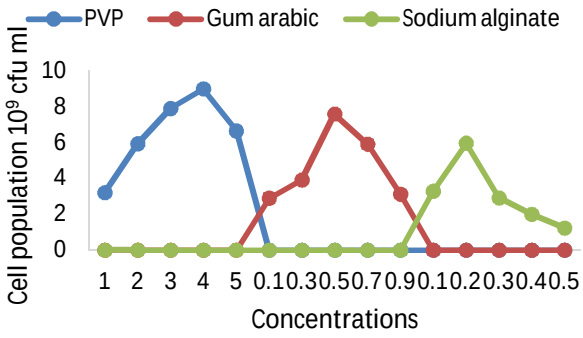

Figure 3: *Azospirillum* strain Asp-7 viable cell population at different concentrations of additives.

Discussion: In the present study a total of nine *Azospirillum* spp. were isolated from the rhizosphere soil of different crop production systems. Out of nine *Azospirillum* strains, strain Asp-7 showed better plant growth promoting traits like phosphate solubilisation, ammonia production, IAA and siderophore production. Physiologically, most active auxin in plant growth and development is IAA.[51] Furthermore, plant studies proved the effect of inoculation of *Azospirillum* Asp-7 with enhanced root length. IAA production may be responsible for increased root growth and/or enhanced formation of lateral roots and root hairs in various plant species.[12] *Azospirillum brasilense* producing nitric oxide, a small diffusible gas act as a signaling molecule in IAA inducing pathway and helps in adventitious root development in tomato plants.[9, 31 & 51] In our study, strain Asp-7

showed highest IAA production and also enhance root formation in maize. The amount of phosphorus in the soil is generally high, most of this phosphorus is insoluble and therefore not available to support plant growth. Solubilisation of phosphorus by phosphate-solubilizing bacteria is an important trait.[39 & 37] Phosphorous solubilizing bacteria (PSB) are used as biofertilizers since 1950's.[25] Many phosphorous solubilizing bacteria are reported as plant growth promoter in several crops like tomato, rice etc.[39, 19 & 24] *Azospirillum* strain Asp-7 showed highest phosphate solubilisation helping plant in better nutrient uptake and also used as a biofertilizer. Siderophore chelate the ferric ions with a high specific activity and serve as vehicles for the transport of irons (Fe^{3+}) into the cell. Siderophore producing organisms will be making the soil fertile and they also have antifungal activity against phytopathogens.[16 & 3] Siderophore producing bacteria improves plant health at different levels like enhance iron nutrition, inhibit the growth of pathogens.[43] *Azospirillum* strain Asp-7 showed siderophore production compared to other isolates. Inoculation of *Azospirillum* strains Asp-7 enhanced plant growth parameters like root length, shoot length and dry mass. These results revealed that the strain Asp-7 can promote plant growth promotion and selected for further studies. Furthermore, *Azospirillum* strain Asp-7 can tolerate minimal water potential of -0.49 MPa and even could grow up to 40°C such strain can be a valuable candidate for dry land crops. Molecular characterization of the strain was done on the basis of 16S rDNA gene sequence analysis[35 & 46] and identified as *Azospirillum brasilense* and the sequence was submitted to Genbank under the accession number KT374217.

To enhance the shelf life of *Azospirillum brasilense* strain Asp-7 in liquid formulation, its growth performance was checked with three different additives such as polyvinyl pyrrolidone (PVP), gum Arabic and sodium alginate. These polymers can be used as additives as well as cell protectants in the medium. These additives will improve quality of the inoculant, such as stabilizing the product, better adhesion to seed, inactivating soluble seed coat toxins and improving cell survival during storage as well as after exposure to different environmental conditions after inoculation and seed planting.[34, 33 & 28]

In our study, among the three additives, PVP at 4% showed good cell population compared to gum arabic and sodium alginate. It could be due to PVP have the ability to limit heat transfer, high water activities and good rheological properties.[44] Polyvinyl pyrrolidone also have a high water binding capacity, which could maintain water around the cells for their metabolism[10] and other properties like sticky nature, which may support cell adherence to seed and their viscous nature may slow the drying process of the inoculant after seed inoculation.[48] The liquid formulation was developed with an initial set population of 7×10^{10} cfu ml^{-1}. The cell population decreased rapidly after six months of incubation in liquid formulations without PVP, whereas the cell population in PVP amended liquid formulation decreased slowly. The obtained results were similar to the liquid formulation developed with PVP.[45, 50 & 15] But in our study, more concentration of PVP was used, it may increase the higher shelf and maintain the good number of cell population than the other studies. This finding suggests the liquid formulation with PVP probably gave higher shelf life because of the high water binding capacity which could maintain water around the cells for their metabolism than without PVP. Further plant studies are required to explore effect of liquid formulation under greenhouse and field conditions.

CONCLUSION: The present study supports that the isolated *Azospirillum brasilense* strain Asp-7 showed plant growth promoting traits, temperature and drought tolerance. After screening with different additives the polyvinyl pyrrolidone selected for the development of liquid formulation. PVP enhanced the shelf life of *Azospirillum brasilense* strain Asp-7 in liquid formulation. Subsequent studies under field conditions are necessary to evaluate the efficacy of the developed liquid formulation.

ACKNOWLEDGEMENT: The authors are thankful to Rashtria Krishi Vikas Yojana (RKVY), Government of Andhra Pradesh for providing the financial assistance in the form of project on Development of liquid bio formulations.

REFERENCES:

1. Akbari, AGh., Seyyed Mehdi, A., Alikhani, H. A., Allahdadi, I., & Arzanesh, M. H. (2007) Isolation and selection of indigenous *Azospirillum* spp. and the IAA of superior strains effects on wheat roots, *World Journal of Agricultural Sciences,* (3), 523-529.
2. Ali, SkZ., Sandhya, V., & Rao, L. V. (2014) Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp, *Annals of Microbiology,* (64), 493–502.
3. Angel Jenifer, M. R., Reena, A., Aysha, O. S., Valli, S., Nirmala, P., & Vinoth kumar, P. (2013) Isolation of siderophore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal plant pathogens, *Interna-*

tional Journal of Current Microbiology and Applied Sciences, (2), 59-65.

4. Bakker, A. W., & Schipper, B. (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* sp. mediated plant growth stimulation, *Soil Biology and Biochemistry,* (19), 451–457.

5. Bhattacharyya, P., & Kumar, R. (2000) Liquid biofertilizer-current Knowledge and Future prospect. *National seminar on development and use of biofertilizers, biopesticides and organic manures,* Bidhan Krishi Viswavidyalaya, Kalyani, West Bengal, 10-12.

6. Bottini, R., Cassan, F., & Picolli, P. (2004) Gibberellin production by bacteria and its involvement in plant growth promotion, *Applied Microbiology and Biotechnology,* (65), 497-503.

7. Brahmaprakash, G. P., & Sahu, P. K. (2012) Biofertilizers for sustainability, *Journal of Indian institute of science,* (92), 1.

8. Chen, W. P., & Kuo, T. T. (1993) A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA, *Nucleic Acids Research,* (21), 2260.

9. Creus, C. M., Graziano, M., Casanovas, E. M., Pereyra, M. A., Simontacchi, M., Puntarulo, S., Barassi, C. A., & Lamattina, L. (2005) Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato, *Planta,* (221), 297–303.

10. Deaker, R., Roughley, R. J., & Kennedy, I. R. (2004) Legume seed inoculation technology-a review, *Soil Biology and Biochemistry,* (36), 1275-1288.

11. Dey, R., Pal, K. K., Bhatt, D. M., & Chauhan, SM. (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L) by application of plant growth promoting rhizobacteria, *Microbiological Research,* (159), 371–394.

12. Dimpka, C., Weinand, T., & Asch, F. (2009) Plant–rhizobacteria interactions alleviate abiotic stress conditions, *Plant, Cell and Environment,* (32), 1682–1694.

13. Dobereiner, J. (1980) *Forage grasses and grain crops," in Methods of evaluating biological nitrogen-fixation,* F. J. Bergersen (eds.), John Wiley and Sons, New York.

14. Felsenstein, J. (1985) Confidence limits on phylogenesis: an approach using the bootstrap, *Evolution,* (39), 783–791.

15. Girisha, H. C., Brahamprakash, G. P., & Mallesha, BC. (2006) Effect of osmo protectant (PVP-40) on survival *Rhizobium* in different inoculants formulation and nitrogen fixation in cowpea, *Geobios,* (33), 151-156.

16. Girish, P. K., Shrikant, S. B., Sunil, A. M., & Manish, N. D. (2010) Exploring the potential of *Pseudomonas* sps as phosphate solublizer, plant growth promoter, Biocontrol agent and pesticide degrader, *Asian Journal of Experimental Biological Sciences,* 40-41.

17. Gordon, S. A., & Weber, R. P. (1951) Colorimetric estimation of indole acetic acid, *Plant Physiology,* (26), 192–195.

18. Gouzou, L., Burtin, G., Philippy, R., Bartoli, F., & Heulin, T. (1993) Effect of inoculation with Bacillus polymyxa on soil aggregation in the wheat Rhizosphere: preliminary examination. *Geoderma,* (56), 479–490.

19. Hafeez, Fy., Safdar, M. E., Chaudhry, A. U., & Malik, K. A. (2004) Rhizobial inoculation improves seedling emergence, nutrient uptake, and growth of cotton, *Australian Journal of Experimental Agriculture,* (446), 617-22.

20. Hegde, S. V. (2008) Liquid bio-fertilizers in Indian agriculture. *Bio-fertilizer newsletter,* 17-22.

21. Hubbell, D. H., & Kidder, G. (2009) Biological Nitrogen Fixation. University of Florida IFAS Extension Publication SL, (16), 1-4.

22. Idris, E. E., Iglesias, D. J., Talon, M., & Borriss, R. (2007) Tryptophan-dependent production of Indole-3- Acetic Acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42, *Molecular Plant-Microbe Interactions,* (20), 619-626.

23. Islam Zaherul, M. D., Soon Young, A. H. N., & Hae Keun, Y. U. N. (2015) Analysis of structure and differential expression of *Pseudomonas syringae* 5-like (RPS5-like) genes in pathogen-infected Vitis flexuosa, *Turkish Journal of Biology,* (39), 775-789.

24. Karpagam, T., & Nagalakshmi, P. K. (2014) Isolation and characterization of phosphate solubilizing microbes from agricultural soil, *International Journal of Current Microbiology and Applied Sciences,* (3), 601-14.

25. Krasilinikov, N. A. (1957) On the role of soil micro-organisms in plant nutrition, *Mikrobiologiya,* (26), 659-72.

26. Kumaresan, G., & Reetha, D. (2011) Survival of *Azospirillum brasilense* in liquid formulation amended with different chemical additives, *Journal of phytology,* (3), 48-51.

27. Leo Daniel Amalraj, E., Maiyappan, S., & John Peter, A. (2012) In vivo and in vitro studies of *Bacillus megaterium* var. *phosphaticum* on nutrient mobilization, antagonism and plant growth

promoting traits, *Journal of ecobiotechnology,* (4), 35-42.

28. Leo Daniel, A. E., Venkateswarlu, B., Suseelendra, D., Praveen Kumar, G., Mir Hassan Ahmed, SK., Meenakshi, T., Uma, S., Sravani, P., & Lakshmi Narasu, M. (2013) Effect of Polymeric Additives, Adjuvants, Surfactants on Survival, Stability and Plant Growth Promoting Ability of Liquid Bioinoculants, *Journal of Plant Physiology and Pathology,* (1), 2.

29. Mehta, S., & Nautiyal, C. S. (2001) An efficient method for qualitative screening of phosphate solubilizing bacteria, *Current Microbiology,* (43), 51–56.

30. Michel, B. E., & Kaufmann, M. R. (1973) The osmotic potential of polyethylene glycol 6000, *Plant Physiology,* (51), 914–916.

31. Molina-Favero, C., Creus, C. M., Simontacchi, M., Puntarulo, S., & Lamattina, L. (2008) Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato, *Molecular Plant Microbe Interactions,* (2), 1001–1009.

32. Mozammel Hossain, Md., Iffat, J., Salina, A., Nazibur Rahman, Md., & Badier Rahman, SM. (2015) Isolation and identification of *Azospirillum* isolates from different paddy fields of North Bengal, *International Journal of Research in Pharmacy and Biosciences,* 3.

33. Mugnier, J., & Jung, G. (1985) Survival of bacteria and fungi in relation to water activity and the solvent properties of water in biopolymer, *Applied Environmental Microbiology,* (50), 108-114.

34. Panlada, T., Payakaponga, P., Teaumroonga, N., Paul, W., Singleton, B., & Boonkerd, N. (2007) Growth, Survival and Field Performance of Bradyrhizobial Liquid Inoculant Formulations with Polymeric Additives, *Science Asia,* (33), 69-77.

35. Pedraza, R. O., Motok, J., Tortora, M. L., Salazar, SM., & Diaz-Ricci, JC. (2007) Natural occurrence of *Azospirillum brasilense* in strawberry plants, *Plant and Soil,* (295), 169–178.

36. Rana, G., Arindam, A., & Tanusri, M. (2014) New primers generated bacterial mapping and bio fertilizing potentiality assessment of *Pseudomonas* sp. isolated from cow dung, *International Journal of Agronomy and Agricultural Research,* (5), 1-12.

37. Richardson, A. E. (2001) Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants, *Australian Journal of Plant Physiology,* (28), 897–906.

38. Richardson, A. E., Barea, J. M., McNeill, A. M., & Prigent-Combaret, C. (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms, *Plant and Soil,* (321), 305-339.

39. Rodriguez, H., & Fraga, R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion, *Biotechnology Advances,* (17), 319–339.

40. Sandhya, V., Ali, SkZ., Minakshi, G., Gopal Reddy, & Venkateswarlu, B. (2009) Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-P45, *Biology and Fertility of Soils,* (46), 17-26.

41. Sandhya, V., Ali, SkZ., Grover, M., Reddy, G., & Venkateswarlu, B. (2010) Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes antioxidant status and plant growth of maize under drought stress, *Plant Growth Regulators,* (62), 21–30.

42. Schwyn, B., & Neilands, J. B. (1987) Universal chemical assay for the detection and determination of siderophores, *Analytical Biochemistry,* (160), 47–56.

43. Shen, X., Hu, H., Peng, H., Wang, W., & Zhang, X. (2013) Comparative genomic analysis of four representative plant growth-promoting rhizobacteria in *Pseudomonas*, *BMC Genomics,* (14), 271.

44. Singleton, P., Keyser, H., & Sande, E. (2002) Development and evaluation of liquid inoculants, *Australian Centre for International Agricultural Research*, Canberra, 52-66.

45. Surendra Gopal, K., Akhila Baby, (2016) Enhanced shelf-life of *Azospirillum* and PSB through addition of chemical additives in liquid formulations, *International journal of Science, Environment*, (5), 2023-2029.

46. Suresh Babu, S., Thangaraju, M., & Santhana Krishnan, P. (2002) Shelf life improvement of *Azospirillum* inoculant by addition of polymers, chemicals and amendments in the lignite carrier, *Journal of Microbial World,* (4), 51-58.

47. Tahir, M., Mirza, M. S., Zaheer, A., Dimitrov, M. R., Smidt, H., & Hameed, S. (2013) Isolation and identification of phosphate solubilizer *Azospirillum*, *Bacillus* and *Enterobacter* strains by 16SrRNA sequence analysis and their effect on growth of wheat (*Triticum aestivum* L.), *Australian Journal of Crop Science,* (7), 1284-1292.

48. Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0, *Molecular Biology Evolution,* (30), 2725-2729.

49. Temprano, F. J., Albareda, M., Camacho, M., Daza, A., Santamaría et al, C. (2002) Survival of several *Rhizobium*/*Bradyrhizobium* strains on different inoculant formulations and inoculated seeds, *International journal of Microbiology,* (5), 81-86.

50. Uma Maheswari, N., & Elakkiya, (2014) Effect of liquid biofertilizers on growth and yield of *Vigna mungo L, International Journal of Pharmaceutical Sciences Review and Research,* 29.

51. Vendan, R. T., & Thangaraju, M. (2006) Development and standardization of liquid formulation for *Azospirillum* bioinoculants, *Indian Journal of microbiology,* (46), 379-387.

52. Vurukonda, S. S. K. P., Sandhya, V., Manjari, S., & Ali, SKZ. (2016) Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria, *Microbiological Research,* (184), 13–24.