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ISOLATION AND CHARACTERIZATION OF PHYTASE ENZYME FROM *Escherichia coli*

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ABSTRACT

Phytic acid was discovered in 1903, and is found to be nearly ubiquitous component in cereals and grains. It is found to be 80% or more in plants, especially in legumes. Monogastric animals feeding on plants or grains are unable to utilize the phosphate which has been bound to phytic acid. Thus, studies have been centered around a number of phytate degrading enzyme, one of them is phytase enzyme. Phytase is also found to be used in the area of nutrition, environment and biotechnology. It has a capability to hydrolyzed phytate to myoinositol and inorganic phosphate. It releases phosphorus, making it available for absorption and utilization by monogastric animals (hens, chickens, pigs, swines, etc.) and thereby reducing bound phosphorus excretion which is in unusable form creates pollution in environment. Dietary inclusion of phytase in monogastric animals has shown that it can decrease total phosphorus level in the manure. It was found that micro organisms have ability to produce phytase enzyme and hence can be utilized by monogastric animals. Thus bacteria were isolated from the different samples of soil available in valsad and nearby area on general media. Afterwords, isolated bacteria that were able to produce the enzyme phytase were isolated on PSM (phytase screening medium). Enzymes were also tested for its activity which was studied by colorimetric method. The isolated enzyme is found to be extracellular in nature and shows the conversion of phytate to inorganic phosphorus and a positive step towards the problem of Eutrophication.

KEYWORDS: Phytate, Phytic acid, Phytase, Eutrophication.

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INTRODUCTION

Phytic acid is partially attributed to the wide-spread human nutritional deficiencies of calcium, iron, and zinc in developing countries where the staple foods are of plant origin.[1] Supplementation of diets with inorganic phosphorus along with the excreted phytate phosphorus, however, imposes global ecological problems when it enters into rivers resulting in cyanobacterial blooms, hypoxia, and death of marine animals.[2] Phytic acid or myo-inositol 1,2,3,4,5,6-hexakisphosphate, is the major phosphorus (P) storage compound in plant seeds and can account for up to 80% of total phosphorus. Soluble inorganic and cellular phosphorus, meaning phosphorus bound in nucleic acids, phosphorylated proteins, phospholipids, phosphor-sugars, represents the remaining phosphorus. Because of the high density of negatively charged phosphate groups, phytate chelates with mineral cations like potassium (K), magnesium (Mg), calcium (Ca), zinc (Zn), iron (Fe), copper (Cu) and forms poorly soluble complexes. Apart from minerals, phytate also forms complexes with proteins and amino acids. The amino group present on the side chain of the amino acids is one of functional groups involved in protein-phytate interaction, thereby decreasing the digestibility of proteins. These salts of phytic acid are known as phytins and their availability/digestibility to monogastric animals including fish is very limited due to the lack of intestinal phytase.[3] Unfortunately, phytates are not hydrolysed in the monogastric gut, and the phytate-associated phosphates remain unabsorbed, requiring the exogenous addition of phosphate to avoid phosphorus deficiency. In addition to increasing basic food costs, the phosphates in the excreted phytates are available to microbial soil consortia, which break them down to create phosphate run-off and serious environmental pollution,[4] phytate-phosphorus is excreted into the environment and is acted upon by microorganisms that release the phosphorus, causing pollution in terms of algal growth. The enzymes that catalyze the conversion of phytic acid to inositol and inorganic phosphate are known as phytases. Phytases (*myo*-inositol hexakisphophate phosphohydrolases, EC 3.1.3.8 and 3.1.3.26) are produced by plants and microorganisms.[5] These enzymes hydrolyze phytate (*myo*-inositol hexakisphophate) in a stepwise formation of myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphate to release inorganic phosphate.[6,7] The present investigation involves isolation of phytase producing bacteria from poultry faeces and different types of soil and characterization of isolates obtained.

MATERIALS AND METHODS

Sodium phytate was purchased from Sigma-Aldrich, USA. All dehydrated media were purchased from Himedia India Pvt Ltd., Mumbai, India. All reagents were of analytical grade and were purchased from Qualigens, Mumbai, India.

Sample Collection

Soil Samples used for isolation of Phytase producing organisms were collected from different areas of Valsad city and its surroundings. The soil samples were collected from areas having mangroves, beach soil, leguminous soil, poultry soil and rhizospheric soil for incorporating a more diversifying microbial growth. Even poultry fecal material was used for isolation of the enzyme.

Isolation of Phytase degrading bacteria

1 gm of soil samples were suspended in distilled water and plated on basal media like Nutrient agar (1% peptone, 2.5% NaCl, 1.5% Meat extract, 3% agar, pH 7.2). Plates were subjected to incubation at RT for 24 hours. Different species were found on the plates which were isolated and primarily identified. The isolated species were further plated onto Phytase producing medium (PSM) (1.5% glucose, 0.5% NH_4SO_4, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 6.5 with 0.5% sodium phytate.[8] Plates were incubated at RT and were observed for 2-3 days. The colonies exhibiting zone of clearance (translucent area) were selected and streaked onto fresh PSM plates.

Characterization and Identification of Isolated Bacteria

The bacterial isolates showing maximum zones were measured and further identified up to genus level based on Gram staining, Motility, Morphology, Physiochemical properties of the isolate according to Bergey's Manual of Determinative Bacteriology.[9] The strains were further evaluated at Saffron Life Sciences, Bilimora for further testing and Molecular Identification. For Sequence analysis, bacterial genomic DNA was extracted and purified using CTAB method.[10] PCR amplification was performed in a final reaction volume (100) µl. The PCR reaction was run for 35cycles in a DNA thermal cycler. The amplified PCR product were than analyzed in (1.0) % w/v Agarose gel, excised from the gel, and purified. The amplified DNA sequence was then sequenced. The 16SrRNA gene sequences of the isolates were aligned with reference 16SrRNA sequence of the GenBank using the BLAST algorithm available in NCBI.

Inoculum Preparation

The inoculum was prepared for production of the enzyme. The pure culture was inoculated into sterile PSM broth & incubated at 30°C in a rotary shaker for overnight. The fresh overnight culture was used as an inoculum for the production of enzyme. After sufficient incubation the sample from the medium is collected, centrifuged and used to study its enzyme activity.

Enzyme activity

Phytase activity was calculated by measuring the rate of increase of inorganic phosphate according to the method of Harland & Harland.[11] Protein quantifications were made by Lowry's Method and compared with the standard (200µg/ml) using Bovin Serum Albumin (BSA).

RESULT AND DISCUSSION

Total 13 different isolates were obtained. The organisms isolated were screened for the production of Phytase enzyme by streaking the organisms on phytase producing plates containing specific substrate Sodium Phytate (Figure 1). Many organisms were found to be

grown on the plates but few showed zone of hydrolysis of sodium phytate. Similar results have also been found in other organisms.[12,13,14] By plate screening, *E coli* (K4) was found to be the best strain and could produce clear zone around the colonies (Figure 2) which showed similar results.[15]

Figure 1
Cultural and Growth Characteristic of Bacteria Isolated from Soil of Different Places

Figure 2
Zone of Hydrolysis of Selected Bacteria K4 (14mm)

Table 1
Cultural & Growth Characteristic, Motility and Gram Reaction of Bacteria Selected for Phytase Production

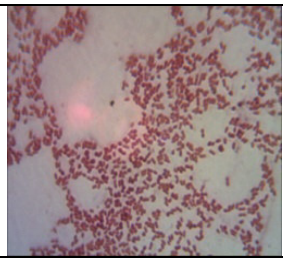
Name of Organism	Characteristics of the Colony	Motility	Gram reaction and Morphology	Figure
K4	Small. Irregular, Smooth, Filamentous, Moist, Translucent, Yellow pigmented colonies	Motile	Gram negative short rods occurring singly	

Figure 3
Biochemical Test results of K4 organism

Primary identification of micro organisms which were able to degrade phytic acid into inorganic phosphate was carried out by studying motility, Gram reaction, Morphological characteristics and biochemical test according to Bergey's manual of Determinative bacteriology (Table 1, Figure 3)[9]. Genus and species confirmation of the organisms was carried out at Saffron Life science, Bilimora for testing of 16S rRNA sequencing. On comparing 100% homology was found with the 100% query K4 to be *Escherichia coli* strain NRC131.Quantitative screening by phytase assay also showed that *E.coli* to be the best strain for production of phytase enzyme. *E.coli* produces phytase enzyme around (39.84U/ml) (Table 2, Figure 4) in 48 hours.

Table 2
Enzyme Activity of K4 Bacteria

Time Duration (Hours)	OPTICAL DENSITY	PROTEIN CONCENTRATION µg/ml	ENZYME ACTIVITY Units/ml
24	0.28	150	36
48	0.31	166	39.84
72	0.31	166	39.84
96	0.29	155	37.2
120	0.22	119	28.56

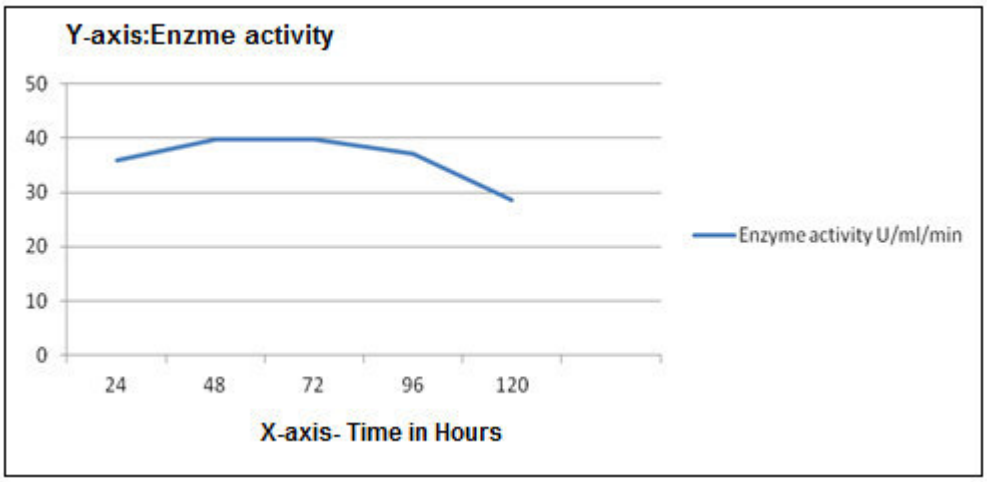


Figure 4
Enzyme Activity of K4 Bacteria

CONCLUSION

From the above results, it can be concluded that phytase producing organisms were successfully isolated and identified on the base of morphological, cultural, biochemical and molecular characteristics (16S r RNA sequencing). Also phytase enzyme has been successfully produced with the help of submerge fermentation. Enzyme activity was found to be satisfactory around 39.84 U/ml/min. Activity of the same phytase enzyme can be increased by optimizing the physical parameters like pH, temperature, NaCl and Sucrose concentration in the medium. In order to increase the economic value of the enzyme, the crude enzyme can be purified using column chromatography and SDS page analysis.

CONFLICT OF INTEREST

Conflict of Interest declared none.

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