INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-4, Issue-4, Oct-Dec-2013 Coden : IJABPT Copyrights@2013

ISSN : 0976-4550

Received: 29th August-2013 *Revised: 08th Sept-2013* *Accepted: 11th Sept-2013*

Research article

ISOLATION, CHARACTERIZATION OF PHYTASE PRODUCING BACILLUS SUBTILS BtRS2 FROM THE RHIZOSPHERE SOIL OF Bt COTTON FIELD

K. Usha Sri[1],

[1]Lecturer in Microbiology, Dept. of Microbiology, S.G.Govt. Degree & P.G College, Piler-517 214, A.P,India

ABSTRACT: Soil samples of Bt Rhizosphere were collected from Bt cotton growing area of Andhra Pradesh, India and was used as a source material for isolation and screening of phytase producing bacteria. 19Bacteria were isolated from Bt Rhizosphere. Phytase enzyme activity of the cultures was screened on modified phytase solubulizing medium (MPSM). The result inferred that six isolates BtRS1 to BtRS6 were strongly positive in enzyme activity than six of other microorganisms while seven isolates were found negative and among all, Isolate BtRS2produced significantly higher phytase yield than other isolates and was chosen for species identification. Preliminary identification by microscopic and biochemical tests identified the isolate BtRS2 as *Bacillus* sp. Further, the identification of the strain was confirmed by subjecting it to 16S rRNA gene sequence analysis. The isolate BtRS2 showed high similarity to *Bacillus subtilis sps* and was thus designated as *Bacillus subtilis* BtRS2 The sequence is deposited under accession no. KC471313 in GeneBank

Key words: Rhizosphere, Transgenic crop, Genetically modified (GM), Mineralization, Biogeochemical cycles, MPSM, Phytase

INTRODUCTION

The transgenic crop, now popularly called Bt cotton, represents about 90% of cotton cultivated area in India .Genetically modified cotton genotypes incorporating a crystal (*Cry*) toxin producing *cry1Ac* gene derived from *Bacillus thuringiensis* (Bt), were introduced in India for commercial cultivation in the year 2002 (Morse et al. 2005). The potential risks of genetically modified (GM) plants to environmental and human health have become a public concern in recent years, due to the release of transgenic crop plants worldwide and their replacement of traditional crops (Nap et al. 2003). The introduction of GM plants into agricultural ecosystems raised a number of questions, including the ecological impact on soil ecosystems. Soil-borne communities are dominated by microorganisms, which account for > 80% of the total biomass in soil (Kowalchuk et al. 2003). They are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens and improvement of soil structure (Bruinsma et al. 2003). Changes in the structure or function of microbial communities have a major impact on soil ecosystems and biogeochemical processes. Microbial community structure and function in rhizosphere soil, which is directly influenced by root exudates of GM plants, are often proposed as an early and dynamic indicator of GM risk assessment on soil ecology, and used increasingly for sensitive responses (Nannipieri et al. 2003). Organic phosphorus is abundant in soils and is an important source of phosphorus for plants in both natural and managed environments. Information on soil organic phosphorus is essential for understanding biogeochemical cycles and ecosystem ecology, because organisms possess a variety of complex mechanisms to access organic phosphorus in their environment. Mineralization of phosphate from soil organic P by phosphatase enzymes is of particular significance, as organic P accounts for a major proportion (generally 40 to 80%) of the total P in most soils, occurring primarily as inositol phosphates (Turner et al., 2007). A more effective utilization of phosphate from soil and fertilizer sources would be particularly beneficial to agriculture throughout the world (Richardson et al., 2005).
The use of phytase -producing microorganisms is seen as a chance to reduce the need of P-fertilizers

Isolation of phytate degrading bacteria

Isolation of the phytase-producing bacteria was carried out by sampling soil from Bt cotton fields in Warangal, Andhra Pradesh. 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. Bacterial strain was isolated from the soils of BtR fields.1 g of these samples was suspended in 5 ml of 0.9% saline solution for dilution plate method. (Cappuccino and Sherman, 2010).

Isolation of phytase producing bacteria;

The colonies of nutrient agar were isolated individually on(MPSM) modified phytase screening media containingNaphytate-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 and characterization of selected isolate

The isolate BtRS2 was identified by Morphological, Cultural and Biochemical characteristics according to the guidelines of Bergey's Manual of Systemic Bacteriology and characterized using 16S rRNA sequencing followed by BLAST

Phytase production by Bacillus subtilis

The six phytase producers BtRS1 to BtRS6 were inoculated in toTryptone 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 malatebuffer to 50 µL supernatant to which 4 µL of sodium phytate was added and incubated at 37ºC for 30 min. 100µLreaction sample was added to the solution containing 10mMammonium 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 subtilis BtRS2 exhibited highest phytase activity in terms of calcium phytate Units.

RESULTS AND DISCUSSION

The plate screening was carried out for 19 isolates. The isolates were incubated on Modified Phytase Solubulizing Medium (MPSM) plates, modified method of Yanke et al. (1998) and clear zone by phytase activity of the colony was visually indicated. The result inferred that six isolates BtRS1 to BtRS6 were strongly positive in enzyme activity than six of other microorganisms while seven isolates were found negative Among the 17 isolates, BtRS2 isolate which showed maximum activity was characterized as BtRS2. Screening of microorganisms for the solubulization of phytate is of importance and it has been intensively pursued for many years by scientists. Bacteria are the most widely distributed groups of microorganisms in nature. Phytase is a useful enzyme in commercial interest. This is used in plant growth promotion and added to animal feed to increase the availability of phosphorus. Until now, it has been reported that phytase is produced by a variety of microorganisms including bacteria such as B. subtilis (Powar and Jagannathan, 1982; Rodriguez et al., 2007) and Pseudomonas spp. (Cosgrove, 1970; Pandeyet al., 2004), yeasts such as Saccharomyces cerevisiae (Barbaricet al., 1984; Latif and Hashem, 2011) and fungi such as Aspergillus sps. Nowadays, commercial production of Microbial phytase for plant growth promotion was broadly applied (Joel et al., 2003). Morphological studies had revealed that the BtRS2was 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. BtRS2 showed positive results for casein hydrolysis, Voges proskauer, Citrate utilization, Urease, H2Sproduction, Starch hydrolysis, Lecithinase, Gelatin liquefaction, Arginine dihydrolysis, and Phosphate solubilization reactions. The BtRS2 was also positive for the utilization of sugars like Starch, Maltose, Glucose, Glycerol Negative towards succinate, ®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 upto 10 (w/v). Bacterial growth was observed in the temperature ranging from4ºC – 55ºC with an optimum growth around 37ºC. Phytase producing bacteria was identified by Morphological, Cultural and Biochemical characterization of the selected bacterial isolate BtRS2 was carried out according to the guidelines of Bergey's Manual of Systemic Bacteriology. The identification of the strain was further done by 16S rRNA gene sequencing analysis. Therefore, it was designated as *Bacillus subtilis* BtRS2. The sequence is deposited under accession no. KC471313 in Gene Bank.

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

Table1: Morphological and biochemical tests for identification of bacterial isolate

Identification tests	Bacterial isolate
Colony morphology	Configuration Round, Concentric, Cream, Wrinkled
Margins Entire	Slightly Raised
Elevation	Surface Butyraceous
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	–
10^0C	–
30^0C	+
37^0C	+
40^0C	+
45^0C	+
50^0C	+
55^0C	+
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	–
H_2S production	–
Gelatin hydrolysis	+
Urea hydrolysis	+
Starch hydrolysis	+
Lectinase	+
Lipase (Tween 80 hydrolysis)	–
Catalase test	+
Oxidase test	–
Denitrification	–

Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

Arginine dihydrolase	+
Phosphate solubilization	+
Chitinase	+
Casein hydrolysis	+
Degradation of Tyrosine	+
Nutritional characteristics	
Starch	+
Maltose	+
Glucose	+
Lactose	+
Mannitol	+
Maltose	+
Sucrose	+
Galactose	+
Xylose	–
Glycerol	+
Succinate	–
®-Alanine	–
L-Histidine	–
L-Lucine	–
D-Alanine	–

The growth of these soil organisms can serve as a basis for the development of living soils by optimizing the potentials of the beneficial biotic populations. 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 BtRS2 isolate was determined according to Tryptic Soya Method.

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 BtRS2 isolate filterate responsible for release of 1µ mole of phytase per min was considered to be one unit of inorganic phosphate. Since Bacillus subtilis BtRS2had been detected to exhibit highest phytase activity in terms of0.06U/ml in subsequent experiments this organism was further exploited to assess the potential phytase producer**.** (Table-1)

CONCLUSION

This study highlights the characterstics of phytase producing bacteria isolated from Bt rhizosphere .The Bacillus subtilis BtRS2 of the present investigation need further optimization studies.

. REFERENCES

Barbaric, S., Kozulic, B., Reis, B., and Mildner, P. (1984). Physicochemical and kinetic properties of acid phosphatase fromSaccharomyces cerevisiae. J. Biol. Chem. 259: 878-879.

Bruinsma M., Kowalchuk G.A., van Veen J.A. (2003): Effects of genetically modified plants on microbial communities and processes in soil. Biology and Fertility of Soils, *37*: 329–337.

Cappuccino, J. G. and Sherman, N. (2010). Phosphate solubulization. In Microbiology a Laboratory Manual. International student edition.8th Ed. Addison Wesely Publication. 343-344

Cosgrove, D. J. (1970). Inositol phosphate phosphatases of microbiological origin. Inositol phosphate intermediates in thedephosphorylation of the hexaphosphates of myo-inositol, scylloinositol,and D-chiroinositol by a bacterial (Pseudomonas sp.) phytase.Aust. J. Biol. Sci. 23: 1207-1220.

Heinonen, J. K. and Lahti, R. J. (1981). A new and convenient colorimetric determination of inorganic orthophosphate and itsapplication to the assay of inorganic pyrophosphatase. Anal. Biochem.113: 313-317.

Joel, R., Cherry, Glenn, E. and Falholt, N.P. (2003). Encyclopedia of environmental microbiology Enzymes: Biotechnological Applications

Kowalchuk G.A., Bruinsma M., Veen J.A. (2003): Assessing responses of soil microorganisms to GM plants. Trends in Ecology and Evolution, *18*: 403–410.

Latif, A. E. and Hashem. (2011). Molecular Genetic Identification of Yeast Strains Isolated from Egyptian Soils for Solubilization of Inorganic Phosphates and Growth Promotion of Corn Plants. J. Microbiol.Biotechnol. 21(1): 55-61.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytical Chemistry. 31: 426-428.

Morse S., Bennett R.M., Ismael Y. (2005): Genetically modified insect resistance in cotton: Some farm level economic impacts in India. Crop Protection, *24*: 433–440

Nannipieri P., Ascher J., Ceccherini M.T., Landi L., Pietramellara G., Renella G. (2003): Microbial diversity and soil functions. European Journal of Soil Sciences, *54*: 655–670.

Nap J.P., Metz P.L.J., Escaler M., Conner A.J. (2003): The release of genetically modified crops into the environment: Part I. Overview of current status and regulations. Plant Journal, *33*: 1–18.

Nelson, T. S. and Peeler, H. T. (1961). The availability of phosphorus from 178 single and combined phosphates to chicks. Poultry. Sci. 40:1321-1328.

Powar, V. K. and Jagannathan, V. (1982). Purification and properties of phytate-specific phosphatase from Bacillus subtilis. J. Bacteriol. 151: 1102-1108.

Pandey, A., Trivedi, P. and Palni, L.M.S. (2004). Carrier-based Preparations of Plant Growth-promoting Bacterial Inoculants Suitable for use in Cooler Regions. World J. Microbiol. and Biotechnology. 21(6-7): 941-945.

Richardson, A. E., George, T. S., Hens, M. and Simpson, R. J. (2005). Utilization of soil organic phosphorus by higher plants. In B.L. Turner, E. Frossard, and D. Baldwin. (Ed.), Organic Phos-phorus in the Environment. CABI Publishing, Wallingford, UK. pp.165-184.

Rodriguez, H., Fraga, R., Gonzalez, T. and Bashan, Y. (2007). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Developments in Plant and Soil Sci. 102: 15-21.

Turner, B. L., Richardson, A. E. and Mullaney, E. J. (2007). Inositol Phosphates:Linking Agriculture and the Environment. CAB International, Wallingford, UK. p. 304.

Yanke, L. J., Bae, H. D., Selinger, L. B. and Cheng, K. J. (1998). Phytase activity of anaerobic ruminal bacteria. Microbiology. 144: 1565-1573.