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Isolation and evaluation of isolated bacteria as potential biofertilizer

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Abstract
This study was conducted to isolate bacteria from garden soil from the college campus in Kalyan, Dist Thane, and evaluate their potential as biofertilizers. Seven morphologically distinguished bacterial isolates (ASH1, SOB1, SOB2, SOB3, SOB4, PS1 and PS2) were isolated. These isolates were studied for their colony characteristics and were found to be Gram negative in nature. These isolates could grow at a broad temperature range, tolerate high concentrations of salt and survive in varying pH concentrations. The isolates were checked for their ability to produce IAA, sulphate ions, organic acids, siderphores, nitrites and solubilise phosphates. Isolate SOB3 was found to produce maximum amount of IAA, nitrites and showed higher sulphate oxidising potential. These isolates if used as biofertilizers can restore the soil's natural nutrient cycle and build soil organic matter, thus providing nutrients to the growing plants.

Keywords: Biofertilizers, Bacterial isolates, IAA production, sulphate ion production, organic acids, siderphores, nitrite production, solubilise phosphates.

1. Introduction
During the last few years, agriculture development has taken a leap which has resulted in the use chemical fertilizers. The constant use of these chemicals has resulted in the decrease in the land fertility and chemical toxicity increasing. For optimum plant growth, nutrients must be available in sufficient and proper quantities (Chen, 2006) [4]. The most important constraint limiting crop yield worldwide, is soil infertility. Unless the fertility is restored, farmers will gain little benefit from the use of improved varieties and more productive cultural practices (Mohammad and Sohrabi, 2012) [22]

Biofertilizers are important components of integrated nutrients management. These potential biological fertilizers would play key role in productivity and sustainability of soil and also protect the environment as ecofriendly and cost effective inputs for the farmers. They are cost effective, ecofriendly and renewable source of plant nutrients to supplement chemical fertilizers in sustainable agricultural system. Biofertilizers are products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form through biological process such as nitrogen fixation and solubilization of rock phosphate (Rokhzadi *et al*., 2008) [28].

Microbial bio-fertilizers have gained attention as an effective alternative against the chemical fertilizers for environment-friendly agriculture. Bacterial mechanisms of plant growth promotion include biological nitrogen fixation (BNF), synthesis of phytohormones, environmental stress relief, synergism with other bacteria-plant interactions, inhibition of plant ethylene synthesis, as well as increasing availability of nutrients like phosphorus, iron and minor elements, and growth enhancement by volatile compounds (Fuentes-Ramirez and Caballero-Mellado, 2005) [10].

It also helps maintain the natural habitat of the soil. Using these bio-fertilizers, healthy plants can be grown, while enhancing the sustainability and the health of the soil. They add It increases crop yield by 20-30%, replaces chemical nitrogen and phosphorus by 25%, and stimulates plant growth. It can also provide protection against drought and some soil-borne diseases (Ghosh, 2007) [11]. Bio-fertilizers are expected to reduce the use of chemical fertilizers and pesticides. In this work, we aim to carry out isolation of soil bacteria which

may have the potential to act as a potential bio fertilizer which may play a vital role in sustainable development of agriculture

2. Materials and methods

2.1. Isolation of bacterial isolates

Garden soil samples were collected from an undisturbed site that was covered by vegetation. The soil pH was checked and was found to be 6.8. One gram of this soil was added to Ashby's mannitol broth containing (g/dl) KH_2PO_4 (0.02); $HgSO_4.7H_2O$ (0.02); $CaCO_3$ (0.5); $CaSO_4$ (0.01); D/W (90 ml); 10 ml of 10% mannitol solution was added after autoclaving. The broth was placed at shaker conditions for 5 days after which the enriched sample was plated on Ashby's mannitol agar plate for the isolation of Azotobacter sp. The soil samples were serially diluted and plated on nutrient agar medium. These plates were incubated at room temperature for 48 hrs. Morphologically distinct colonies were selected for further analysis.

The bacterial isolates were studied for their Gram nature and the effect of temperature, pH and osmotic pressure on their growth.

2.2. Screening for Phosphate solubilising bacteria

The bacterial isolates were spot inoculated on Pikovaskaya's phosphate solubilising agar. The plates were incubated for 48 hrs at room temperature (Rashid *et al*, 2004) [25].

2.3. Screening and quantification of IAA production

The bacterial isolates were screened for IAA production. The isolates were spot inoculated on a nutritive agar medium containing (g/l) peptone (1.0); glucose (2.0); tryptophan (1mM); agar (15%). The plates were incubated at 37 °C for 48 hrs. After incubation the plates were flooded with Salkowsky's reagent (35% perchloric acid and 0.5 M FeCl3) and incubated in the dark for 25 mins.

The bacterial isolates were grown in nutritive medium containing (g/l) peptone (1.0); glucose (2.0); tryptophan (1%). The broth was incubated at 37 °C. Daily 2ml of the broth was taken and Salkowsky's reagent was added. The tubes were incubated at room temperature for 25 mins and centrifuged at 5000 rpm for 5 mins. The reddish supernatant obtained was colorimetrically read at 530 nm [CL 157 (ELICO)]. Standard IAA graph was plotted using 100mcg/ml of IAA as a standard(Gordon and Weber, 1951) [12].

2.4. Screening for Sulphate oxidizing bacteria

The bacterial isolates were grown in Thiosulphate broth containing 0.01% bromothymol blue dye. The tubes were incubated at room temperature for 11 days. The pH was noted daily (Visser *et al*, 1997) [33].

2.5. Sulphate ion production ability

The bacterial isolates were grown in Thiosulphate broth for 24 hr sat 37 °C. The broth was centrifuged at 5000rpm for 5mins. The supernatant was mixed with Barium chloride (10%w/v) in the ratio of 1:1, followed by vigorous mixing. The white turbity obtained was color imetrically read at 450nm [CL 157 (ELICO)]. Standard sulphate graph was obtained by using Barium sulphate solution (1mg/ml) (Visser*et al*, 1997) [33].

2.6. Nitrite production ability

The isolated bacteria were grown in Winogradsky's non – nitrate medium and incubated for 24 hrs at 37 °C. The supernatant was treated with 0.8% sulphanilic acid solution and 0.8% α naphthylamine solution. The resulting pink colour developed was colorimetrically read at 520 nm [CL157 (ELICO)].Standard nitrite graph was obtained by using $NaNO_3$ solution having a final concentration of $1mgNO_2^-N/L$.

2.7. Organic acid production

The bacterial cultures were grown on MM9 agar medium (g/L) Na_2HPO_4(6.8); KH_2PO_4(0.3); NaCl (0.5); NH_4Cl (1.0); Glucose (4% w/v); Agar (3%, w/v). After incubation at 37 °C for 24 hrs, the plates were flooded with methyl red dye (1% w/v) solution. The colony colour changed to pink indicating the production of organic acids (Visser *et al*, 1997) [33].

2.8. Siderophores detection

CAS-blue agar was prepared according to Schwyn and Neilands (Shin *et al*., 2001) [31] method, where 60.5 mg Chrome azurol S (CAS) dissolved in 50 ml distilled water and mixed with 10 ml $FeCl_3$ (1 mmol/lit) + HCL (10 mmol/lit) solution. While constantly stirring, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml distilled water. The resultant dark blue mixture was diluted 20-fold and autoclaved at 121°C for 15 min. Agar (3%, w/v) was used as gelling agent.

Petri dishes (10 cm in diameter) were prepared with CAS-blue agar (dye solution 10 ml) as bottom agar plate. After solidifying, the CAS- blue agar was overlayed with an appropriate MM9 agar (7ml) containing (g/L) Na_2HPO_4 (6.8); KH_2PO_4 (0.3); NaCl (0.5); NH_4Cl (1.0); Glucose (4% w/v); Agar (3%, w/v). These plates were spot inoculated with 24 hr old culture suspensions of the bacterial isolates. The plates were incubated at room temperature for 24 to 48 hrs (Hu and Xu, 2011) [13].

2.9. Application as biofertilizers

Bacterial isolates were individually and in consortia grown in nutrient broth at 37 °C till their OD reached 0.2 extinction units. The culture was centrifuged and the pellet was washed free of any medium. The pellet was resuspended in sterile Saline (0.8% w/v). The 10 ml of the culture was added to 20 gms of sterile cocopeat.

Fig 1: Moong plantlets grown in biofertilizers.

Moong seeds were sterilized using bavistin and sowed in the inoculated cocopeat. The seeds were kept at room temperatures and watered daily. After 7 days, the grown plantlets were removed, washed to remove the traces of soil

(Fig 1). The protein levels and Fe content of the plantlets were checked using Folin Lowry method of protein estimation and ICP-AES (SAIF, IIT Bombay) analysis resp. Untreated cocopeat was used as blank for growing moong seeds

3. Results and Discussion

One morphologically distinguished bacterial isolate (isolate ASH 1) was isolated from Ashby's agar plate, whereas six different bacterial isolates were obtained from nutrient agar plate- SOB1, SOB2, SOB3, SOB4, PS1and PS2.

Colony characters were studied and the organisms were found to be Gram negative in nature. The optimum temperature for all the isolates as found to be in the range of 28 to 37 °C. Isolates ASH1, SOB2 and SOB 3 could grow well even at 55 °C. The effect of pH was studied on the isolates and it was seen that isolates ASH1, SOB1, SOB2, SOB 3, SOB 4 could grow well at a pH range from 6.0 to 10.0 whereas isolates PS1 and PS2 could grow at a pH range from 2.0 to 10.0.

The effect of osmotic pressure was seen on the bacterial isolates and isolates ASH1, SOB2, SOB3 and SOB 4 could tolerate NaCl concentrations upto 6% whereas isolates SOB2, PS1 and PS2 could tolerate NaCl concentrations upto 10%.

Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa *et al*. 2002) [9]. Root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001) [27]. The isolates were screened for their phosphate solubilising ability using Pikovaskaya's phosphate solubilising agar. Isolates SOB2, SOB4 PS1 and PS2 showed clearance around the colony indicating that these isolates could solubilise phosphates (Fig 2).

Fig 2: Phosphate solubilising bacteria

Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (Kudashev, 1956; Krasilinikov, 1957) [18, 17]. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation (Khan and Joergesen, 2009) [14].

Microbial community influences soil fertility through soil processes viz. decomposition, mineralization, and storage/release of nutrients. Microorganisms enhance the P availability to plants by mineralizing organic P in soil and by solubilizing precipitated phosphates (Chen *et al*., 2006; Pradhan and Sukla, 2005) [5, 24]. These bacteria in the presence of labile carbon serve as a sink for P by rapidly immobilizing it even in low P soils (Bünemann *et al*., 2004) [3].

Indole-3-acetic acid (IAA) is now regarded as the most significant auxin and regulates many aspects of plant growth and development (Thimann, 1972; Leopold and Kriedemann, 1975) [32, 19]. Isolates were checked for the production of IAA and it was observed that most of the isolates produced IAA but maximum production was seen in isolate SOB3 (140.53mcg/ml), PS1 (37.43 mcg/ml) and PS2 (22.32mcg/ml) (Fig3). The day wise production of IAA was checked. It was seen that a steady increase in the production was seen for all isolates. Maximum production was seen in isolate SOB3 (Fig 4).

The conversion of tryptophane to Indole Acetic Acid is carried out by the action of Nitrilase enzymes IAA can be synthesized from tryptophan in plants (Schneider and Wightman, 1978; Cohen and Bialek, 1984) [29, 6]. A predominant biosynthesis route of IAA has been found, in which tryptophan is converted into indole-3-pyruvic acid or tryptamine and then transformed into IAA via indole-3-acetaldehyde. In another pathway, IAA is formed from tryptophan via indole-3-acetaldoxime in Cruciferae:

Tryptophan → indole-3-acetaldoxime → indole-3-acetonitrile → IAA

Naturally occurring in plants, indole3-acetaldoxime (Kindl, 1968) [15] can be metabolized to IAA by indole-3acetaldoxime dehydratase (EC 4.2.1.29) (Mahadevan, 1963) [21], leading to the formation of indole-3-acetonitrile, which can be hydrolyzed to IAA by nitrilase (EC 3.5.5.1). The enzyme activity of the Nitrilase enzyme was carried out (Kobayashi *et al*., 1993) [16].

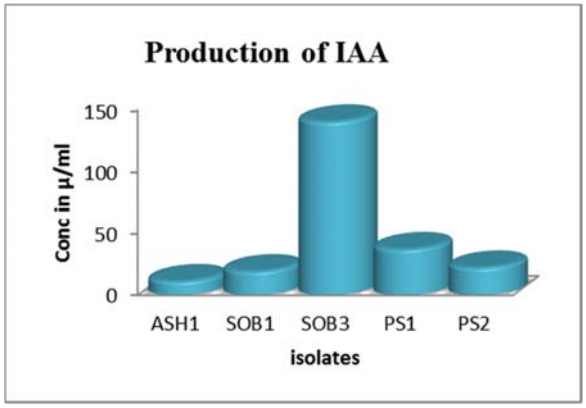

Fig 3: Production of IAA by bacterial isolates

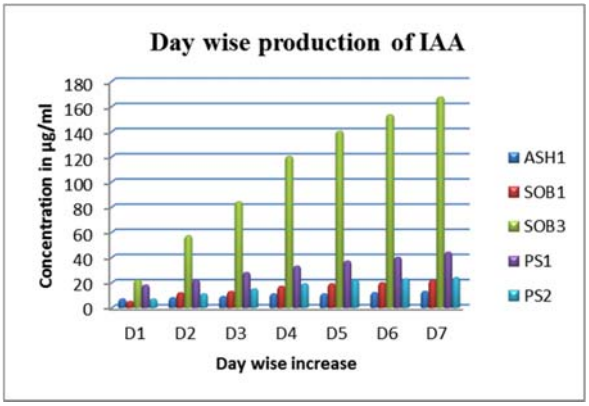

Fig 4: Day –wise production of IAA by bacterial isolates

The enzyme activity was different for different isolates. SOB3 showed maximum activity on Day 4 (0.126 mM/ml/min), decreasing down after Day 5. ASH 1, SOB1, PS1 and PS2 showed maximum activity after 24 hrs of incubation (Day 1) with the substrate and subsequent decline in the activity after 48 hrs of incubation (Fig 5).

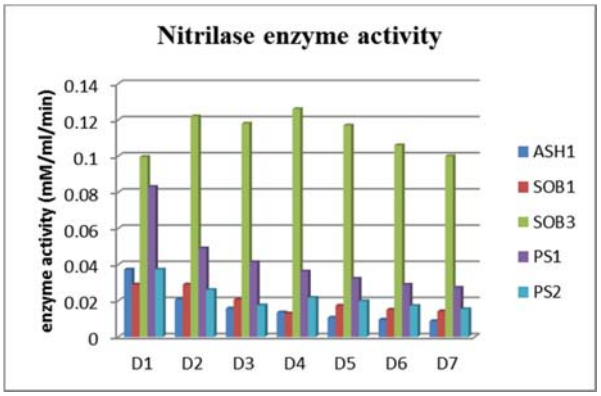

Fig 5: Nitrilase enzyme activity for the production of IAA

The soil microbial biomass is the key driving force behind all sulphur transformation. Beside their important contribution in agriculture these microbes also play significant role in removal of toxic H2S from the environment. Most of the known sulphur oxidising bacteria (SOB) belongs to the genera *Thiobacillus, Thiothrix, Thiomicrospira, Achromatium* and *Desulfuromonas* (Das, 1996) [7]. Sulphate ion production was checked for the bacterial isolates. The organism was grown in thiosulphate broth containing bromothymol blue. The pH of the broth was checked daily for 11 days. It was seen that there was a change in the pH shifting towards the alkaline side. Sulphate ion production was seen for the isolates. Reduction in pH of the growth medium by sulphur oxidizing bacteria was also reported by Donati *et al* (1996) [8]. The pH reduction of the medium was due to the production of sulphuric acid. Isolates SOB 3 (0.744%) and PS2 (0.66%) showing maximum production in 48 hrs (Fig 6).

The sulphate oxidising enzyme activity of the isolates was determined and it was seen that isolates SOB3 (0.0134 M/ml/min) and PS2 (0.012 M/ml/min) showed maximum enzyme activities (Fig 7). Ravichandra *et al*. (2007) [26] reported the maximum sufate ion production from 14150mg/ml by a *Thiobacillus* spp. Similarly Babana *et al*. (2011) reported the highest sulphuric acid concentration (243mg/l) by a strain ATTC55128 followed by (230 mg/l) by another strain, AHB436.

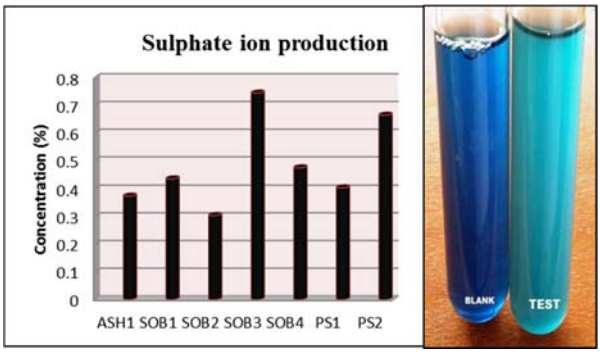

Fig 6: Sulphate ion production in the bacterial isolates

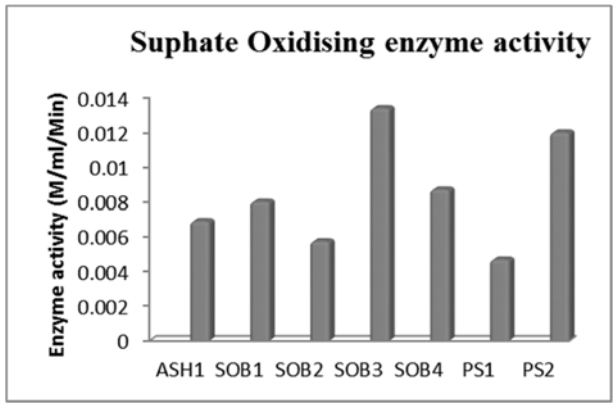

Fig 7: Sulphate oxidising enzyme activity seen in bacterial isolates

The bacterial isolates were able to produce nitrates in the medium which was estimated colorimetrically at 520 nm. Bacterial isolate SOB3 (1.17 mg NO_2^-N/lit) was seen to produce maximum amount of nitrates, followed by isolate ASH1 (0.74 mg NO_2^-N/lit) (Fig 8).

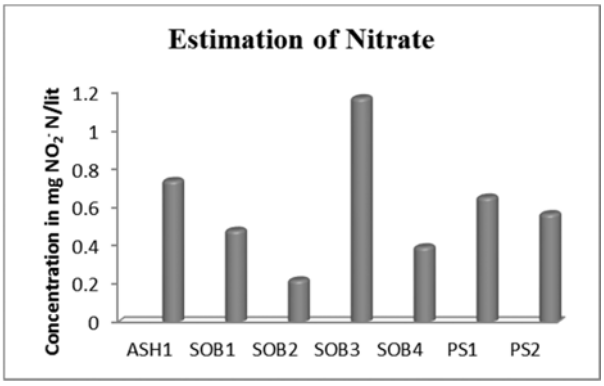

Fig 8: Nitrate production in bacterial isolates

Many species of nitrifying bacteria have complex internal membrane systems that are the location for key enzymes in nitrification: ammonia mono oxygenase which oxidizes ammonia to hydroxylamine and nitrite oxidoreductase, which oxidizes nitrite to nitrate. The enzyme activity was found to be maximum in bacterial isolate SOB3 (0.0044 mM/ml/min) followed by isolate ASH1 (0.0034 mM/ml/min) (Fig 9).

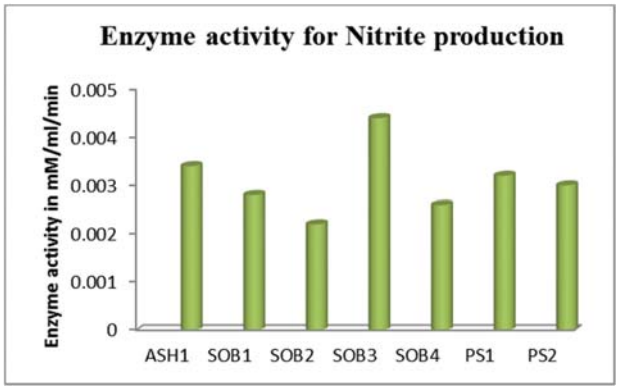

Fig 9: Enzyme activity for nitrite production

The isolates were checked for the production of organic acid. The isolates were grown on MM9 agar medium. After incubation at 37 °C for 24 hrs, the plates were flooded with

methyl red dye (1% w/v) solution. The bacterial isolates SOB2 and SOB4 showed the presence of colour change to pink colour indicating that the isolates produced organic acids (Fig 10A).

The bacterial isolates were screens for the production of siderophores. In agricultural microbiology, these siderophores have been used as plant growth promoting bacteria that control root pathogens and also make iron available to the plants thus increasing the content of iron in plants (Loper and Buyer, 1991) [20]. The isolates were spot inoculated on CAS agar medium and incubated at room temperature for 24 to 28 hrs. Pink colonies were seen indicating the presence of isolates producing siderophores (Fig 10B). The siderophore production of *Staphylococcus* strains was detected by the CAS assay (Alexander and Zuberer, 1991; Schwyn and Neilands, 1987) [1, 30]. Two of the siderophore producing strains, MBTU_PB2 and MBTU_PB3 belonged to the *Staphylococcus* sp. The distinct responses of colour change of CAS reaction (orange, purple, or purplish-red) with the different microorganisms, could be related to structural differences in the types of siderophores secreted (Neilands, 1984) [23].

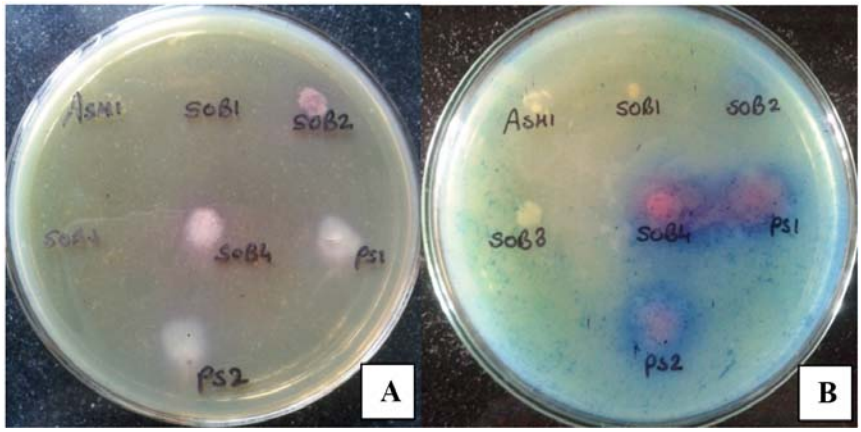

Fig 10: Screening of bacterial isolates for the production of
(A) Organic acids
(B) Siderophores

The protein analysis of the treated and untreated plantlets showed a difference in the protein content. Maximum protein concentration was seen when the plantlets were grown in the presence of the mix consortia (0.34 mg/ml) followed by the plants grown in the presence of PS2 (0.31 mg/ml) (Fig 11).

The Iron (Fe) content in the plants was estimated by ICP-AES analysis (IIT- SAIF, Bombay) and was seen that there was considerable increase in the iron content grown in the presence of bacterial isolates producing siderophores. Maximum Fe content was found in the plantlets grown in the presence of isolate PS2 (30.002 ppm) followed by the mix consortia (27.27 ppm) (Fig 12).

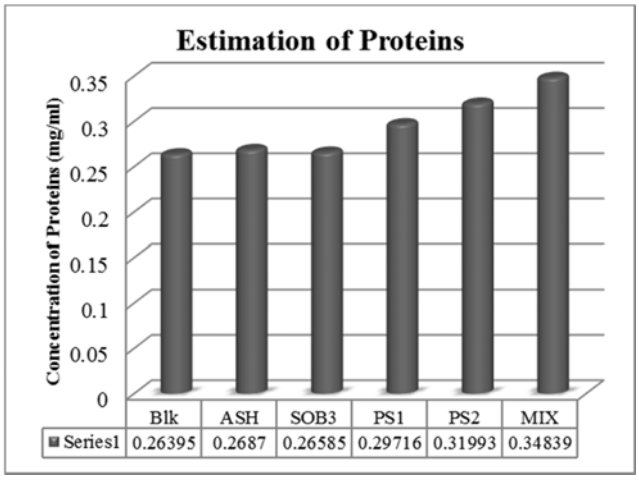

Fig 11: Protein estimation of moong plantlets after growing in biofertilizers.

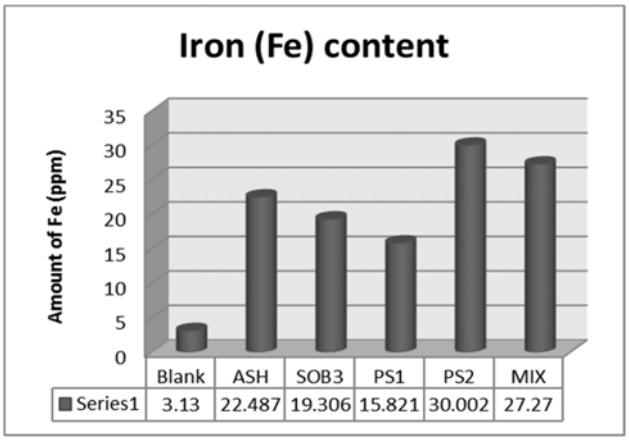

Fig 12: Iron estimation of moong plantlets after growing in biofertilizers.

4. Conclusion

With the increasing use of chemical pesticides, and the resultant increasing pollution, natural biofertilizers would prove to be a boon for the agricultural industry. The microorganisms present in soil have tremendous capacity to produce various compounds which are useful for the soil fertility and growth of plants. The isolates obtained were screened for IAA, Organic acids, Siderophores, Phosphate solubilisation and Sulphate ion production. These isolates could grow in high salt and varied pH concentrations, making them useful in salt-affected areas. Further work on utilizing these isolates to make a potential biofertilizers is to be carried out. These potential biological fertilizers would

play key role in productivity and sustainability of soil. It will also protect the environment as eco-friendly and cost effective inputs for the farmers. With using the biological and organic fertilizers, a low input system can be carried out and it can be help achieving sustainability of farms.

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