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ANALYSIS OF SOIL FERTILIZING CAPABILITIES, GROWTH AND ENZYME PRODUCTON STATISTICS FOR SYMBIOTIC NITROGEN FIXING BACTERIA VITSS5 SCREENED FROM PALAR REGION, VELLORE

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ABSTRACT

In this study, the nitrogen fixing bacteria *VIT SS 1-6* were screened from the root nodules of Groundnut (Family: *Fabaceae*, Species: *Arachis hypogaea*) collected from Vellore district were examined for their plant growth promoting properties. Of the six isolates obtained, five were fast growing and one was slow growing. The slow growing species *VIT SS5* was further examined for its nitrogen fixing, potassium and phosphorus solubilising capabilities to identify its level of efficiency over the traditionally used *N-nitrogen P-phosphorus K-potassium fertilizer*. This particular strain was chosen because it was identified in most of the isolates. Tentative identification was done using biochemical tests and microscopic examination. The screened organism was tested for its enzyme production, utilization of carbohydrate sources, salt, pH and temperature tolerance and statistical analysis was performed. Further studies on preparation of liquid fertiliser will be done.

KEYWORDS:Plant growth promoting bacteria (PGPB), Biochemical tests, Statistical growth optimization, Enzyme assays and NPK-fertilizer.

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INTRODUCTION

The symbiotic relationship between the root nodules of the leguminous plants of *Fabaceae* and diazotrophic bacteria like *Rhizobium* enables the fixation of atmospheric nitrogen. The plant makes carbon source-malate and succinate available for the bacteria for its kerb cycle and in turn the bacteria facilitates fixation of atmospheric nitrogen into ammonium ions which is used by the plant for amino acid and nitrogenous carbon compound synthesis. Nitrogen fixation is brought about by a complex of dinitrogenase and dinitrogenase reductase known as Nitrogenase. Leghemoglobin in the plant cytosol maintains low free oxygen concentrations but mediates high oxygen flux to the bacteroids. Thus the relationship is highly effective and conserved[1]. Legume roots and germinating seedlings exude flavonoid compounds that induce specific rhizobia in the vicinity to produce and secrete a glycolipid, the Nod factor. The capability to incite nodules on host legumes has been shown to depend on rhizobial Nod factors (lipooligochitosaccharides). The process of infection occurs through the production of an infection thread. During the last stage of infection, bacterial release into certain plant cells that become filled with rhizobia. During this endocytosis at the unwalled tips of infection threads the bacteria get surrounded with plasma membrane from the plant. The bacteria differentiate into nitrogen-fixing cells known as bacteroids, and the surrounding plant membrane becomes the specialized peribacteroid membrane. These are often termed symbiosomes. Bacterial Surface Polysaccharides (ex: cyclic glucans, acidic exopolysaccharides, etc.) are essential for the completion of the infection process. Rhizobial nif genes are those that are homologous to genes required for synthesis and function of nitrogenase in nonsymbiotic bacteria. nifKD genes encode the protein subunits of dinitrogenase, and nifH encodes the polypeptide of nitrogenase reductase. nifBEN genes specify the proteins needed to produce the iron–molybdenum cofactor of dinitrogenase. In addition, as in all Proteobacterial nitrogen-fixing bacteria, the regulatory gene nifA is invariably present. fix genes are additional genes needed for symbiotic nitrogen fixation. Dct genes are needed for uptake of most of the carbon supplied by the plant, which is in the form of Krebs-cycle dicarboxylates, mainly succinate and malate. All rhizobial strains identified upto date have larger than average bacterial genomes, ranging from 6.5 to 9.1Mb. In some rhizobia nod, nif, and fix genes are found on a plasmid termed the symbiotic plasmid. These were among the first bacteria in which a high rate of duplications was recognized and studied in detail[2]. Some rhizobial strains are capable of solublizing phosphorus and some have the ability of symbiosis with phosphate solubilising bacteria[3]. *Rhizobium, Pseudomonas* and *Bacillus* species are among the most powerful solubilizers, while tricalcium phosphate and hydroxyapatite seem to be more degradable substrates than rock phosphate[4]. Seed inoculation with effective strains of Rhizobium can meet the requirements of the legume to achieve increased yields. Traditionally, leguminous plants are cultivated to replenish soil fertility during crop rotation and also for their high protein and calorific content food produce. They can also be used for intercropping to minimize the application of fertilizers.

MATERIALS AND METHODS

1.1 Collection of root nodules[5]

Groundnut crops were collected from fields near the Palar region, Vellore, Tamilnadu. The crops were 15 days old and were grown in humid and saline conditions. The plants collected were thoroughly washed off soil and the root nodules were isolated using forceps. Root nodules were occasional on lateral roots and in distant from tap root. The nodules were repeatedly washed with 1% sodium hypochlorite solution and then distilled water, stored in a vial containing anhydrous calcium carbonate or silica gel overlaid by cotton and tightly screwed. In this way nodules collected can be preserved for 6-12 months.

1.2 Culturing and screening of Bacterium[6]

Root nodules were crushed using mortar and pastel and the paste was spread plated on Yeast Extract Mannitol Agar (YEMA) composed of: Mannitol-10g, Di potassium hydrogen phosphate-0.5g, Magnesium sulphate hepta hydrate-0.2g, Sodium chloride-0.1g, Calcium carbonate-3g, Yeast extract-0.5g, agar-15g, Distilled water-1000ml with Bromothymol blue as indicator. However, some articles state that 1g/1000ml of mannitol is sufficient for the culture of rhizobium. Appearance of colonies was observed for after 3 to 5 days. Isolated colonies were obtained which were used for inoculation in the production media (YEMA).

1.3 Staining and Biochemical testing

The bacteria isolated were observed microscopically using Gram and Spore staining. The bacteria isolated are tested using Indole test using SIM agar for tryptophan, Methyl red test using MR-VP agar for acid production by glucose oxidation, Voges-Proskauer using Trypticase soy agar for Organic acid production from glucose metabolism, Citrate test using Simmon citrate agar for citrate utilization, Triple Sugar Iron agar test for identification of glucose, lactose or sucrose utilization and hydrogen sulphide gas production[7, 8, 9, 10 and 11]. The bacteria were also tested for utilization of different carbohydrate sources viz. ribose, arabinose, glucosamine, glucose, fructose, galactose, maltose, sucrose, lactose, trehalose and cellobiose. Salt tolerance was tested for with 2, 3 and 4% Sodium chloride concentrations. Growth in pH 4.5, 7.0 and 9.0 and temperatures 28°C, 37°C and 42°C were observed. Statistical analysis for growth and enzyme production was made using software, Origin 7.1v[12, 13, 14, 15, 16, and 17].

1.4 Enzyme assay[18, 19, 20 and 21]

α-amylase (EC 3.2.1.1) assay was studied using colorimetric stop method (540 nm). Amount of maltose liberated from starch in 3 minutes at 20°C and pH 6.9 was determined.

Protease assay was determined by colorimetric method (280nm). With casein as substrate hydrolysis was measured at 37°C and pH 7.5. Lipase (EC 3.1.1.3) assay was examined using titrimetric method. Amount of fatty acid from triglyceride in 1 hour at 37°C and pH 7.7 was determined. Trioleoylglycerol is used as substrate.

1.5 Analysis of soil fertilizing capabilities

Nitrogenase activity is analysed using Nitrate reduction media composed of Beef or Meat extract-3g, Gelatin peptone-5g, Potassium nitrate-1g and Distilled water-1000ml. Coloration formed by the addition of Solution-A (sulfanilic acid), Solution-B(alpha-naphthylamine) and Zinc powder is used to identify and analyse the nitrate reduction process. Phosphate solubilisation is analysed using Sperber media which is prepared using Glucose-10g, Yeast extract-0.5g, Magnesium sulphate hepta hydrate-0.25g, Calcium chloride-0.1g; Calcium phoshate-2.5g, Agar-15g and Distilled water-1000ml. Zones represent positive result[22, 23 and 24]. Potassium solubilisation is analysed using Alexandrov's media which is made up of Sucrose-10g, Di potassium hydrogen phosphate-2g, Magnesium sulphate hepta hydrate-0.5gm, Calcium carbonate-1g, Agar-20g and Distilled water-1000ml. Zones indicate positive test[25].

RESULTS

Colony characteristics and Microscopic observation

The cells of isolates were rod shaped, gram negative, non-spore forming and motile. Their size approximately ranged between 0.6-0.8 X 1.8-2.2µm. The cells gave a banded appearance under phase contrast microscopy due to the presence of Poly-β-hydroxybutyrate granules. The strains were pleomorphic under adverse conditions of growth. The colonies of the slow growing species were smooth, slightly raised, cream coloured, opaque and had less exopolysaccharide secretion. Their size ranged from 1-3mm in diameter. Alkaline reaction was observed on the media. The fast growing species produced slimy, convex, raised, white and opaque colonies with considerable exopolysaccharide production. Their size varied from 5-7mm in diameter. The slow growing species were isolated and sub-cultured for further studies.

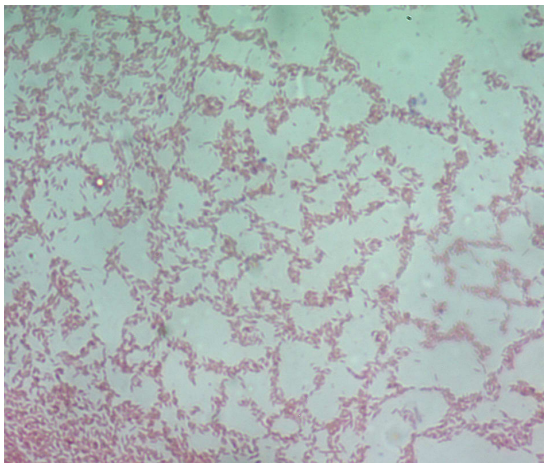

Figure 1.1
Rhizobium (40 X)

Biochemical tests

S.No	Biochemical Test	Result
1.	Indole test	_
2.	Methyl red test	_
3.	Voges-Proskauer test	_
4.	Hydrogen sulphide gas production	_
5.	Citrate utilization	_
6.	Ribose utilization	+
7.	Arabinose utilization	++
8.	Glucosamine utilization	+
9.	Glucose utilization	+++
10.	Fructose utilization	+++
11.	Galactose utilization	++
12.	Maltose utilization	_
13.	Sucrose utilization	_
14.	Lactose utilization	_
15.	Cellobiose utilization	_
16.	Trehalose utilization	_

+ - Poor growth, ++ - Moderate growth, +++ - Well defined growth and - =No growth.

The amount of growth is defined based on factors such as number of colonies, size of the colonies, time taken for the appearance of colonies and exopolysaccharide production.

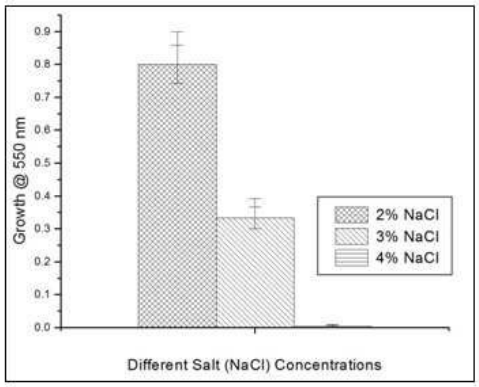

Figure 1.2
Optimum NaCl concentration for growth

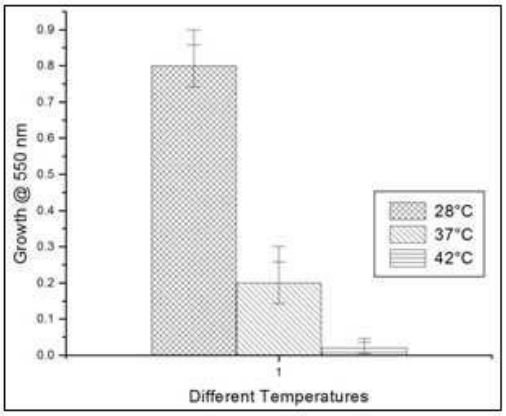

Figure 1.3
Optimum temperature for growth

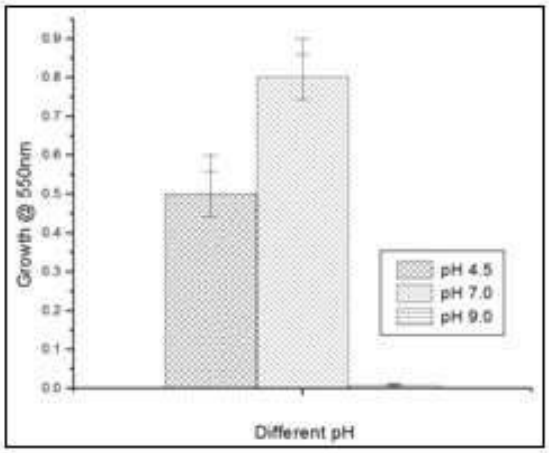

Figure 1.4
Optimum pH for growth
(iii) Enzyme production

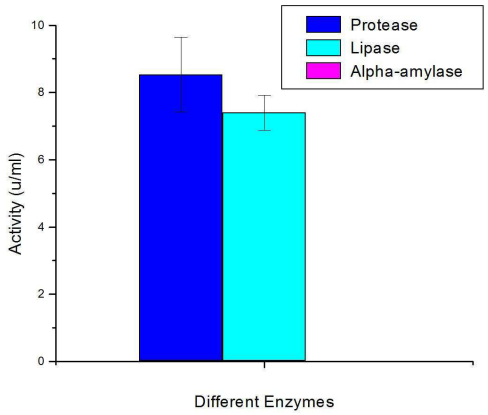

Figure 1.5
Enzyme activity

(iv) Soil fertilizing capabilities

The isolate obtained confirmed nitrogenase activity by reduction of nitrate to ammonia. Phosphate solubilisation was observed as positive since zones were formed in Sperber's media indicating solubilisation of inorganic phosphate source-Calcium phosphate. No zones were present in Alexandrov's media indicating lack of ability for Potassium solubilisation.

DISCUSSION

The slow growing rhizobium strains were identified as *Bradyrhizobium* species since they produced an alkaline reaction on Yeast-Mannitol agar with Bromothymol Blue indicator, slow growth period and also through the various biochemical tests performed. They had moderate tolerance towards high salinity and temperatures but they were highly tolerant to acidic conditions. They were capable of utilizing a wide range of carbohydrate sources mostly mono- and di-saccharides and did not require any vitamins for growth. Protease and lipase enzymes are highly essential for nodule formation process. The isolates showed considerable protease and lipase activity but no α-amylase activity which showed that it had efficient nodulation capability. They were positive for nitrate reduction and phosphate solubilisation but failed to solubilise potassium. Since these bacterial species are non-acid producers, phosphate solubilisation should be due to the synthesis of Phosphatase enzyme.

CONCLUSION

The Bradyrhizobium species screened will be further studied for its compatibility with other plant growth promoting bacteria to produce an efficient Biofertilizer which can be used as a source of Nitrogen, Phosphorus and Potassium for plant growth. Abilities to mobilize other soil nutrients will be identified. Studies will be made on its host range of infection and process. Strain improvement techniques will be conducted to improve its tolerance to environmental conditions and increase its efficiency of nodulation. Further attempts will be made for production of Liquid Biofertilizer.

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