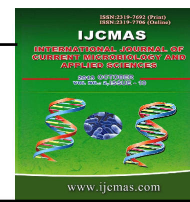
Int.J.Curr.Microbiol.App.Sci (2013) 2(10): 142-149



International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 2 Number 10 (2013) pp. 142-149**
http://www.ijcmas.com

Original Research Article

Isolation, characterization of phytase producing *Bacillus* sps NBtRS6 from the rhizospere soil of NBt cotton field

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A B S T R A C T

Keywords

Immobilized P; Rhizosphere; Phosphatases; Phospho-rylation; Tropical Soils.

Soil samples of Bt Rhizosphere were collected from NBt cotton growing area of Andhra Pradesh, India and was used as a source material for isolation and screening of phytase producing bacteria. 21 Bacteria were isolated from NBt Rhizosphere. Phytase enzyme activity of the cultures was screened on Modified phytase solubulizing medium (MPSM). The result inferred that six isolates NBtRS1 to NBtRS6 were strongly positive in enzyme activity than six of other microorganisms while nine isolates were found negative and among all ,Isolate NBtRS6 produced significantly higher phytase yield than other isolates and was chosen for species identification. Preliminary identification by microscopic and biochemical tests identified the isolate NBtRS6 as *Bacillus* sp and designated as *Bacillus* sp NBtRS6.

Introduction

In nature, phosphorus cycle plays an important role in the survival of living organisms (Stewart and Mckercher, 1982). There are two components of P in soil, organic and inorganic phosphates. A large proportion is present in insoluble forms, and therefore, not available for plant nutrition. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of them appearing after the application of chemical fertilizers. These precipitated forms cannot be absorbed by plants. Organic matter, on the other hand, is an important reservoir of immobilized P that accounts for20–80% of soil P (Richardson, 1994). To convert insoluble (both organic and inorganic) to a form accessible to the plants, like orthophosphate, is an important trait for a PGPB for increasing plant yields. Phosphatase is an enzyme that release inorganic phosphate from organic moiety and complex inorganic materials. It is known to play an essential role in phosphorus cycle, even though; roles of other various physical factors cannot be ignored. Phosphorus is the maker of the energy currency and it plays important roles in enumerable metabolic pathways in living systems (Rasol and Reshi, 2010).Cellular signaling events cascaded with phosphorylation and

dephosphorylation, are associated with an enzyme called phosphatase (EC 3.1.3.-). Soil receives various phosphatases from living organisms that play important roles in the solubilization of inorganic phosphates (Acosta-Mortinez and Tabatabai, 2000). Enzymatic activities of a soil sample are critical index of soil fertility because enzymes play an important role in nutrient cycles (Dick *et al.,* 1996). In particular, phosphatases play a key role in phosphorous cycle by solubilizing organic and inorganic phosphates into available forms that support growth of crop plants (Wyszkowska and Wyszkowski, 2010). Released inorganic form of phosphate is readily soluble in soil and plant system can easily uptake it as nutrient source. Soil phosphatases are heterogeneous in nature and the enzymes have tribal names, according to their substrates (Alvear *et al.,* 2005), such as phosphoric monoester hydrolases, (EC 3.1.3.) and phosphoric diester hydrolases (EC 3.1.4.). Importantly the first group is composed of phytase, nucleotidases, sugar phosphatases, glycerophosphatase (Cohen, 1989). However, the second group contains nucleases and phospholipases (Speir and Ross, 1978). The present study investigated the best phytase producer from rhizosphere of conventional cotton field.

Materials and Methods

Isolation of phytatase producing bacteria

Isolation of the phytase-producing bacteria was carried out by sampling soil from NBt cotton fields in Andhraprdesh. Soil samples (0-15 cm depth) were collected using a sterile stainless steel spatula into a sterile jar. Three replicate samples were randomly collected from three sites (1 m apart) to make a composite sample and this was used for bacterial screening. One gram of each sample was suspended in 10 ml of sterile distilled water & was serially diluted and 10^{-3}&10^{-4} dilutions of each sample were spread on to nutrient agar medium and incubated for two days.

Screening for best phytase producing isolate

The plate screening was carried out for 21 isolates from nutrient agar medium The colonies of nutrient agar were isolated individually on(MPSM) modified phytase screening media containing Na phytate-2g/L, NH4NO3- 5g/L, MgSO4-0.5g/L, KCl -0.5g/L,FeSO4-0.1g/L, glucose-15g/L, bactoagar-15g/L, CaCl2-8% and PH 6.5 was adjusted. The plates were incubated 37ºC for 24h. To visualize the Clear zone equal volumes of 6.25% ammonium molybdate and 0.42% ammonium vanadate solution are flooded, in the plate and incubated and can be examined for zones of clearing indicative of phytase activity. Efficient phytate solubulizer was selected based on the formation of larger clearing zones on MPSM agar (Yanke *et al.,* 1998).

Identification of selected isolate

Morphological and Biochemical tests

The strain was initially examined for cell morphologies and cell arrangement by gram staining, presence or absence of spores and capsules and motility using microscopy. Various morphological and biochemical tests were carried out by the techniques described in the Mackie and McCartney Practical Medical Microbiology .The various biochemical tests carried out were Indole test, Methyl

red test, Voges proskauer test, Citrate utilization tests, catalase, oxidase, urease, nitrate reduction, starch hydrolysis, gelatin hydrolysis, H2S production and carbohydrate fermentation tests. The isolate was tested for fermentation of various sugars like Glucose, Lactose, Mannitol, Maltose, Sucrose, Xylose and Galactose, growth at various temperatures (4, 10, 30, 37, 40, 45, 50, 55), NaCl requirement (2,4,6,8,10),growth at different pH (5,6,7,8,9).

Phytase production by the isolate

The six phytase producers NBtRS1 to NBtRS6 were inoculated in to Tryptone Soya broth and incubated at 37ºC for 24 h. 40 µL calcium phytate was added as an inducer. The phosphate liberated was quantified after 2 days. Culture broth was centrifuged at 5,000 rpm for 5 min and 350 µL of 0.1 M Tris malate buffer to 50µL supernatant to which 4 µL of sodium phytate was added and incubated at 37ºC for 30 min. 100µ L reaction sample was added to the solution containing 10mM ammonium molybdate solution: 5 N H2SO4 : acetone in the ratio of 1:1:2. Enzyme reaction was allowed for 30 min and the observance of sample was measured at 405 nm (Heinonenand Lahti, 1981).

The liberation of reducing sugar was measured by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit (U) of phytase was defined as the amount of enzyme required to liberate one micromole inorganic phosphate per min under the given assay conditions. Bacillus sps NBtRS6 exhibited highest phytase activity in terms of calcium phytate Units.

Result and Discussion

Phytase producing bacteria was identified by Morphological, Cultural and Biochemical characteristics of the selected bacterial isolate NBtRS6 was carried out according to the Guidelines of Bergey's Manual of Systemic Bacteriology. Morphological studies had revealed that the NBtRS6 was was aerobic endospore forming, non pigmented and wrinkled with concentric rings. The organism was positive for growth under anaerobic conditions. The growing cells were Gram positive, motile with rod shape. BtRS6 showed positive results for casein hydrolysis, Voges proskauer, Citrate utilization, Urease, H2S production, Starch hydrolysis, Lecithinase, Gelatin liquefaction, Arginine dihydrolysis, and Phosphate solubilization reactions. The NBtRS6 was also positive for the utilization of sugars like Starch, Maltose, Glucose, Lactose, Mannitol ,Maltose, Sucrose, Galactose, Glycerol. Negative towards suucinate, R -Alanine, L-Histidine, L-Lucine, D-Alanine.

The isolate grew well in nutrient broth at pH range of 7.0 to 9.0 and showed salt tolerance at NaCl concentration up to 10 (w/v) .Bacterial growths was observed in the temperature ranging from 4ºC – 55ºC with an optimum growth around 37ºC. It was identified as *Bacillus* sps and designated as *Bacillus* NBtRS6.After Identification of bacterial culture, the efficacy of the organism for phytase production was determined using the basal mineral salts medium. Phytase activity was of NBtRS6 isolate was determined according to Tryptic Soya Method.

Table.1 Hydrolysis efficiency of the isolates

Isolate	Colony diameter, C (mm)	Halo diameter, Z (mm)	Hydrolysis Efficiency, Z-C/C(%)
NBtRS1	11	22	100
NBtRS2	12	27	125
NBtRS3	10	21	110
NBtRS4	15	32	113
NBtRS5	11	23	109
NBtRS6	12	30	150
NBtRS7	30	35	17
NBtRS8	8	9	12.5
NBtRS9	33	36	9
NBtRS10	30	32	7
NBtRS11	28	33	18
NBtRS12	20	24	20

A total of 21 colonies showed growth on nutrient agar media plates on incubation. Out of 21 colonies, six were strongly positive in enzyme activity than six of other microorganisms, as indicated by the clear zone of hydrolysis around them. While NINE isolates were found negative for phytase production on MPSM. And are designated as NBtRS1-NBtRS21. Among the 21 isolates, NBtRS6 isolate which showed maximum activity was characterized as *Bacillus* sps NBtRS6.

The amount of soluble reducing sugars that was glucose released from production sugars was determined. Phytase activity was expressed in terms of inorganic phosphate released. The volume of NBtRS6 isolate filterate responsible for release of 1µ mole of phytase per min was considered to be one unit of inorganic phosphate. Since *Bacillus* NBtRS6 had been detected to exhibit highest phytase activity in terms of 0.08U/ml .

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). Root development, stalk and stem strength, flower and seed formation, crop maturity and production-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus nutrition.. Soil P dynamics is characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes.

Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001). Release of organic anions, and production of siderophores and acid phosphatase by Plant roots / microbes (Yadaf and Tarafdar, 2001) or alkaline phosphatase (Tarafdar and Claasen, 1988) enzymes hydrolyze the soil organic P or split P from organic residues. The largest portion of extracellular soil phosphatases is derived from the microbial population (Dodor and Tabatabai, 2003

Table.2 Morphological and biochemical tests for identification of Bacterial isolate

Identification tests Bacterial isolate
Colony morphology
Configuration Round, Concentric, Cream, Wrinkled Margins Entire Surface Butyraceous Elevation Slightly Raised Pigmentation - Opacity Opaque Gram's reaction Positive Cell shape Rods Size(µm) 3-5µm in length, width 1.0 -1.2 µm in width Spores + Motility +
Physiological tests
Growth at temperature 4^0C - 10^0 C - 30^0 C + 37^0 C + 40^0 C + 45^0 C + 50^0 C + 55^0 C +
Growth in NaCl (%)
2 + 4 + 6 + 8 + 10 +
Growth at Ph
5 - 6 - 7 + 8 + 9 + Growth under anaerobic condition +

Biochemical tests

Indole test –
Methyl red test -
Voges proskauer test +
Citrate utilization test -
H2S production -
Gelatin hydrolysis +
Urea hydrolysis +
Starch hydrolysis +
Lectinase +
Lipase (Tween 80 hydrolysis) -
Catalase test +
Oxidase test -
Denitrification -
Arginine dihydrolase +
Phosphate solubilization +
Chitinase +
Casein hydrolysis +
Degradation of Tyrosine +
Nutritional characteristics
Starch +
Maltose +
Glucose +
Lactose+
Mannitol +
Maltose+
Sucrose+
Galactose+
Xylose-
Glycerol +
suucinate -
L-Alanine -
L-histidine -
L-lucine -
D-alanine –

Microorganisms are integral to the soil phosphorus (P) cycle and as such play an important role in mediating the availability of P to plants. Organic phosphate can be solubilized by bacterial Organic acids, after which free phosphates may sometimes be liberated by hydrolysis or the dephosphorylating action on organic compounds by a wide spectrum of enzymes released from microorganisms. It is essential to bring about some microbial transformations of both inorganic and organic compounds in soil to make available of this element to plants.

Enterobacter agglomerans solubilizes hydroxyapatite and hydrolyze the organic P (Kim *et al* ., 1998). Mixed cultures of PSMs (*Bacillus, Streptomyces, and Pseudomonas* etc.) are most effective in mineralizing organic phosphate (Molla *et al.,* 1984). The mineralization of organically bound phosphorus, which forms a major fraction of the soil P capital (Harrison, 1979), is essential to the maintenance of the phosphorus cycle and the Replenishment of the available P in the soil in forest ecosystems (Harrison, 1985).

The insightful microbial contribution to plant P nutrition and opportunities for manipulating specific microorganisms to enhance P availability in soil has therefore been of considerable interest over many decades. This interest is accentuated by P deficiency being common in weathered and tropical soils throughout the world, by rising costs of P fertilizer, and because the efficiency of P use by plants from soil and fertilizer sources is often poor despite many soils containing a relatively large amount of total P that is only sparingly available to plants.. Exploitation of microorganism's to increase the availability of P in soil therefore is an attractive suggestion for developing a more sustainable agriculture.

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