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Research Article

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Isolation of plant growth promoting substance producing bacteria from Niligiri hills with special reference to *Phosphatase* enzyme

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ABSTRACT

The present study isolated plant growth promoting substance producing bacteria from Niligiri hills, Ooty, Tamilnadu. Totally 17 morpholigaclly different bacterial isolates were isolated. All the isolates were screened for plant growth promoting substances. Among 17 isolates, 5 (29.4%), 9(33.3%) and 11(40.7%) shows positive results for ammonia, acetoin and phosphate solubilization, 9 isolates (33.3%) produced indole acetic acid and 7 (25.9%) isolates shows positive for N_2 fixation. The strain NG7 selected as potent strain for the production of phosphatase since the strain showed best activity among other strains. The NG7 strain produce 27.6U/ml of phosphatase enzyme in production. The strain NG7 showed good production of phosphatase when glucose (carbon source), beef extract (nitrogen source), pH 7, temperature 25ºC at 72hrs of incubation of production medium. The potent strain NG7 was identified as Bacillus sp.

Keywords: Phosphatase, Niligiri, Plant growth, Optimization.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) is a group of rhizosphere-colonizing bacteria producing substances which increase the growth of plants and/or protect them against pathogens. These bacteria have the potential to be mycorrhizal helper bacteria (MHB), enhancing their importance on forest management [1]. Plant growth promoting rhizobacteria enhance the growth of plant either directly or indirectly. The direct mechanism involves nitrogen fixation, phosphate solubilization, HCN production, production of phytohormone, such as auxin, cytokinin, and gibberlins and lowering of ethylene concentration. The capacity to produce the phytohormone Indole Acetic Acid (IAA) has widespread among bacteria that inhabitat diverse environments such as soils, fresh and marine waters, plants and animal hosts [2].

Many soil microorganisms possess multiple beneficial traits of nutrient mobilization, production of plant growth promoting substances and biocontrol activity. Among the plant growth promoting rhizobacteria (PGPR), Pseudomonas fluroscens and Bacillus subtilis have emerged as the largest and potentially the most promising group of PGPR with their rapid growth, simple nutritional requirements, ability to utilize diverse organic substrates and mobility [3]. Bacillus is the most abundant genus in the rhizosphere and the PGPRs activity of some of these strains has been known for many years, resulting in a broad knowledge of the mechanisms involved. There are a number

of metabolites that are released by these strains [4]. Phosphate solubilizing Bacillus spp., stimulates plant growth through enhanced Phosphate nutrition increasing the uptake of nitrogen, phosphorous, potassium and iron [5].

Enzymes are proteins that accelerate and mediate the rate of bio chemical reactions in the living system. Phosphatase are enzymes which hydrolyze the complex organic phosphates in to inorganic phosphates(Pi) by the process dephosphorylation which will be further used by the cell to construct the Nucleic acid, Phospholipids, ATP energy molecule etc.

Some free living microorganisms in soil have capability to produce extracellular enzymes such as phosphatase. This enzyme is able to mineralize organic phosphates into inorganic phosphates that provides high P for plant. Soil phosphatases play a major role in the mineralization processes (dephosphorilation) of organic P substrates [6]. Assimilation of phosphate from organic compounds by plants and microorganisms take place through the enzyme "phosphatase" which is present in a wide variety of soil microorganisms. Plant can absorb phosphate only in soluble form [7]. Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation which enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability crop [8]. With this view, the present study focuses towards plant growth promote substance producing bacteria from less explored ecosystem with special reference to phosphatase enzyme..

EXPERIMENTAL SECTION

Collection of sample and Isolation of Bacteria

In this present study, for the isolation of bacteria, soil samples were collected from Niligiri hills, Ooty, Tamilnadu. All the samples were collected aseptically using sterile polythene bags and transport to the laboratory. About one gram of soil sample was diluted in 100 ml of sterile distilled water blank (10^{-2} dilution) and further serially diluted 10^{-3},10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilution using 9ml sterile distilled water blank . Nutrient agar was used for isolation of bacteria. After sterilization, filter sterilized nystatin 20µg/ml was added to the medium, to prevent fungal growth. About 0.1ml of aliquot from 10^{-5}, 10^{-6}, 10^{-7} dilution was plated on nutrient agar plates. Plating was done in triplicate and all the plates were incubated at 28ºC for 48hrs [9]. After incubation, morphologically different colonies were selected for further studies

Screening for plant growth promoting substances

Acetoin production: The Voges-Proskauer test was used as a qualitative method for the detection of acetoin, a precursor for 2,3 butanediol. All the bacterial isolates were inoculated on each 2ml of MR-VP broth and incubated at 28ºc for 5 days. After incubation, to the 1ml bacterial culture 3ml of freshly prepared 5% α-Napthol in absolute ethanol and 1ml of 40% KOH were added and the mixture was stirred vigorously. The formation of a red colour is indicative of the presence of acetoin [9].

Ammonia Production: For the screening of ammonia production, all the bacterial isolates were inoculated into each 5ml of sterile peptone broth and incubated at 28ºc for 48 hours. After incubation, about 0.5ml of Nessler's reagent was added into all the tubes. The production of ammonia is indicated by the appearance of pink colour[10].

Phosphate solubilization: All the isolates were screened for phosphate solubilization by the method described [10]. All the bacterial isolates were spot inoculated on Pikovoskaya's agar plates and incubated at 28ºCfor 2-3days. After incubation the plates were observed for clear halo formation around the bacterial growth for every 24hours.

Nitrogen fixing bacteria: Nitrogen fixing activity of bacterial isolates was studied by inoculating them into Jensen medium and incubating them into 5 days for 28ºc. Presence of growth on Jensen medium indicates nitrogen fixation [10].

Indole acetic acid (IAA): For the screening of IAA production, all the bacterial isolates were inoculated into each 5ml of sterile nutrient broth supplemented with 3mg/ml of L-tryptophan and incubated at 28ºc for 5 days. After incubation, cell free supernatant was obtained by centrifuging at 10,000rpm for 10min and to that a drop of orthophosphoric acid and 2ml of Solwaski's reagent was added and kept for 20min at room temperature. The development of pink colour indicates the production of IAA [9].

Selection of potential phosphatase producing strain by plate method:
All the selected bacterial strain was further screened for confirmation of phosphatase activity by plate method. The bacterial strains were spotted on Pikovoskaya's agar. The plates were incubated at 28˚C for 48 hrs. After incubation, the plates were observed for clear halo formation around the bacterial growth. Strain which showed maximum zone of clearance of their preliminary screening was selected as potential strain for further studies.

Production of phosphatase enzyme from potent strain
Ten ml of nutrient broth was prepared and inoculated with one loopful of potent bacterial culture and incubated at 28˚C for 18hrs and used as inoculums of production. For the production of phosphatase the composition of production medium was used (containing 1% glucose, 0.5% peptone, 0.1% yeast extract, 0.02% MgSO4.7H2O, 0.002% KH2PO4, 0.5% NaCl,). About 5ml of bacterial inoculums was transferred to 50ml of production medium supplemented with 0.3% agar as a sole source of carbon was prepared in 250ml Erlenmeyer flask and incubated at 28˚C for 72hrs at 170rpm [11].

Enzyme recovery and semi quantitative assay of crude phosphatase enzyme
The fermented broth was centrifuged at 6000 rpm for 30min and the cell free supernatant obtained was collected and used as crude phosphatase enzyme. The semi quantitative assay of the crude phosphatase enzyme produced from test strain was done by agar well diffusion method on Pikovoskaya's agar. About 20µl of crude enzyme was added into the well and incubated at 28˚C for 24hrs [12]. After incubation the plates were observed for zone of clearance around the well. The size of zone of clearance was directly proportional to the quantity of crude enzyme present in the crude preparation.

Partial purification and assay of phosphatase
The separation and dialysis of phosphatase was carried out by adopting the protocol described [13]. About 50 ml of crude enzyme produced from the potent strain was taken and stirred with magnetic beads. To this 50 ml of 80% ammonium sulphate solution was added and slowly mixed for about one hour. The precipitate was allowed to form at 4ºC for 24 hours. Then the whole solution was centrifuged at 4,000 rpm for 10 minutes at 4ºC. The precipitate obtained after ammonium sulphate precipitation was dialysed using dialysis membrane against phosphate buffered saline (pH-7) for 24 hours. The buffer was changed occasionally. Then the dialysate was tested for protein assay by Lowry's method [12].

Optimization of phosphatase Production
Effect of critical medium components on phosphatase production was studied by adopting classical one factor at a time method. Factors which are studied include carbon source, nitrogen source, incubation period, temperature and pH [13].

Characterization and identification of potential strain
Microscopic observation, cultural and biochemical characteristic of potential phosphatase enzyme producing strain was studied by adopting standard procedure and the potential strain was identified with the help of Bergey's Manual of systematic bacteriology.

RESULTS AND DISCUSSION

Isolation of Bacteria
Totally 17 morphologically different bacterial colonies were selected from forest soil samples after incubation. All the strains were selected for further studies based on their fast growth. Saravanan [9] isolated microorganisms from Kodaikannal hills and studied various activity of isolated microorganisms. Many reports are there for forest ecosystem from various plants but there is less report of Western Ghats.

Plant growth promoting substances
Plant growth promoting substances of bacterial isolates in this study shows positive results. Out of 17 isolates 5 (29.4%), 9(33.3%) and 11(40.7%) shows positive results for ammonia, acetoin and phosphate solubilization. Nine isolates (33.3%) produced indole acetic acid and 7 (25.9%) isolates shows positive for N_2 fixation. (Fig 1). Saravanan [9] isolated plant growth promoting substances and reported from Kodaikanl hills.

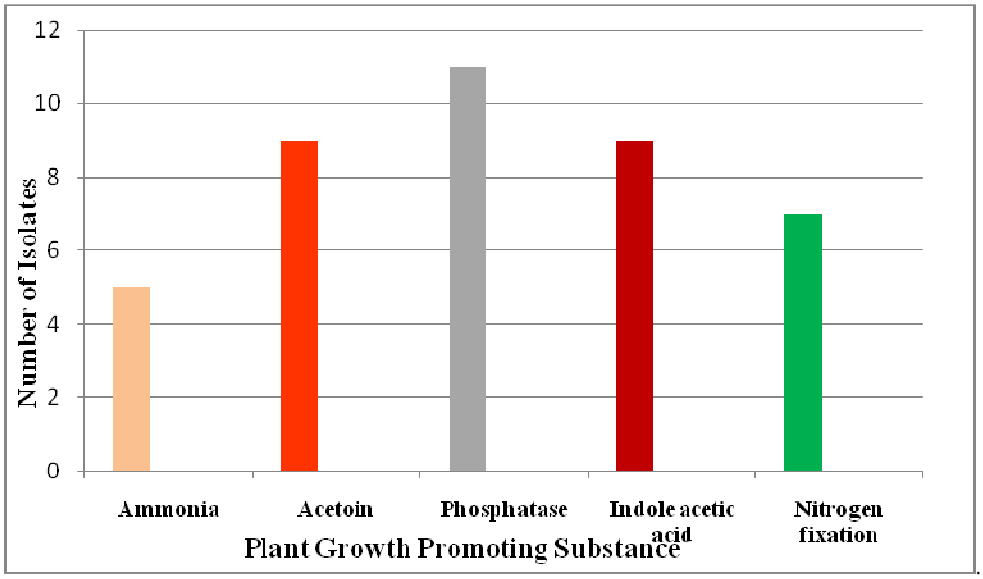

Figure 1. Number of isolates producing plant growth promoting substances

Selection of potent phosphatase strain

Among the 11 isolates producing phosphatase enzyme the strain NG7 showed maximum zone of clearance (21mm) in plate, hence the strain NG7 selected as potent strain for further studies.

Radhakrishnan [14] concluded that the size of zone of clearance around well is that the proportional to the enzymatic activity of the test organism (Table 1).

Table 1. Screening of phosphatase enzyme for bacterial isolates

S. No	Zone of Clearance (in mm in diameter)
NG1	12
NG 2	14
NG 3	10
NG 4	09
NG 5	15
NG 6	16
NG 7	21
NG 8	14
NG 9	16
NG 10	18
NG 11	18
NG 12	16
NG 13	14
NG 14	19
NG 15	14
NG 16	16
NG 17	11

Partial purification and quantitative assay of phosphatase enzyme

The strain NG7 was selected as potent strain that could produce large amount of phosphatase into the production medium and the quantity of the crude enzyme was found to be 18.4U/ml. Omran [15] used *Bacillus sp.*, strain for screening of phosphatase production and reported from different strains. In this present study partial purification of the enzyme by ammonium sulphate precipitation and dialysis showed 27.6 U/ml of enzyme activity. Saravanan et al., [9] concluded that the size of zone of clearance around well is that the proportional to the enzymatic activity of the test organism[14]. In this present study, strain NG7 showed more zone of clearance then other strains. Dialysate

of NG7 strain showed 32mm of zone of clearance in semi quantitative assay which shows increase of activity than crude enzyme.

Optimization of phosphatase production
The optimization study was carried out by one factor at a time. Priya [16] isolated and studied optimization of alkaline phosphatase from *Bacillus megaterium*. In this present study also optimization study was carried out.

Effects of carbon source on phosphatase production
The maximum activity 24mm was obtained when glucose (1%) was used as the carbon source and lactose (1%) showed the second maximal enzyme production 19mm. The minimal production 17mm was obtained when fructose (1%) was used as carbon source and sucrose showed the second minimal enzyme production 15mm.

Effects of nitrogen source on phosphatase production
The maximum activity 30mm was obtained when beef extract (1%) was used as the nitrogen source and peptone (1%) showed the second maximal enzyme production 27mm. The minimal production 25mm was obtained when yeast extract (1%) was used as nitrogen source and malt extract showed the second minimal enzyme production 21mm.

Effects of pH on phosphatase production
The maximum activity 32mm was obtained when pH 7 was used, 27mm was obtained when pH 7 was used which shows second maximal enzyme production. The minimal production 24mm was obtained when pH 6 was used and 21mm was obtained when pH 5 and 19mm was obtained when pH 4 was used showed the minimal enzyme production.

Effects of temperature on phosphatase production
The maximum activity 24mm was obtained when temperature was at 25˚C and 22mm was obtained when pH 30˚C was showed maximal enzyme production. The minimal production 18mm was obtained when temperature 35˚C and no zone was observed at 45˚C was used as temperature.

Effects of incubation period on phosphatase production
The maximum activity 26mm was obtained when incubation period was at 72 hours and 21mm was obtained in 48hrs incubation. Second minimal production 14mm zone of clearance was obtained at 24 hours incubation. There is no increased zone in 96 hrs and 130hours incubation period.

Characterization of Potent strain
Based on the morphological characters, cultural characters and biochemical characters the potent strain was identified as *Bacillus* sp.

CONCLUSION

All these conditions are favorable to industrial application, avoiding microbial contamination and reducing the formation of undesirable products. The phosphatase produced by the *Bacillus sp.,* NG7 isolated in this study will have wide applications. Optimization studies using statistical methods like response surface methodology is in progress to prove its phosphatase producing potential further.

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