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ISOLATION, CHARACTERIZATION OF PHYTASE PRODUCING *BACILLUS SUBTILLS* MTCC NO.9878 FROM THE GUT OF *EISENIA FOETIDA*

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ABSTRACT
17 Bacteria were isolated from earthworm gut (*Eisenia foetida*). Phytase enzyme activity of the cultures from gut was screened on modified phytase solubulizing medium (MPSM). The result inferred that four isolates PSB1 to PSB4 were strongly positive in enzyme activity than six of other microorganisms while seven isolates were found negative and among all PSB1 isolate which showed maximum activity was characterized as *Bacillus subtilis* MTCC No. 9878 based on cultural, morphological, physiological and biochemical characterstics.

INTRODUCTION

The present-day cultivation technology requires very high input of synthetic fertilizers to intensify the farming, besides the loads of insecticides, fungicides and herbicides. This causes soil degradation which eventually affects adversely the agricultural productivity (Hasan, 1996). Phosphate solubilization is a complex process involving both organism and soil, which needs more attention between laboratory methods to soil tests and seed inoculation experiments (Jacobsen *et al*., 2005). 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-secreting micro-organisms is seen as a chance to reduce the need of P-fertilizers. The cost of Phosphate (P) -fertilizer application is dependent on soil and crop; for example, for groundnut a need of about 25 kg P_2O_5 / ha, has been calculated, which corresponds approximately to Rs 400 / ha. By using phytase-secreting soil micro-organisms and/or phytase secreting crops the input costs are expected to be reduced by 50 to 75%. Through local groups and community based organizations, farmers understand that "soil is a community resource and an active reservoir" and "green manures is nature's tonic for the soil". These notions inspire and enlighten the concept and help in building knowledge and skills for developing a more integrated soil fertility management.

The present research is going to introduce a sustainable soil fertility system, evaluate the combined effect of biofertilizers and organic manure such as green manure, compost and farmyard manure. Microbial phytases can be exploited for efficient conversions of phytate to phosphates when given as green manure to the soil for improved yield. The present study concludes the gut flora of earthworms contribute phosphates to crops when supplemented to soil to replace the chemical fertilizer.

Earthworm, the friend of the farmer, can play a variety of important roles in agroecosystems (Gajalakshmi and Abbasi, 2002). Their feeding and burrowing activities incorporate organic residues and amendments into the soil, enhancing decomposition, humus formation, nutrient cycling and soil structural development. Earthworms are increasingly recognized as indicators of health of the agroecosystem and as important tools for ensuring soil improvement and efficient nutrient cycling.

The role of microbial activity in the earthworm gut, cast and soil is very essential for the degradation of organic wastes for the release of nutrients to plants. During vermicomposting organic matter undergoes, physico-chemical and bio-chemical changes by the combined effect of earthworm gut flora and also other microbial activities. Earthworms transform organic waste constituents by their grinding and also through digestion of aerobic and anaerobic microflora into a nutrient rich vermicompost.

Introduction of earthworms to areas not previously populated has led to improvement of soil quality and productivity (Gajalakshmi and Abbasi, 2004). Earthworms increase the amount of phosphorous mineralized from organic matter in soil as reflected in the casts which is the source of nutrients for plants (Rombke *et al.,* 2005).

Phytase is a useful enzyme in commercial interest. Phytase is also an important industrial enzyme hydrolyzing phytate to release inorganic phosphate (Nelson and Peeler, 1961; Ullah and Gibson, 1987). Phytic acid (myo- inositol 1, 2, 3, 4, 5, 6-hexa kis hydrogen phosphate) and mixed cation salts of phytic acid, designated as phytates, these are a group of organic phosphorous (P) compounds found widely in nature, derived from a number of sources like plants, animal tissues and microorganisms. Studies have shown that microbial phytases are most promising for biotechnological applications and plant growth promotion in agriculture and added to animal feed to increase the availability of phosphorus. Phytase producing bacteria are substrate specific. They show greater resistant to proteolysis and better catalytic efficiency. phytase has broad applications therapeutics and in reducing environmental pollution.

Until now, These are a group of organic phosphorous (P) compounds found widely in nature these are derived from a number of sources like plants (Lolas and Markakis, 1977; Ullah and Gibson, 1987), animal tissues (Bitar and Reinhold, 1972) and microorganisms including bacteria, yeasts, and filamentous fungi (Shieh and Ware, 1968; Howson and Davis, 1983; Suziki *et al*.,1997). The hydrolysis of phytic acid to myo-inositol and phosphoric acid is a very important metabolic process in many biological systems. It has been reported that phytase is produced in a variety of microorganisms including bacteria such as *B. subtilis* (Powar and Jagannathan, 1982) and *Pseudomonas* (Cosgrove, 1970), yeasts such as *Saccharomyces cerevisiae* (Barbaric *et al.*, 1984) and fungi such as *Aspergillus* species. Nowadays, commercial production of microbial phytase for plant growth promotion was broadly applied.

In the present study owing to importance of earthworms on effective phytate solubulization, bacteria were isolated from gut of *Eisenia foetida* and screened for phytase enzyme activity.

MATERIALS AND METHODS

Collection of earthworms and sampling procedure

Earthworms (*Eisenia foetida)* were collected from Farmyard Manure (FYM) around Tirupati region, Andhra Pradesh, India. Earthworms were washed with sterile tap water and then placed on a sterile petriplate moistened with filter paper and subjected to starvation for 24 h, further they were disinfected with 70% ethanol, gut was dissected out, weighed and homogenized (for 5 min with a vortex mixture) in sterile 0.85% NaCl 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 containing Naphytate-2g/L, NH_4NO_3- 5g/L, $MgSO_4$-0.5g/L, KCl -0.5g/L, $FeSO_4$-0.1g/L, glucose-15g/L, bactoagar-15g/L, $CaCl_2$-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

Based on Morphological and Biochemical characterstics the isolate PSB1 was identified as a strain of *Bacillus* in our laboratory. Subsequently the isolate was submitted to Instittute of Microbial Technology (Chandigarh).

Phytase production by *Bacillus subtilis*

The four phytase producers PSB1-PSB4 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 50 μL supernatant to which 4 μL of sodium phytate was added and incubated at 37ºC for 30 min. 100μL reaction sample was added to the solution containing 10mM ammonium molybdate solution: 5 N H_2SO_4 : 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 (Heinonen and Lahti, 1981).

The liberation of reducing sugar was measured by dinitro salicylic 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 MTCC NO.9878 exhibited highest phytase activity in terms of calcium phytate Units.

RESULTS AND DISCUSSION

The plate screening was carried out for 17 isolates. The isolates were incubated on Modified Phytase Solubulizing Medium MPSM plates, modified method of Yanke *et al*.(1998) and clear zone by phyase activity of the colony was visually indicated. The result inferred that four isolates PSB1 to PSB4 were strongly positive in enzyme activity than six of other microorganisms while seven isolates were found negative The result of description on the size of clear zone was as follows: 4 isolates were for + + + +, 3 for + + +, 2 for + +, and 1 for +. 7 isolates did not make clear zone. Most microorganisms making clear zone were bacteria. Among the 17 isolates, PSB1 isolate which showed maximum activity was characterized as *Bacillus subtilis* MTCC NO.9878.

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

Table1: 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^{0}C	-
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	-
H_2S 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	+
Glycerol	+
suucinate	-
β-Alanine	-
L-histidine	-

Cont....Table1: Morphological and biochemical tests for identification of bacterial isolate

Identification tests	Bacterial isolate
L-lucine	-
D-alanine	-
Antibiotic resistance	
Penicillin G	-
Ampicillin	+
Chloramphenicol	+
Erythromycin	+
Streptomycin	-
Tetracycline	-
Gentamycin	+
Tobramycin	+
Rifampicin	+
Polymyxin	+

et al., 2007) and *Pseudomonas spp.* (Cosgrove, 1970; Pandey *et al*., 2004), yeasts such as *Saccharomyces cerevisiae* (Barbaric *et 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 PSB1 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. PSB 1 showed positive results for casein hydrolysis, Voges proskauer, Citrate utilization, Urease, H_2S production, Starch hydrolysis, Lecithinase, Gelatin liquefaction, Arginine dihydrolysis, and Phosphate solubilization reactions. The PSB- 1 was also positive for the utilization of sugars like Starch, Maltose, Glucose, Glycerol. Negative towards suucinate, β -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 from 4ºC – 55ºC with an optimum growth around 37ºC. The PSB1 was sensitive to antibiotics like Penicillin G, Streptomycin, Tetracycline, and showed resistance to Ampicillin, Chloramphenicol, Erythromycin, Gentamycin, Tobramycin, Rifampicin, Polymyxin (Table 1).

Phytase producing bacteria was identified by Morphological, Cultural and Biochemical characterization of the selected bacterial isolate PSB1 was carried out according to the guidelines of Bergey's Manual of Systemic Bacteriology. Identified and characterized by IMTECH, Chandigarh table 1 and identified as *Bacillus subtilis* MTCC NO. 9878 and the phylogenetic relationship revealed that 99.9% identity was with *Bacillus subtilis*. Based on the above characteristics *Bacillus subtilis* was able to solubulise phytate. The finding of the present study reinforces the general concept that the gut and casts of earthworms tend to be much more microbiologically active than the surrounding soil. Enhancing 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 and diagnosis of bacterial culture, the efficacy of the organism for phytase production was determined using the basal mineral salts medium. Phytase activity was of PSB1 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 PSB1 isolate filterate responsible for release of 1μ mole of phytase per min was considered to be one unit of inorganic phosphate. Since *Bacillus subtilis* MTCC No. 9878 had been detected to exhibit highest phytase activity in terms of 0. 0.09U/mL in subsequent experiments this organism was further exploited to assess the potential phytase producer.

The *Bacillus subtilis* MTCC No. 9878 of the present investigation need to be further studied in depth of its potential phytase producing capacity for actual application in conversion of insoluble phytate. Microbial phytases can be exploited for efficient conversions of phytate to phosphates when given as greenmanure to the soil for improved yield. The present study concludes the gut flora of earthworms contribute phosphates to crops when supplemented to soil to replace the chemical fertilizer.

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