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ISOLATION AND CHARACTERIZATION OF NITROGEN *FIXING BACILLUS SUBTILIS STRAIN* AS-4 FROM AGRICULTURAL SOIL

Satapute, P. P., Olekar, H. S., Shetti, A. A., Kulkarni, A. G., Hiremath, G. B., Patagundi, B. I., Shivsharan, C. T and *Kaliwal, B. B

Post Graduate Department of Biotechnology and Microbiology, Karnatak University, Dharwad- 580003

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ABSTRACT

The present study was conducted with the goal of isolation, screening and characterization of most potential nitrogen fixers from the agricultural soil. Five bacterial isolates were isolated and characterized from rhizosphere of ground nut plant. The potential isolate was identified as *Bacillus subtilis strain* AS-4 based on the morphological, biochemical, cultural and 16s rDNA identification. The organism grew well in SM basal salt medium and nitrogen-free semi-solid LGI medium, tolerated 10-15% salt concentration and the optimum growth was found at 27°C and pH 7.0. The present investigation proves that the soil isolate *Bacillus subtilis strain* AS-4 is salt tolerant, free living nitrogen fixing bacteria that could be exploited as soil inoculants and can be used for nitrogen fixation in soil with high concentration of salt, which is of long run, eco-friendly and cost ineffective.

INTRODUCTION

Soil microorganisms constitute the world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. The plant diversity significantly enhances the rates of microbial processes that mediate ecosystem via C and N_2 cycling, this effect being more strongly dependent on plant production than on species richness (Mukerji *et al.,* 2006). The first nitrogen-fixing microbe discovered was *Clostridium pasteurium,* by a famous Microbiologist S. Winogradsky in Paris in 1893. The biogeochemical cycle of nitrogen is essential for agriculture, as well as for the productivity of natural ecosystems. Nitrogen being of major importance for all living organisms is often a limiting growth factor in soil ecosystem. Soil reservoirs are fed by biologically and/or by chemically fixed nitrogen (in the form of fertilizers), but the balance is frequently negative due to significant losses through denitrification, erosion, leaching and volatilization. Biological nitrogen fixation is considered as the limiting step of the nitrogen cycle, as this process is functioned by a restricted number of prokaryotes, including bacteria of the genus *Azospirillum* and specific symbiotic associations (Postgate, 1998: Bartha, 1993).

Biological fixation of the atmospheric nitrogen can be estimated at about 175 million metric tons per year or about 70% of all nitrogen fixed on the Earth per year, the remaining is by some micro-organisms, autotrophs orheterotrophs 'free' fixers (Peter *et al.,* 2002). The transformation, or 'fixation' of nitrogen from the unavailable gaseous form in the atmosphere to forms that plants and other organisms can use (either *NH* or *NO*) is mediated by (i) bacteria in symbiotic relation- ships with vascular plants, (ii) symbioses between *cyanobacteria* and fungi (lichens) or plants, (iii) free living heterotrophic or autotrophic bacteria that are typically associated with soil or detritus and (iv) abiotic reactions occur without microbes and (iv) abiotic reactions occur without microbes in the atmosphere associated with lightening (Timothy, 1999) . Nearly 40% of world's surface has salinity problems with most of the salinic areas are confined to the Tropics and Mediterranean region and has made the salt tolerance and urgent priority for the future of agriculture (Corodovilla *et al*., 1994; Gisbert *et al.*, 2000). The success of symbiotic biological nitrogen fixation in saline soil depends on the survival and growth of the nitrogen fixing bacteria introduced (Vincent, 1974). Therefore, salt tolerance in nitrogen fixing bacteria such as *rhizobium spp*., *Bacillus subtilis*, Azotobacter, Azospirllum etc. is a desirable agronomic trait. Therefore, the present investigation was carried out to isolate and characterize the most potential strain of nitrogen fixing bacteria from agricultural soil.

MATERIALS AND METHODS

Soil Sampling

Rhizosphere soil of groundnut plants was collected from the agricultural fields of University agricultural sciences, Dharwad. The samples were passed through a sieve of 2 mm to remove stones and plant debris.

Isolation of nitrogen fixing bacteria

Individual samples were suspended in 1 mL sterile distilled water and its 0.1 mL aliquot was inoculated on SM basal medium (Per liter) Na_2HPO_4 4.5 g, KH_2PO_4 1.5 g, NH_4Cl 0.3 g, $MgSO_4.7H_2O$ 0.1g, $Na_2S_2O_3$ (100 ml) and trace metal solution 5.0 mL (per liter composition trace metal solution: EDTA 50 g, $ZnSO_4.7H_2O$ 22 g, $CaCl_2$ 5.54 g, $MnCl_2.4H_2O$ 5.06 g, $FeSO_4.7H_2O$ 4.99 g, $CoCl_2.H_2O$ 1.61 g, $CuSO_4.5H_2O$

* Corresponding author: +91 (0) 836-2779533
E-mail address: b_kaliwal@yahoo.com

1.57 g and $(NH_4)6Mo_7O_{24}$. $2H_2O$ 1.1 g) (Loganathan and Nair, 2004) and incubated at 37°C for 48 hrs. The cultures obtained were transferred on nitrogen free medium LGI medium (per liter) $CaCO_3$ 1.0g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g, $FeSO_2.7H_2O$ 0.1 g, $Na_2MoO_4.2H_2O$ 5.0 mg, Sucrose 5 g and bromophenol blue solution 5mL (Add bromophenol blue 0.5 g to 100 mL of 0.2N KOH) incubated at 37°C for 24 to 48 hrs and maintained on yeast extract mannitol agar (YEMA) medium (Cavalcante and Dobereiner 1988).

Morphological and biochemical Characterization

The isolate showing high nitrogen fixation was selected for morphological and biochemical characterization, To detect the physiological activities of the selected strains oxidase, catalase, motility, indole, methyl red (MR), Voges-Proskauer (VP), citrate utilization, urease, nitrate reduction, hydrogen sulfide (H_2S) production, gelation liquefaction and carbohydrate fermentation tests were conducted (Collee and Miles, 1989).

16S rDNA sequencing for bacterial isolate

DNA was isolated from the culture and 16S rDNA gene was isolated. The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the Phylogenetics tree was constructed using MEGA 4.

Effect of Temperature on growth

The bacterium was grown in YEM broth at temperature ranging from 04-55°c to study the effect of temperature on growth and nitrogen fixing capability of soil isolates. The optical density was measured at 660 nm to study the growth of soil isolate.

Effect of pH on growth

The influence pH on Nitrogen fixing activity of bacteria was studied by growing the bacteria in a YEM broth with pH ranging 4.0- 10.0. The optical density was measured at 660 nm.

Effect of Salt on growth

The bacterium was incubated in YEM with 10 and 15% of NaCl and incubated at 26°C for 30 hrs to study the effect of salt on growth and nitrogen fixing of soil isolates (Jadav *et al.*, 2010). Samples were taken at intervals of 5, 10, 15, and 25hrs. The optical density was measured at 660 nm.

RESULTS AND DISCUSSION

The interactions between plants and the rhizosphere microorganisms are diverse. Nitrogen compounds released from plant roots into the soil drives to a great microbial development in the rhizosphere. Rhizobacteria living on the root surface have many positive effects; they can promote the plant growth, crop production, and play an important role in providing resistance to microbial diseases. Most plant species interacts with the rhizobacteria to obtain the essential mineral elements such as N_2 or P. Some groups of microorganisms are capable to solubilise or to transform these elements, making them available to the plants (Simon, 2003). Symbiotic N_2-fixation is a well-known process, constituting the interaction of the bacteria with legume roots which leads to the formation of N_2 fixing nodules.

Table 1 Biochemical characteristics of soil isolate *Bacillus subtilis strain* AS-4

Test	Result
Indole	Negative
Methyl red	Negative
Vogues Prausker	Positive
Citrate	Positive
Catalase	Positive
Urease	Positive
Oxidase	Positive
Starch hydrolysis	Positive
Gelatin hydrolysis	Positive

Isolation and purification of Microorganisms

The present study encompassed the isolation and characterization of nitrogen-fixing bacteria from rhizosphere soil of the ground nut, from the fields of University of agricultural sciences, Dharwad. Ten samples were collected and screened. Among 25 isolates obtained on SM basal medium, only 5 isolates (S-1 to S-5) were found to grow on nitrogen-free LGI medium (Azospirillum amazonense medium) indicating that they could fix nitrogen. Total five bacteria colonies were isolated out of which two were gram positive bacillus and three gram negative bacilli.

Table 2 Effect of temperature on growth of the soil isolate Bacillus *subtilis strain* AS-4

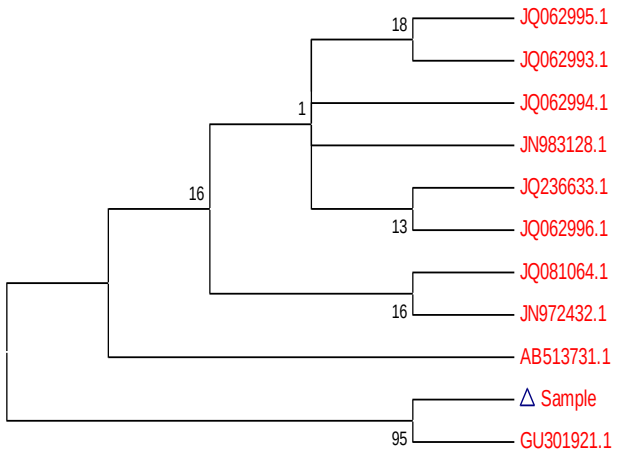
Sl. No	Temperature (°C)	Optical density (660nm)
1.	4	0.02
2.	17	0.11
3.	27	0.39
4.	37	0.28
5.	45	0.03
6.	55	0.01

The soil isolate S-3 was showing consistent growth in YEMA medium and was selected for further studies. The strain S-3 was a rod-shaped and gram positive bacterium. The isolated colony was rough, creamy off white with umbonate edge, the biochemical characteristics were as depicted (Table 1). By 16s rDNA gene sequencing it was observed that, the sequence of strain displayed the highest identity (100%) with the 16s rDNA gene of *Bacillus subtilis strain* AS-4 (GenBank Accession Number: GU301921.1) based on nucleotide homology and phylogenetic analysis (Fig. 1).

The nitrogen fixing bacteria can be isolated directly from the root nodules of the host plant or from the soil, using yeast extract mannitol selective culture media (Egorov, 2003; Matthew *et al.*, 2008). Based on the morphological attributes and biochemical characteristics the isolates S-3 found to be a member of the genus *Bacillus.* The results of sequencing of 16S rDNA authenticated that the isolates was *Bacillus subtilis strain* AS-4. It has been reported that *Bacillus subtilis* is a sporulating soil bacterium often found to be associated with decaying organic matter. Sporulation in *Bacillus subtilis* is said to be initiated by nutrient depletion, this developmental process provides a survival mechanism when nutrients become scarce in the environment (Hoch, 1993). Despite the different ecological habitats and life cycles of *Bacillus subtilis* and

enteric bacteria, there are only minor differences in the physiology of ammonium assimilation between these bacteria (Schreier, 1993). *Bacillus subtilis* has no assimilatory glutamate dehydrogenase activity, ammonium assimilation occurs solely by the GS–glutamate synthase pathway. Ammonium is also a good nitrogen source for *Bacillus subtilis*, because the expression of glutamine synthesis is partially repressed in ammonium-grown cells (Atkinson and Fisher, 1991).

Fig: 1 Phylogenic tree for soil isolate



Effect of temperature on growth of the soil isolate Bacillus *subtilis strain* AS-4

The effect of temperature on growth of *Bacillus subtilis strain* AS-4 was studied in various temperatures 04, 17, 27, 37, 45 and 55 °C. The results indicate that the optimum temperature for the growth was at 27°C and growth was hampered at very low and high temperatures of 4 and 55°C (Table 2). A similar result has been obtained in the study of two isolates *Bacillus sonorensis and Bacillus subtilis* that showed growth between 10-40 °C (Jadhav *et al.*, 2010).

Table 3 Effect of pH on growth of soil isolate *Bacillus subtilis strain* AS-4

Sl. No	pH	Optical density (660nm)
1.	4	0.05
2.	5	0.07
3.	6	0.18
4.	7	0.35
5.	8	0.26
6.	9	0.05
7.	10	0.02

Effect of pH on growth of soil isolate *Bacillus subtilis strain* AS-4

The effect of pH on growth of *Bacillus subtilis strain* AS-4 was studied at different pH range 5, 6, 7, 8, 9, and 10. The results indicate that the optimum pH for the growth was at 7.0 (Table 3). The growth was hampered at very acidic and alkaline pH as indicated by growth of the soil isolate. In a study *Bacillus subtilis was* able to tolerate pH in arrange of 5.0-10 with optimal growth at 7.0-7.5 (Jadhav *et al.*, 2010).

Effect of salt on growth of soil isolate *Bacillus subtilis strain AS-4*

The growth in medium with both 10 and 15 % NaCl indicated that soil isolate *Bacillus subtilis strain* AS-4 was able to tolerate high concentration of salt. However, the growth was observed solely in the initial hours up to 25 hours of incubation and later on it decreased (Table 4). The reduction of N_2-fixing activity and photosynthetic activity by salt stress is usually attributed to reduction in (i) respiration of nodules (Delgado *et al.* 1994). (ii) cytosolic protein production (Delgado *et al*., 1993) (iii) dry weight and (iv) nitrogen content in the shoot (Georgiev and Atkias 1993; Cordovilla *et al*., 1995). Thus, the halotolerant novel Bacillus sp. prove promising and can be further exploited as soil inoculants. The *Bacillus subtilis strain* AS-4 isolated in the present investigation is to high salt concentration. The strain can be used as an inoculant for nitrogen-fixation in soils with high salt concentration.

Table 4 Effect of Salt concentration (NaCl) on growth of isolate *Bacillus subtilis strain* AS-4

Sl. No	Hours	Optical density (660nm) 10% NaCl	Optical density (660nm) 15% NaCl
1.	5	0.35	0.08
2.	10	0.55	0.30
3.	15	0.70	0.40
4.	20	0.70	0.61
5.	25	0.55	0.70
6.	30	0.45	0.70

CONCLUSION

The results suggested that the soil isolate *Bacillus subtilis strain* AS-4 has nitrogen fixing ability and can tolerate high salt concentration. Further, it suggests that *Bacillus subtilis strain* AS-4 could be exploited as soil inoculants and can be used for nitrogen fixation in soils with high concentration of salt, which is of long run, eco-friendly and cost ineffective. Further, it has a potential for use as bio-fertilizer in soil with high concentration of salt.

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