Current Trends in Biotechnology and Pharmacy
Vol. 17(4) 1448-1456,October 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
DOI: 10.5530/ctbp.2023.4.80

Identification of Phytate Degrading Probiotic *Bacillus Sp.* – potent Source of Phytase for Phosphate Bioavailability

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Abstract

Phytate, an anti-nutrient, limits the bioavailability of phosphorus and other minerals in feedstocks of monogastric animals. An extrinsic phytase, a phosphatase enzyme, is supplemented in their diets to increase the absorption of minerals like calcium, iron, and zinc found in many grains and oil seeds. A viable source for the screening and isolation of bacterial phytase producers was found in the current investigation to be bacteria-rich food-grade probiotics. Although fungi and yeast based phytases are popular, bacterial phytases have emerged as potential source of phytase with their biochemical properties. Probiotic with variety of formulations containing spores of bacillus species was used as potential source for phytase secreting bacillus and its identification. Upon screening of these strains, PS4 strain showed 6.2mm clear hydrolytic zone on 1.5 % PSM agar plate. Biochemical assays and 16s rRNA sequencing identified the phytase producing strain to be *Bacillus* sp BAB 3372. The phytase enzyme activity assay showed 1.15 U/ml. Genomic DNA from the phytase producing isolates was 19.773 ng/µl and its purity assessed by measuring the absorbance ratio of 260/280 was found to be 1.8. 16srRNA sequencing identified the isolate as bacillus BAB 3372 Accession No. KF952780.1. This preliminary screening has helped in the identification of bacterial β-Propeller phytases that can be used as feed supplement in livestock and also for reduction of Eutrophication in the areas livestock production.

Keywords: *Bacillus,* β-Propeller phytase, Phytate, Probiotics, 16s rRNA, Phytase Screening Medium (PSM)

Introduction

Cereals and legume seeds are the main source of phosphorus. In these crops phytate is involved in controlling key enzymatic and regulation of metabolic pathways (1). Phytate is available in the form of phytic acid and has six phosphate groups (inositol hexaphosphate or 6 phosphate inositol) which are polyanionic, and complexes with cations such as calcium (Ca^{2+}), iron (Fe^{2+}), zinc (Zn^{2+}), magnesium (Mg^{2+}), potassium (K^{+}) and manganese (Mn2+) and these complexes are Known as Phytates. By chelating these minerals, phytate reduces the bioavailability of these important minerals among the monogastric animals (2). Phytate also forms insoluble complex with peptides, starch vitamins

and lipids which results in their reduced absorption and bioavailability (3). Due to its poor availability in monogastric organisms like chicken, pigs and fish it is considered as anti-nutrient feed. The undigested phosphorus in these organisms are released into environment and cause eutrophication of lakes (4).

Phytase (Myo-inositol hexakisphosphate phosphohydrolases), belongs to phosphatase are capable of hydrolyzing organic phytate and release inorganic phosphorus. (P5-P4 ester). It is found in plants, animals, and microbes (5) and are classified as HAP:Histidine acid phosphatases; BPP: β-propeller phytases, PAP: Purple acid phytases, CP: cysteine phosphatases and the Enzyme database classified phytases based on the carbon in the myo-inositol ring of phytate at which dephosphorylation is initiated into 3-phytases (EC. 3.1.3.8), 6-phytases (EC. 3.1.3.26) and 5-phytases (EC. 3.1.3.72). Commercialization of phytases for feed was started in 1991 with the launch of Histidine acid phosphatases from *Aspergillus niger*(6). In 1999 researchers firmed that acid phosphatases from *E. coli* are effective than phytases from fungal sources (7). Applications of phytases in food processing and feed is still limited due to low thermal stability problems (8-9). Thus, there is a great need for phytases with good thermostability, high activity at low pH that would find applications in food and feed industry.

Primary screening methods have been attempted for isolation of extracellular phytase from bacteria, yeast, plants and fungi (10). However, production of phytase from plants is reported to suffer from cumbersome and uneconomical pretreatment processes (11).The phytases from fungi and yeast have been reported to exhibit variable glycosylation patterns (12). There are several challenges while culturing filamentous fungi like high viscosity, clumpy growth, insufficient oxygen, and mass transfer limitations resulting in low yields (13). Bacterial phytases on the other hand are easy to produce due to their fast production rates (14). Bacteria do not grow in the form of pellets as may be seen in the case of fungi, and therefore, the problems of mass transfer are generally not observed, and due to simple cellular structure and simple metabolic processes can easy be manipulated for fermentative production of enzymes (15).

Bacterial phytases have been reported to be isolated from various phosphorus rich phytate and low pH habitats, (Acidophiles). Probiotics are known for their ability to survive and thrive in various harsh conditions, especially in the gut environment like Bile resistance, Tolerance to low pH. Probiotics are known to secrete many hydrolytic enzymes and are naturally designed to be active at gut environment. Commercially available probiotics are known to host upto 16 types of *Bacillus* spp. These species are predominantly gram-positive spore forming facultative aerobes viz., *B. subtilis, B. coagulans, B. cereus, Lactobacillus sp.,* etc. Hence, the authors hypothesize that these commercially available food-grade probiotics formulations may have good potential to host phytase producing bacteria with good catalytic activity at Low pH and thermal stability. This has been used to screen probiotic formulations for phytase producers on a screening media followed by their isolation and identification with 16S rRNA sequencing.

Material and Methods

Microbial media like Luria bertani (LB) media, nutrient agar, MRS **(**De Man, Rogosa and Sharpe**)** and other chemicals and reagents were procured from HiMedia, India which were of analytical grade.

Sampling and bacterial culture

Commercially available probiotic sachets (Sporlac Sanzyme Ltd., India,) were used to screen and isolate bacterial strains with high phytase producing ability. The probiotic powder (4 g) was dissolved in ten ml of 0.85% sterile sodium chloride and serially diluted. Sample containing 100µl was inoculated in Petri plates

with nutrient agar, LB agar, and MRS media for enrichment, cultivation, and isolation of all species of *Lactobacillus.* These Petri plates were incubated at 37°C, for 36–48 hrs. Morphologically distinct colonies obtained were biochemically analyzed for identification. Single colonies were chosen from plates with selective media and stored in glycerol stock. The colonies obtained were sub-cultured in LB broth and incubated in orbital shaker at 200 rpm for 36 to 48 hrs at 37°C.

Qualitative and quantitative screening for phytase-producing isolates qualitative phytase assay

The pure bacterial cultures grown aerobically were assessed for production of extracellular phytase on Phytase Screening Medium as mentioned by Howson (1983). Its composition is expressed in (g 1-1) and the detail is as below: D-glucose, 10.0$CaCl_2$ 2.0; sodium phytate, 4.0; NH_4NO_3, 0.5; $MgSO_4.7H_20$, 0.01; KC1, 0.5; $FeSO_4.7H_2O$, 0.01; $MnSO_4.4H_2O$, 0.01, Agar 15.0.pH 6.0 adjusted with 1 M HCl before autoclaving at121°C for 20 min. Replicate plates were inoculated with 0.1 ml sample by spread plate method and incubated at 37°C for 4 days. The opaque region in the culture is a visual indication for production of extracellular phytase indicating the utilization of phytate. Hydrolytic zone was measured for selected colonies in millimeters using a ruler.

Quantitative phytase assay

Quantification of Phytase activity was done as described by Heinonen et al. (16) with some modifications. (Figure 1) The isolated strains were inoculated in culture media for phytase activity with (% w/v): peptone 1; Dextrose 0.5; Yeast Extract 0.5; Sodium phytate ($C_6H_6Na_{12}O_{24}P_6$) 0.1, Magnesium Sulphate ($MgSO_4$) 0.1; Calcium chloride ($CaCl_2$), 0.1, and pH 7.0 in 250ml flask and incubated for one week at 37°C, in orbital shaker at 200 RPM. The suspension cultures were centrifuged in refrigerated centrifuge at 4°C for 20 m, with 5000 rpm. The supernatant was analyzed for phytase activity and expressed in U/ml.

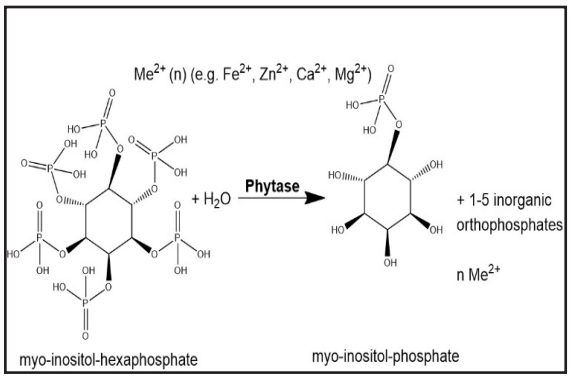

Figure 1: Phytase Activity: Hydrolysis of phytate into myo-inositol phosphate.

Phytase Assay: Aliquot of 0.5 ml supernatant was mixed with 4ml of AAM (Acetone-Acid-Molybdate) colorimetric solution which consists of 10 mM $(NH_4)6Mo7O_{24}$, 2.5M $H_2SO_4$and C_3H_6O in 1:1:2 V/V/V. The reaction mixture was incubated at 37 °C for 10min and the absorbance was measured at 400nm. Positive control was carried out under same conditions by adding 0.5ml of a Commercial phytase. The reaction was inhibited by addition of 1 ml 10% trichloroacetic acid (TCA). Calibration curve was prepared with inorganic phosphate (KH_2PO_4) in the range of 0.15–2.5 µmol. Phytase activity U/mg of protein is defined as the µmol of phosphate released per minute under the standard assay conditions of pH 5.5 at 37 °C for 10 min. This method was chosen since it uses the direct detection of the yellow phosphomolybdic acid, without reduction to molybdenum blue.

Characterization and identification:

The three most promising phytase-producing isolates were analyzed based on the phenotypic characters and biochemical tests as described by Holt et al.(17) The morphological characteristics, colony morphology, color, shape, motility, Gram staining reaction, physiological (temperature, oxygen requirement) and biochemical characterization (indole test, methyl red, citrate utilization, voges proskauer, urease, nitrate reduction, catalase, oxidase).has

been carried out for the tested bacteria.

DNA extraction, amplification, and sequencing

An aliquot of 2 ml suspension culture was taken and centrifuged for isolating the genomic DNA by Qiagen All Prep kit.PCR mix with 2.0 µL of 1.25 mM each dNTPs mixture , 1.0 µL of 20 mM F&R primer, DNA 2.5µL, Taq polymerase 1.0µL and sterile deionized water and made upto 100 µL. It was denatured at at 95°C for 4 minm. PCR thermal outline include Denaturation at 95°C for 60 s, Annealing at 50°C for 45 s and Primer extension at 72°C for 90 s 30 cycles and the final extension is at 72°C, for 10 min The products of PCR were performed using (1%) agarose gel electrophoresis and visualized by staining with ethidium bromide. Its amplicon was purified to remove contaminants. Sequencing was analyzed by BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. More taxonomic insights on the isolated strains were done by Marchesi et al.(18) 16S rRNA sequencing and phylogenetic analysis.16S rRNA bacterial primers 27F and 1492R was used to amplify the gene with 10 ng of genomic DNA isolated from strain. From the forward & reverse sequence data obtained by Clustal W, the 16S rDNA gene Consensus sequence was derived.

The sequenced data obtained from PCR products was aligned and evaluated to find closest homolog of isolates using mixture of Genbank database using the BLAST program of NCBI, Clustal analysis was carried out using Clustal W, Multiple Clustal Alignment software. The best hits of ten sequences were identified and aligned using maximum identity score Clustal W. phylogenetic tree and distance matrix was generated using MEGA 7. Using the Ribosomal Database Project (RDP) database16S rRNA classifier Wang et al.(19) close relationships at the genus was established by Clarridge etal. (20) and at species level Conlan et al. (21). Moreover, dedicated 16S rRNA databases Cole et al. (22) for taxonomic placement of sequences exists.

Results and Discussion

Qualitative phytase screening

Out of the five strains isolated, qualitative screening carried out with PS4 and PS5strains showed transparent regions around their colonies, is an indication of its phytase activity extracellularly. The other isolates, PS1, PS2, PS3 did not show any ability to produce phytase in the qualitative assay. Isolate PS4 produced clear peripheral hydrolytic zones of (6.2 mm) and PS5 showed(4.4mm), were found to be extracellular phytase producers. (Figure 2)

The observation of clear zones surrounding the colonies of some of the isolates is in concurrence with earlier researchers of Demirkan et al.(23) Abd-Alhadi et. al.(24) and Onawola et al. (25-26) who had isolated phytase-producing bacteria strains. Quantitative enzyme assay was conducted for all the five isolates. The PS4 and PS5isolatesexhibited highest transparent zones in quantification for phytase activity. These two phytase-producing isolates were selected for further study. During the incubation period of 5-7 days, the first three days of culture showed low levels of phytase activity and in the stationary phase ie 4thday showed highest activity and gradually declined in its activity and cell density. The bacterial isolates PS 4 had and highest phytase activity over other isolates.(Table 1) Maximum phytase activity was observed in stationary phase was in lines with phytase of *Bacillus sps*.KHU-10 by Choi et al.(27) *B.subtilis* (natto) and Shimizu et al.(28).Earlier scientific findings show probiotic bacteria like *weissella kimchii* R-3 (1.77 U/Ml) has been reported for extracellular phytase production by Andrabi et al.(29).Morphological and Biochemical characterization studies on phytase producing bacteria such as *Bacillus* sp., as described by Kumar et al.(30).In this study, the authors report a maximum production of phytase enzyme to be 1.15 U/ml which was in accordance with earlier researchers reported from *L.penstoses* Amritha et al.(31)

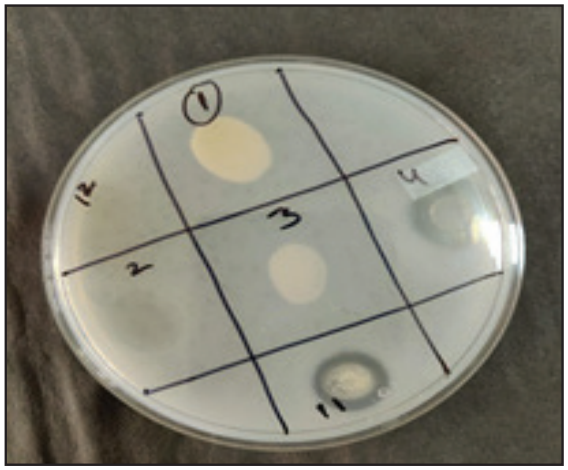

Figure 2: Screening of Phytate hydrolyzing bacteria

Table 1: Phytase enzyme hydrolytic zones of isolates and its Activity

S.NO	Bacterial Isolates	Hydrolytic zone (mm)	Phytase Activity (U/ml)
1	PS1	0.4	0.16
2	PS2	0.6	0.12
3	PS3	0.8	0.29
4	PS4	6.2	1.15
5	PS5	4.4	0.94

Characterization and identification of isolates

Out of the five isolates, four strains were Gram-positive, rod shaped. They also showed similarity in colony morphological characteristics like PS1, PS3, PS5 circular, creamy white and non-motile, all the five isolates showed positive rod shaped to Grams reaction. All colonies were shown smooth surface and entire margin, but differ in their opacity likePS2 and PS4 showed opaque colonies. All the isolates were aerobic and grew well between 25°C – 40°C and did not show any growth below 20°C. Based on the phenotypic characters, three isolates were identified as *Bacillus sp*. as described in Williams et al. (32).

Biochemical characterization for the three Phytase producing isolates PS3, PS4 and PS5 showed that except indole test all the test showed positive results identifying as them as *Bacillus* spp. Genomic DNA from the phytase producing isolates (PS3, PS4 and PS5) was 19.773 ng/µl and its purity assessed by measuring the absorbance ratio of 260/280 was found to be 1.8. Visualized band at 1350bp in Agarose gel electrophoresis with 1% agarose stained with ethidium bromide. (Figure 3)

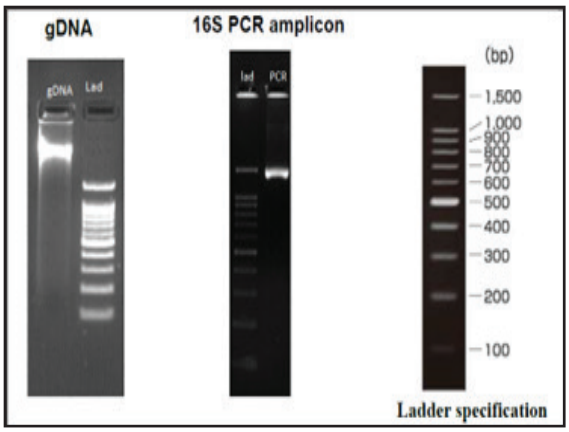

Figure 3: Gel Analysis of the amplified 16S PCR amplicon of the phytase-producing Isolate PS4 and Genomic DNA

The phylogenetic relationship of B12 SL, AB362708.1 and KX986311.1 with closest relatives as seen in dendogram (Figure 4) AB362798.1,KX986311.1 are more closely related. Sample which was labelled as B12_SL displayed high similarity with *Bacillus sp.* based on nucleotide homology & phylogenetic analysis. (Table 2) Evolutionary history of *Bacillus sp,* was conducted by Maximum Likelihood based on model described in Kimura et al.(33). Bootstrap tree consensus was inferred by taxa clustered together in the bootstrap test of 1000 replicates were displayed beside the branches by Felsenstein et al.(34). Gaps and missing positions were rejected and 1432 positions in final dataset were observed. It was executed in MEGA7 software by Kumar et al. (35).

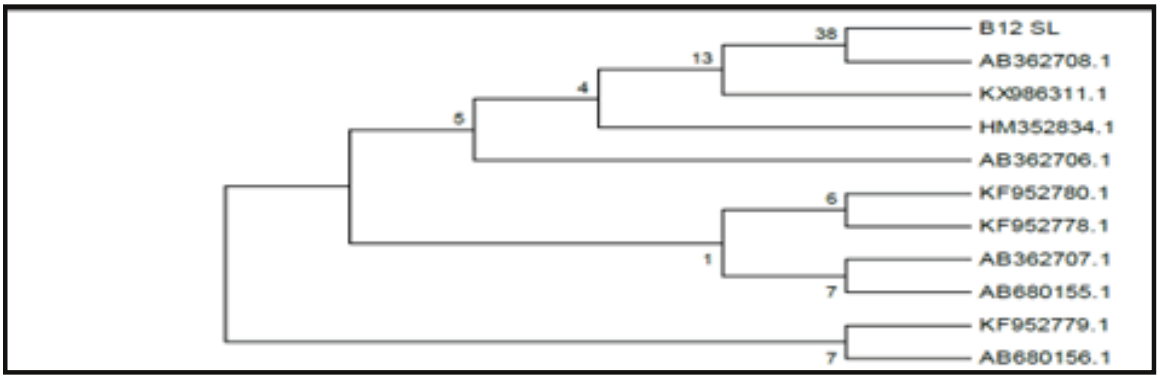

Figure 4: Dendogram: Phylogenetic relationships of isolates B12 SL, AB362708.1 with close homologs; horizontal bars signify branch length; The neighbouring sequences are specified with values of bootstrap

Table 2. Estimates of Evolutionary Divergence between Sequences

B12_SL		0.087	0.087	0.087	0.087	0.086	0.087	0.087	0.087	0.085	0.086
KF952780.1	1.268		0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.001
KF952779.1	1.268	0.000		0.000	0.000	0.001	0.000	0.000	0.000	0.001	0.001
KF952778.1	1.268	0.000	0.000		0.000	0.001	0.000	0.000	0.000	0.001	0.001
AB362707.1	1.268	0.000	0.000	0.000		0.001	0.000	0.000	0.000	0.001	0.001
KX986311.1	1.264	0.001	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001
AB362706.1	1.268	0.000	0.000	0.000	0.000	0.001		0.000	0.000	0.001	0.001
AB680156.1	1.268	0.000	0.000	0.000	0.000	0.001	0.000		0.000	0.001	0.001
AB680155.1	1.268	0.000	0.000	0.000	0.000	0.001	0.000	0.000		0.001	0.001
HM352834.1	1.263	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001		0.001
AB362708.1	1.264	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	

Table 3 : web BLAST Description Table with scores and Accession Numbers

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. BAB-3372 16S ribosomal RNA gene, partial sequence	2621	2621	100%	0	100%	KF952780.1
Bacillus sp. BAB-3371 16S ribosomal RNA gene, partial sequence	2621	2621	100%	0	100%	KF952779.1
Bacillus sp. BAB-3370 16S ribosomal RNA gene, partial sequence	2621	2621	100%	0	100%	KF952778.1
Bacillus coagulans gene for 16S rRNA, partial sequence, strain: NRIC 1527	2621	2621	100%	0	100%	AB362707.1
Bacillus coagulans strain E21 16S ribosomal RNA gene, partial sequence	2615	2615	100%	0	99%	KX986311.1
Bacillus coagulans gene for 16S rRNA, partial sequence, strain: NRIC 1526	2615	2615	100%	0	99%	AB362706.1
Bacillus coagulans gene for 16S rRNA, partial sequence, strain: NBRC 3887	2614	2614	100%	0	99%	AB680156.1
Bacillus coagulans gene for 16S rRNA, partial sequence, strain: NBRC 3886	2610	2610	100%	0	99%	AB680155.1
Bacillus coagulans strain A05 16S ribosomal RNA gene, partial sequence	2610	2610	100%	0	99%	HM352834.1
Bacillus coagulans gene for 16S rRNA, partial sequence, strain: NRIC 1528	2610	2610	100%	0	99%	AB362708.1

Distance matrix

Analyses were conducted using the Kimura 2-parameter model Kimura et al.(33). The Base substitutions per site between sequences are shown. The Standard error estimates are represented diagonally. It involved eleven nucleotide sequences.

Primary screening analysis revealed that PS4 isolate showed the highest phytase activity. It was identified by 16S rRNA sequencing as *Bacillus* sp BAB 3372 (GenBank Accession No. KF952780.1). Four strains displayed 100 % similarity in NCBI database and other sequences showed 99 identity with the *Bacillus coagulans* strain E21 having the GenBank Accession No. KX986311.1. (Table 3).The study of microbial diversity in several habitats is done by 16s rDNA analysis Kushwaha et al. (36). Earlier researchers Abdolshahi et al. (37) and Ahmad et al. (38) established the presence of phytase activity in *B. subtilis*. Sheikh Rizwanuddin (39) reported phytase producing microbes for phytate solubilization

Conclusion

Phytase-producing *Bacillus* strains BAB 3372 has been reported on isolation and identification of phytase producing *Bacillus* strains from Probiotics is scarce and seems to have been reported by the authors. In addition, *Bacillus* isolates showed higher phytase activities than observed with some similar strains. As such, they have the potential for food and feed, and for the reduction of environmental phosphorus pollution or in the solubilization of organic phosphorus in soil. *Bacillus sp.* reported to be the highest phytase-producing bacteria extracellularly and they effectively mineralize phytate. Identification of microorganism is a prerequisite to isolate the gene. The wild type isolates of Bacterial phytase show very low expression levels and its induction is regulated either by energy and nutrient availability and phosphate limitation. Exploitation of microorganisms to increase the bioavailability of Phosphorus in soil and in feedstocks for monogastric animals therefore is an attractive suggestion for developing a more sustainable agriculture and environment.

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