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Isolation of Phytase Producing Bacteria and Optimization of Phytase Production Parameters

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Background: Cereals, legumes, and oilseed crops are very important crops as nutrition for human and animals. Phytate (myo inositol hexa kis phosphate) is the main storage form of phosphorus in these crops. These crops are major source of nutrients for humans and animals including fish, poultry and pig. Phytic acid is the nutritional constituent of animal diet but it is not digested by monogastric animals because they do not contain phytase enzyme in their intestines to break the phytic acid and due to this, phytic acid acts as an antinutritional chelating agent for various metal ions like Ca, Mg, Fe, Zn, and etc., so that reduced the nutritive quality of food.

Objectives: Phytase is an important enzyme in the food/feed industry; therefore, isolation of phytase producing bacteria and optimization of phytase production on different parameters were performed in this study.

Materials and Methods: The present study was conducted in Biotechnology laboratory, Motilal Nehru National Institute of Technology, Allahabad, Uttar Pradesh, India. To isolate phytase producing bacteria from different soil samples like cattle shed, pulse crop field, poultry farms, and etc. 0.1 gr of the soil samples were streaked on phytase screening medium. The qualitative screening of the isolates was performed on phytase screening medium plate with 1.5% agar, and phytase activity was determined by using shaking flask method. The best phytase producer was optimized using different parameters of phytase production.

Results: We isolated 32 phytase producing bacteria on phytase screening media. Upon screening of these strains, one of the best strain (DR6) which showed a 39 mm clear zone on phytase specific medium (PSM) was identified as *Bacillus subtilis*. So this strain was selected for further enzymatic assay and optimization. This strain showed 378U/mL enzymatic activity upon enzymatic assay, the result of optimization of this best strain was performed at different parameters, and this strain showed best results at pH 5.5, Temp 50°Cwith Glucose + Sucrose as Carbon source and Yeast extract as Nitrogen source.

Conclusions: The present study suggests that the enzyme obtained from strain *B. subtilis* can be used as feed supplement in animal diet also for reduction of phosphorus pollution problem in areas of livestock production.

Keywords: Phytase; Phytic Acid; *Bacillus subtilis*

1. Background

Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexa kis dihydrogen phosphate) is the storage form of phosphate in nature, and phytic acid is mostly present in cereals, nuts, legumes, and oil seeds (1-4). Most foods of plant origin contain 50% to 80% of their total phosphorus as phytate (5). Phytic acid reduced the availability of various metal ions such as Fe, Zn, Mg, Ca, and etc., thus reducing the nutritive quality of food (6, 7). Due to this, phytase enzyme is required to hydrolyze phytic acid. Phytases (myo-inositol hexa kis phosphate phosphohydrolase) also known as phytate hydrolyzing enzymes. It hydrolyses phytate and releases inorganic phosphate (3, 7-9).

Phytase enzyme is widely produced in nature by bacteria, fungi, yeast, plants, and animals. Phytase producing microorganisms are mostly present in rhizospheric soil of crop plants (10, 11). Monogastric animals like fish, poultry, pig, human,and etc., do not have sufficient levels of phytate-degrading enzymes in their digestive tracts to digest phytic acid, due to this phosphorus is not available to them (12). So feed is supplemented with inorganic phosphorus to meet phosphorous requirement. Hence Phytic acid acts as antinutrient constituent in plant-derived food and feed, because it forms complexes with proteins, amino acids, and various metal ions (13).

This is the main reason of human nutritional deficiencies of different metals like copper, zinc, iron, and etc., where plant food is staple food (14). The undigested phytate is excreted in excretions and poses a serious phosphorus pollution problem and this contributes to the eutrophication of water in areas of intensive livestock production. Due to this scenario phytase enzyme is re-

Implication for health policy/practice/research/medical education:
Isolation and identification of phytase producing bacteria and optimization of enzyme production would be helpful in enhancing bioavailable micronutrients in food grains and reducing phosphorus pollution.

quired to overcome aforementioned problems as food and feed additive. The first commercial phytase product, which was available 10 years ago, offered animal nutritionists the tool to drastically reduce phosphorus excretion of monogastric animals (15).

According to Kornegay (16), phosphorus excretion can be reduced between 25% and 50% depending on diet, species and level of phytase supplementation. Nowadays there is no single phytase that is able to meet the diverse needs for all commercial and environmental applications. Therefore, there is an ongoing interest in screening microorganisms, including bacteria for novel phytases and also phytases play significant role in improving the nutritive quality of food and feed that contain phytic acid (17, 18). Hence phytase has a great industrial significance, and there is an ongoing interest in isolation of new microbial strain producing phytase and optimization of this enzyme (19).

2. Objectives

The principal goal of this study is to isolate the bacteria from different soil samples and optimize the production parameter to get the best bacterial strain.

3. Materials and Methods

3.1. Isolation of Phytate Degrading Bacteria

Bacterial strains were isolated from the soil samples collected from pulse fields, poultry farms, cattle shade, andetc., approximately 0.1 g of these samples were suspended in 5 ml of 0.8% saline solution, and 0.1 ml of this suspension was streaked on to phytase specific medium containing 1.5% glucose, 0.5% (NH4)2SO4, 0.01% NaCl, 0.05% KCl, 0.001% FeSO4, 0.01% MgSO4.7H2O, 0.01% CaCl2.2H2O, 0.001% MnSO4, pH 6.5 with 0.5% calcium phytate. 32 microbial colonies capable to hydrolyze calcium phytate which can be recognized by their surrounding clear halo were obtained by replating single colonies on phytase specific medium agar plate (4).

3.2. Screening for Phytate Degrading Strains

3.2.1. Qualitative Screening of Phytase Producing Bacterial Strains

Bacterial strains were assayed for qualitative screening by plate assay using phytase specific medium with 1.5% agar in petri plates. The halo zone and colony diameters were measured from 3 to 14 days of incubation at 28°C (20).

3.2.2. Quantitative Screening of Phytase Producing Microorganisms

The isolated strains were inoculated in 50 ml of phytase specific medium, and were cultured in a rotary shaker (200 rpm) at 30°C for 3 days. Supernatant from 1 ml of culture after centrifugation at 10000 g for 10 min at 4°C, the clear supernatant was used as the source of extracellular phytases and used for the phytase activity assay. Calcium phytate was used as substrate for phytase activity assay. Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted of 0.5% calcium phytate prepared in sodium acetate buffer (0.1 M, pH 5.5), and 0.1 ml of supernatant. After incubation at 45°C for 30 min, the reaction was stopped by adding 5% trichloroacetic acid. The liberated phosphate ions were quantified by 500µl of 10N H2SO4, 10% ammonium molybdate and 5% FeSO4 (4). After 30 min of incubation at 45°C, absorbance was measured at 660 nm. One enzyme unit (IU) was defined as the amount of enzyme liberating 1 µmol of inorganic phosphate in 1min under the assay conditions.

3.3. Optimization of Culture Condition

For determination of the optimum growth condition and phytase production, the best strain was inoculated into 50 ml of liquid broth and incubated on shaker at 30°C for 5 days. Different carbon sources were tested such as 1.5% glucose, maltose, sucrose, fructose, lactose, and 0.75% glucose + 0.75% sucrose. Nitrogen sources were 0.5% yeast extract, ammonium sulphate, sodium nitrate, urea and ammonium acetate. pH gradient ranged from 3.5 to 8.5, and temperature from 20°C to 70°C (19, 21).

3.3.1. Effect of Incubation Temperature

To study optimal incubation temperature for maximum phytase production, the flask containing production medium was incubated at 20°C, 30°C, 40°C, 50°C, 60°C, and 70°C for 5 days keeping all other conditions at their optimum level.

3.3.2. Effect of pH

To optimize the initial pH of production medium the pH was 3.5. 4.5, 5.5, 6.5, 7.5 and 8.5 with HCl or NaOH.

3.4. Identification of Phytase Producing Bacteria

Identification of best phytase producer bacterium was performed by both phenotypic and genotypic methods. Phenotypic method includes morphology and gram staining of bacteria. Genotypic identification of bacterium was performed by 16S rDNA. Amplification of the 16SrDNA region was performed using primers designed in the conserved region; the primers used were FD1 and RP2. Sequencing of the ~1.5kb region was performed using internal sequencing primers. Sequencing was performed with 5 different primers designed in the conserved regions on 16SrDNA, primers were as follows; 16SEQ2R, INS16SREV, 16SEQ3F, 16SEQ4F, and 16SEQ 4R.

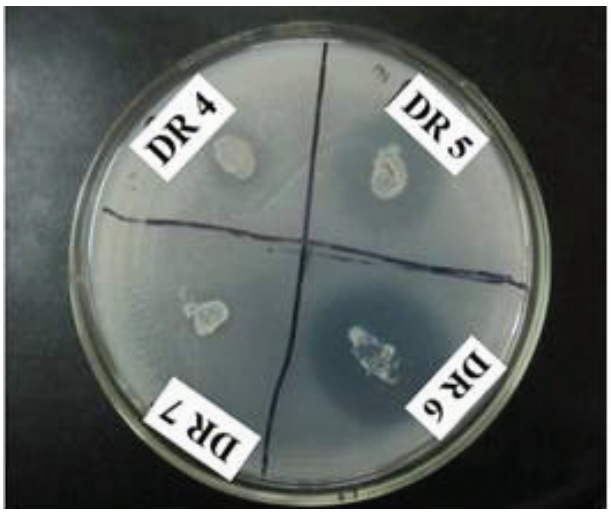

Figure 1. Strains Forming Peripheral Halo Zone Around Isolated Bacterial Colony on Phytase Specific Medium With Agar.

4. Results

4.1. Isolation of Phytase Producing Bacteria

The phytase producing Bacterial strains were isolated from phytase screening medium containing calcium phytate. 32 bacterial strains were isolated on the basis of clear halo zone on phytase specific agar medium around colonies. Strain DR6 was found to be the best strain and could produce about 39 mm clear halo zone around the colony (Figure 1).

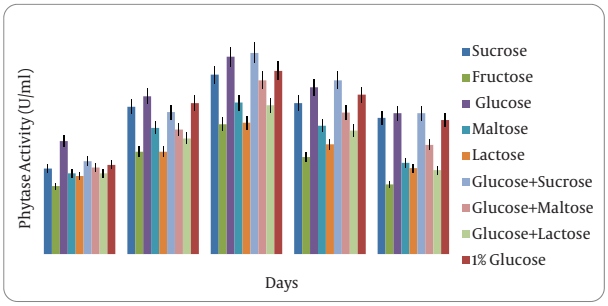

Figure 2. Optimization of Carbon Source for Phytase Production From Best Strain Showed That the Best Carbon Source Was Glucose + Sucrose Which Produced 381 U/mL Phytase Activity.

These isolated strains were subjected to quantitative screening by phytase assay. Strain DR6 showed maximum phytase activity of 378 U/mL on the third day of incubation (Table 1).

Table 1. Qualitative and Quantitative Screening of Different Phytase Producing Isolated Strains

	Enzyme activity U/mL				
Carbon, Days	**1**	**2**	**3**	**4**	**5**
1% glucose	169 ± 0.87	286 ± 2.4	347 ± 1.6	302 ± 1.6	254 ± 1.4
Sucrose	162 ± 1.6	279 ± 2	340 ± 2.3	286 ± 1.7	258 ± 1.4
Fructose	129 ± 1.6	194 ± 1.3	246 ± 1	184 ± 1.5	132 ± 2.6
Glucose	214 ± 1.6	299 ± 2	374 ± 1.5	316 ± 1.8	267 ± 2.1
Maltose	153 ± 1.3	239 ± 1.7	287 ± 1	243 ± 2.6	173 ± 2.8
Lactose	148 ± 2	194 ± 2	249 ± 2	208 ± 1.6	163 ± 2.4
Glucose + Sucrose	176 ± 1.7	269 ± 1.7	381 ± 1.8	329 ± 1.3	267 ± 2.2
Glucose + Maltose	164 ± 2.2	236 ± 2	329 ± 2.1	268 ± 1.1	207 ± 2.4
Glucose + Lactose	153 ± 1.6	219 ± 2.5	282 ± 1.5	234 ± 1.2	159 ± 2.2

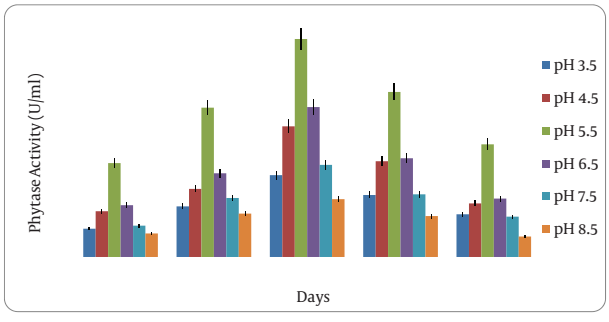

Figure 3. Optimization of pH Parameter for Phytase Production From Best Strain Showed That at pH 5.5 of Medium, P activity Was 362 U/mL.

4.2. Optimization of Culture Condition

The parameters used for optimization of culture conditions were Carbon source, Nitrogen source, pH, and temperature. Carbon sources like glucose, maltose, lactose, sucrose, fructose, and 0.75 % glucose + 0.75 % of different disaccharides were used for optimization. Strain showed optimum phytase activity of 381 U/mL on 0.75 % glucose + 0.75 % sucrose (Figure 2). pH gradient was taken from 3.5 to 8.5 in different flasks. On pH 5.5 this strain showed enzyme activity of 362 U/mL (Figure 3). Nitrogen sources used were 0.5% yeast extract, sodium nitrate, ammonium sulphate, urea and ammonium acetate. On yeast extract phytase activity was 398 U/mL (Figure 4). Temperature parameter was tested by using temperature range from

20°C to 70°C. At 50°C, strain showed enzyme activity of 387 U/ml phytase activity.

4.3. Effect of Carbon Sources on Phytase Production: Carbon Isa Major Component of Cell

The influence of different carbon sources on phytase production was shown in Table 2. Among different carbon sources studied, the highest yield of phytase was obtained with Glucose + Sucrose (381 U/mL).

4.4. Effect of Nitrogen Sources on Phytase Production

Among various organic and inorganic nitrogen sources better phytase production (398 U/mL) exhibited by yeast extract (Table 3).

4.5. Effect of pH on Phytase Production

It was found that maximum phytase production (362 U/mL) was obtained at pH 6 (Table 4). As the metabolic activities of microorganisms are sensitive to pH changes, phytase production was affected if the pH level was higher or lower compared to the optimum value.

4.6. Effect of Temperature on Phytase Production

The effect of incubation temperature was studied on phytase production (Table 5) and 50°C (387 U/mL) was found to be optimum temperature.

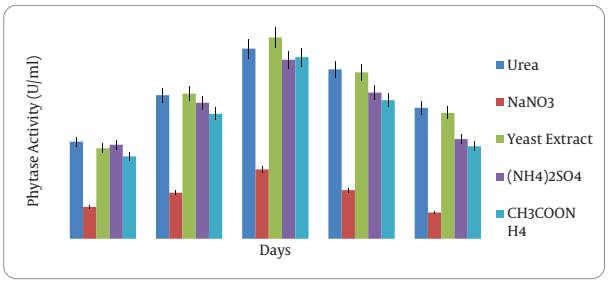

Figure 4. Optimization of Nitrogen Source for Phytase Production From Best Strain Showed That in Medium With Yeast Extract as N2 Source, Phytase Production Was 398 U/mL

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1    accttgttac gacttcaccc caatcatttg tcccaccttc ggcggctggc tcctaaaagg
61   ttacctcacc gacttcgggt gttacaaact ctcgtggtgt gacgggcggt gtgtacaagg
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181  cagtcgagtt gcagactgcg atccgaactg agaacagatt tgtgggattg cttaacctc
241  gcggtttcgc tgccctttgt tctgtccata gtagcacgtg tgtagcccag tcataaggg
301  gcatgatgat ttgacgtcat ccccaccttc ctccggtttg tcaccggcag tcaccttaga
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481  ggacgtccta tctctaggat tgtcagagga tgtcaagacc tggtaaggtt cttcgcgttg
541  cttcgaatta aaccacatgc tccaccgctt gtgcgggccc ccgtcaattc ctttgagttt
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661  gggcggaaac cccctaacac ttagcactca tcgtttacgg cgtggactac cagggtatct
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781  ttcgccactg gtgttcctcc acatctctac gcatttcacc gctacacgtg gaattccact
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1441 ccatctgtcc gctcgacttg catgtattag gcacgccgcc agcgttcgtc ctgagccggg
1501 atcaaac
```

Figure 5. Identification by Genotypic Method (Molecular Characterization) 16S rDNA Sequence of Sample-DR 6

Table 2. Effect of Carbon Sources on Phytase Production

Enzyme Activity, U/mL					
Carbon, Days	**1**	**2**	**3**	**4**	**5**
1% glucose	**169 ± 0.87**	**286 ± 2.4**	**347 ± 1.6**	**302 ± 1.6**	**254 ± 1.4**
Sucrose	162 ± 1.6	279 ± 2	340 ± 2.3	286 ± 1.7	258 ± 1.4
Fructose	129 ± 1.6	194 ± 1.3	246 ± 1	184 ± 1.5	132 ± 2.6
Glucose	214 ± 1.6	299 ± 2	374 ± 1.5	316 ± 1.8	267 ± 2.1
Maltose	153 ± 1.3	239 ± 1.7	287 ± 1	243 ± 2.6	173 ± 2.8
Lactose	148 ± 2	194 ± 2	249 ± 2	208 ± 1.6	163 ± 2.4
Glucose + Sucrose	176 ± 1.7	269 ± 1.7	381 ± 1.8	329 ± 1.3	267 ± 2.2
Glucose + Maltose	164 ± 2.2	236 ± 2	329 ± 2.1	268 ± 1.1	207 ± 2.4
Glucose + Lactose	153 ± 1.6	219 ± 2.5	282 ± 1.5	234 ± 1.2	159 ± 2.2

Table 3. Effect of Nitrogen Sources on Phytase Production

Enzyme activity U/ml					
N2Source, Days	**Urea**	**NaNO3**	**Yeast Extract**	**(NH4)2SO4**	**CH3COONH4**
1	**192 ± 1.7**	**63 ± 1.3**	**179 ± 1.7**	**186±0.6**	**163 ± 1.2**
2	284 ± 3.3	91 ± 1.2	287 ± 1.7	269±1.2	247 ± 1.3
3	376 ± 1.2	137 ± 1.3	398 ± 0.3	354±1.5	359 ± 1.9
4	335 ± 3.2	96 ± 1.5	329 ± 1.3	289±1.3	274 ± 1
5	259 ± 2	52 ± 1.5	249 ± 0.9	197±1.3	183 ± 1.5

Table 4. Effect of pH on Phytase Production

	Enzyme activity U/mL					
pH, Days	**3.5**	**4.5**	**5.5**	**6.5**	**7.5**	**8.5**
1	**47 ± 1.3**	**76 ± 1.3**	**156 ± 1.3**	**86 ± 1.6**	**52 ± 1.5**	**39 ± 1.4**
2	84 ± 1.5	113 ± 1.6	248 ± 2	139 ± 2.3	98 ± 1.3	72 ± 2.1
3	136 ± 1.4	217 ± 1.4	362 ± 1.4	249 ± 2.5	153 ± 1.5	96 ± 1.6
4	103 ± 1.3	159 ± 2	274 ± 1.5	164 ± 1.6	104 ± 2	68 ± 1.4
5	71 ± 1.4	89 ± 2.4	187 ± 1.7	97 ± 1.3	67 ± 1.4	34 ± 1.4

Table 5. Effect of Temperature on Phytase Production

Temperature , ˚C	Activity, U/mL
20	**69 ± 1.7**
30	127 ± 1.4
40	264 ± 2.3
50	387 ± 2
60	219 ± 2.2
70	83 ± 1.4

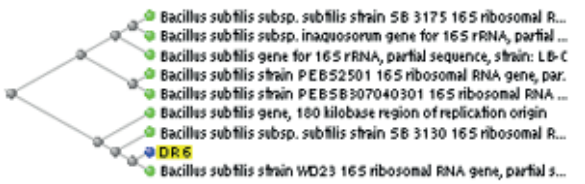

Figure 6. Phylogenetic Dendrogram16S rDNA Sequence of *B. subtilis* DR6

4.7. Identification by Phenotypic Method (Morpho¬logical Characterization) and Genotypic (Molecular Characterization)

Bacillus subtilis DR6 is a Gram positive and rod shaped bacterium. The isolated strain DR6 has been characterized by 16SrDNA sequence. The DR6 was showed nearest relationship with Bacillus sp. on the basis of phylogenetic analysis (Figure 5 and 6)

5. Discussion

In the present study, we isolated 32 bacterial strains on the basis of formation of zone around bacterial colony. One of the best strains showing highest phytase activity was selected for optimization of phytase production. On the basis of results of optimization on different parameters, this strain can be used for phytase production on industrial scale. The optimum productivity of phytase by our best bacterial isolate (*B. subtilis* DR6) was achieved with optimized process parameter such as Glucose + Sucrose as carbon source, yeast extract as nitrogen source, incubation temperature of 500˚C, initial pH of 5.5, and incubation period of 3 days. As well as phytase produced by this strain can be used as feed supplement in animal diet, also for reduction of phosphorus pollution problem in areas of livestock production.

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Authors' Contribution

None declared.

Financial Disclosure

There is no Financial Disclosure for this study.

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Department of Science and Technology (DST), New Delhi.

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