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PURIFICATION STRATEGIES FOR MICROBIAL PHYTASE

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ABSTRACT: Phytase catalyzes the formation and release of inorganic phosphate from phytic acid. A few monogastric animals that lack phytase is incapable of digesting phytate obtained from plants and gets excreted, which results in the accumulation of phosphorus in the form of phytate in the environment which has a detrimental effect. In order to combat this problem, researchers have focused on production and purification of phytase from different microbial sources, which converts phytate to useful form of phosphorous that facilitates plant growth. This review paper summaries the various methods adopted for isolation, production, and purification of phytases from various sources.

INTRODUCTION: Phytase is an enzyme that produces mineral residues and inorganic phosphate from phytic acid (phytate), which is the primary phosphate storage form in plants. Inorganic phosphate is a key mineral element for the growth, reproduction, and metabolism of animals ¹. The bioavailability of minerals and proteins was enhanced by the supplementation of phytase ^{2, 3}. This reduced the risk of eutrophication by reducing the feed additive phosphate ⁴. Due to extensive application of phytase, it has attracted the attention of scientists and entrepreneurs in the areas of nutrition, environmental protection and agriculture ^{5, 6}. For commercial use, microbial production of phytase has been proven more effective than sources like plants and animals, due to high production yields and acid tolerance.

However, the amount of phosphate released may differ based on its source and physicochemical parameters ^{7, 8, 9, 10, 11}. There are different techniques for phytase production including solid-state and submerged fermentation process ^{12, 13, 14, 15, 16}.

Screening and Production of Phytase: Phytases have been produced from many sources, mostly microbial sources like bacteria and fungi. Phosphate-solubilizing bacteria were isolated from aerobic soil after serially diluting the soil samples. Three different populations were used for the isolation purpose- rhizophore, non-rhizophore and endophytic populations. For rhizophore plants, 3 g of rice plant roots along with adhering soil was transferred to a conical flask containing 9 ml of sterile distilled water and contents very subjected to vortexing. Further 10-fold serial dilution was performed, and 0.1 ml aliquots were spread on a selective media plate and incubated at 28 °C in an incubator. For endophytic population, fresh roots were taken and sterilized with 70% alcohol for 5 min and treated with colorox for 30 sec.

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These surface-sterilized roots were further homogenized using mortar and pestle, and the total plate count method was used to determine population count¹⁷.

It was also found that acidic thermophilic bacteria, *Geobacillus stearothermophilus*, produced microbial phytase, which was isolated by inoculating 5 ml of hot spring water in 50 ml of phytase production medium and was kept for incubation at 65 °C for 3 days. After this, the sample was streaked onto a phytase growth media and incubated at 65 °C for 2 days. The bacterial colonies which produced phytase were found to have a clearance zone around it¹. The phytase enzyme was also produced from *Schizophyllum commune* by providing soya bran rice, coffee husk, rye, barleycorn and citric pulp as substrates in solid-state fermentation. The effect of different washes was studied on the production of phosphorus. WB (wheat bran without wash) wb1 (one-time wash), wb2 (2-time wash) and wb3 (3-time wash). The washes were done by ultra-pure water at 50 °C and later filtered using a Whatman filter paper 1. The bran was further dried at 60 °C for 4 h, 80 °C for 4 h and 100 °C for 15 min to reduce the microbial contamination¹⁸. The moisture content was set to 50% w/w. The flasks were inoculated after pretreatment with pellets of *S. commune* and the contents were incubated for 72 h at 30 °C. After fermentation, the fermented material was collected and checked for phytase activity.

A special kind of phytase called thermostable beta-propeller phytase was produced from *Bacillus licheniformis*. The collected soil samples were diluted and were spread onto a phytase screening culture medium. The colonies which showed a clear zone were again screened using submerged fermentation medium. The sterile production medium was inoculated with 12 h old cultures which were grown on a 2% LB medium and were incubated at 55 °C for 24 h. The fermented broth was cleared by centrifugation at 10000 rpm for 10 min and was assayed to check phytase activity¹⁹. Phytase was produced using 3 strains of *Bacillus subtilis*. The collected soil sample was dissolved in 0.9 % of saline and was filtered using Whatman filter paper. 100 µl was taken from the filtered sample and spread on a phytate screening agar medium and incubated for 2 days at 30 °C.

The bacterial colony which produced phytase enzyme had a clearing zone around them. Ones with the highest clearing zone were re-plated onto phytase producing media. A single colony was streaked on LB media and kept for overnight incubation at 30 °C. A loop of this culture was then transferred into phytase producing medium and incubated at 30 °C in an orbital shaker at 600 rpm for 6 days, after which the culture was centrifuged, and the supernatant was used as an enzyme source to study phytase activity²⁰. Phytase was also extracted from the *Aeromonas* species which was isolated from soil sample²¹.

Phytase produced from *Anoxybacillus* sp. MHW14, was isolated from hot spring in the Chiang Mai area located in Thailand, was stored in 15% (v/v) glycerol at -80 °C. It was cultured in nutrient broth and streaked to single colony prior to utilizing. Then utilized for the production by using modified Atlas media of 0.1% ammonium sulphate, 0.01% magnesium sulphate, 0.1% D-glucose, 0.01% Na citrate and 1.0% phytic acid. The liquid medium was adjusted to pH 7.0. Then the fermentation was carried out on 150 rpm rotary shaker at 45 °C for 24 h. The culture broth was taken to determine phytase activity²².

Phytase was also produced from *Shigella* by inoculating in PSM broth and was incubated for 37 ± 11 °C at 120 rpm for 5 days, and then the broth was centrifuged for 10 min at 10,000 rpm. The cell-free supernatant was separated, tested for phytase activity. Accordingly, the reaction mixture consisting of 0.8 ml acetate buffer (0.2M, pH 5.5 containing 10 mM sodium phytate) and 0.2 ml of supernatant. This was incubated for 30 min at 37 °C, and the reaction was stopped by adding 1 ml of 10% trichloroacetic acid. Then the assay mixture of 0.5 ml was taken in fresh set of tubes, mixed with 4 ml of 2:1:1 v/v of acetone, 10 mM ammonium molybdate and 5 N sulfuric acid and 0.4 milliliters of citric acid (1M).

The amount of free phosphate released was determined (spectrophotometrically) at about 355 nm, 1 unit of phytase activity was defined as 1 mol of phosphate produced per min per milliliter of culture filtrate under the defined assay conditions²³. Enteric bacteria from cow-dung also produce phytase.

The isolation of pure cultures from different collected samples was done by the serial dilution method, then by plating onto phytase screening medium (PSM) at about 30 °C. Based on the results of qualitative and quantitative enzymatic analysis, two potent phytase producing strains were selected. The Pure cultures were isolated, screened for phytase production in the PPM at about 37 °C, under shake flask culture and analyzed for intracellular, extracellular phytase activity². Phytase was also isolated from *Schizophyllum*¹⁸.

Phytase was produced using straw mushrooms grown on various substrates such as soil, grains cereals and fruits. 19 fungal strains from soil were isolated using serial dilutions. 1 g of soil from the municipality of Rionegro (Antioquia, Colombia) was dissolved in 9 ml of sterile distilled water then the dilutions of 10^{-2} , 10^{-3} and 10^{-4} were plated onto sterilized potato dextrose agar (300 g potato, 20g dextrose, 20 g agar per liter) containing 100 µg per ml of ampicillin, incubated at 25 °C in the laboratory for about 1 week. Fungi growing on agar plates were subcultured in fresh PDA medium until pure colonies were observed. About 7 environmental isolates from various locations in Medellín (Universidad Nacional de Colombia Sede Medellín and district of Belen) were collected in moist chambers using 250 ml Styrofoam cups with a layer of wet filter paper at the bottom, the grains (rice, wheat and a mix of wheat, barley, and oat flakes) and fruits (lemon and orange) were used as various substrates. These chambers were left open at the site of collection for about 1 day and then closed, incubated at room temperature.

Fungal growth was observed within 1 week of incubation. Isolation and purification were done on PDA plates. About 26 fungal isolates were obtained in pure cultures by single spore transfer onto PDA plates, stored at 4 °C. The isolates were preserved by immersing the mycelium in 20% sterile glycerol and stored in -80 °C freezer. The isolated fungi were further identified on the basis of morphological characteristics by using various updated taxonomical keys¹⁴.

Phytase was extracted from fruiting bodies using cold distilled water for 4 h, followed by centrifugation at 12000 rpm for 15 min and supernatant was collected and subjected to

ultrafiltration. Ion exchange chromatography was performed with NH_4HCO_3 buffer (pH 9.5). Fraction showing phytase activity was dialyzed against NH_4OAc buffer and again subjected to ion exchange chromatography. Other fractions with phytase activity were subjected gel-filtration by FPLC. The molecular mass of the purified enzyme was found using SDS-PAGE and FPLC-gel filtration. Various buffers of different pH values (3.0-9.0) were used to find the pH of maximum activity. The reaction mixtures were incubated at 20, 30, 37, 45, 50, 60, 70, 80 and 100 °C for 15 min. A 20.7% enzyme recovery was obtained with 34.6 fold purification. The phytase activity was found to be 3.11 U/mg. The molecular mass was found to be 14kDa using SDS-PAGE and FPLC. The optimum pH was found to be 5.0 and the optimum temperature was 37 °C¹⁸.

A new phytase was produced from *Aspergillus niger* using citric pulp by solid-state fermentation. The pH was fixed using sodium citrate buffer which contained ammonium citrate, potassium chloride, magnesium sulfate and zinc sulfate. The inoculum used was pellet suspension of *A. niger*. The columns were incubated at 30 °C for 97 h in a water bath. The crude phytase obtained upon fermentation was subjected to centrifugation to remove precipitate²⁵.

The rhizosphere soil samples were collected during the rainy season were cultured in nutrient broth media containing 0.004% w/v, calcium phytate, 0.05% w/v Potassium chloride, 0.05% w/v MgSO_4 , 0.001% w/v ferrous sulphate. 001% w/v manganese sulfate as trace elements required for growth, for about 24-30 h, at 30 °C for mesophilic isolates. Then to the 50 ml of enzyme production medium was added with 4 ml of culture. Then, the cultures were incubated at 30 °C for about 24-48 h in an incubator shaker. Culture contents were then centrifuged at 5000 g for 15 min at 2 or 4 °C.

Accordingly, the supernatant and the cell pellet were collected; both were used to check phytase activity. For the fungal isolates, the supernatant was assayed for three consecutive days. To work out the effect of metal ions and inhibitors on enzyme activity of crude phytase different metals and inhibitors *i.e.* ferric chloride, magnesium sulphate, zinc sulphate, cobalt chloride, copper

sulphate, sodium chloride, silver nitrate, sarium chloride, mercury chloride, sodium nitrite, calcium chloride, lead nitrate, manganese chloride, urea, dithiothreitol, ethylene di-amine tetra acetic acid, phenylmethyl sulphonyl fluoride and polyethylene glycol were all tested at final concentration of about 1mM⁸.

Purification of Phytase: After the production of phytase from the source, it will be in its crude form which has to be purified. There are several purification techniques for phytase and the techniques may vary for phytase isolated from different sources. The phytase obtained from *Flammulina velutipes* was given for SDS-PAGE analysis and it was found that the isolated phytase had a molecular mass of 14.8 kDa. The phytase activity was measured by incubating the enzyme solution with Tris-HCl buffer (pH = 7), which was assayed at 37 °C and stopped with the addition of 5% trichloroacetic acid. This phosphate was measured at 700 nm after the addition of a colour reagent. The amount of enzyme which was required to release 1µmol of Pi per min under standard assay conditions was defined as 1 unit of enzyme activity. The protein determination was also done using Bradford using a protein assay kit with bovine serum albumin as the standard. The results suggest that the activity of phytase extracted has a much lower enzyme activity than other fungal phytases. Various phosphorylated substrates were used to determine the substrate specificity of the purified phytase. 5mM concentrations were added to the assay buffer separately which included AMP, ADP, ATP, fructose-6-phosphate, glucose-6-phosphate, and β-glycerophosphate. The release of inorganic phosphate was determined as mentioned earlier. The specificity of the enzyme was found to be much low. The enzyme activity was tested over a range of temperatures (20-100 °C) and pH (3-9). The optimal temperature and pH were determined by subjecting the solutions to different temperatures and pH respectively. The optimum temperature was found to be 45 °C and optimum pH was found to be 5.0¹⁴.

Crude protein extracts from 9B (*Bacillus*) and 15C (*Geobacillus*) were used for analyzing the phytase properties. Diluted crude protein extracts were used to determine the optimum temperature (subjected to 30-90 °C), thermal stability at standard assay

conditions (at 37 °C), optimum pH (by using buffers of pH ranging from 1.0-9.0, along with sodium phytate) and pH stability. Molecular effectors like ethylenediaminetetraacetic acid and CaCl₂ were used to study the enzyme activity. A combination of anion exchange chromatography and high-performance liquid chromatography was used to evaluate the phytase action according to Sandberg and Adrienne. Crude extract along with CH₃COONH₄⁺ buffer (pH 5.0) and sodium phytate as substrate was incubated & taken out at different timings, cooled and stored at -20 °C.

By AEC inositol phosphates were extracted from the samples and stored at 4 °C, solvents were evaporated & inositol phosphates were resuspended, homogenized, filtered & stored at -20 °C. Reverse-phase HPLC was done for separation and quantification of inositol phosphates with an HPLC chromatograph. The optimum temperatures were found to be 60 °C and 50 °C, respectively. Phytase from *Geobacillus* isolate showed low thermal coefficient and energy of activation than that of *Bacillus* phytase. The results suggest that *Geobacillus* phytase has more ability to work at high temperatures. The optimum pH was found to be 5.0 for both the protein isolates. Relatively higher phytase activity was observed in the presence of molecular effector, CaCl₂ than that of EDTA. The appearance of partially phosphorylated *myo*-inositol phosphates was observed in the first hour in 9B, whereas, in 15 °C isolate, these were observed in the fourth hour, indicating lower degradation rate²⁶.

From *Anoxybacillus*, they grew the bacteria in an optimized medium, on 150 rpm rotary shaker at 37, 45, 50, 55, 60 and 65 °C for 12 h. To determine the effect of pH, the bacterium was grown in the optimized medium, with pH ranging from 5.0-10.0, on 150 rpm rotary shaker at 45 °C for 12 h. The collected samples were used to find the phytase activity. The highest phytase activity was found in the culture incubated at 45 & 50 °C, whereas those that are incubated at 37, 60, and 65 °C showed less activity. The optimum pH range was found to be from 6.0-9.0^{1,20}.

Ascorbic acid method modified from Kim *et al.*, (1998) was used to determine the phytase activity with sodium phytate as a substrate. Phytase

solution and sodium phytate in 0.1 M Tris-HCl buffer (pH 7.5) was taken and incubated at 55 °C for half an hour and stopped by adding 10% (w/v) trichloroacetic acid (TCA). The Pi released was measured by incubating with a color reagent for 55 °C for half an hour. The absorbance was measured at 820 nm. The amount of enzyme required to release 1 μmol of Pi per min under standard assay conditions was defined as 1 unit of enzyme activity. The maximum phytase activity obtained from the optimized media was found to be 0.20 U/ml²². For *Aspergillus niger*, an unoptimized medium with agricultural residues was moistened in an Erlenmeyer flask and subjected to sterilization at 121 °C for 30 min, cooled and inoculated with 1% spore suspension and incubated for a week at 30 °C. The optimized fermentation medium contained wheat bran in Erlenmeyer flask which also contains glucose, dextrin, sodium nitrate, magnesium sulfate moistened with water was subjected to sterilization at 121 °C for half an hour. The medium was cooled and inoculated with 1% spore suspension and incubated at 30 °C for 4 days. Optimized media along with wheat bran were moistened, sterilized and inoculated with spore suspension in enamel-coated metallic trays and incubated at 30 °C for 5 days.

Phytase enzyme activity was calculated at 50 °C. Cellulase, xylanase activities were determined. α-amylase activity was determined using McCleary and Sheehan's method. The presence of reducing sugars was determined using DNS method. Protein concentration was calculated using Lowry's method using BSA as standard. Biomass was determined by measuring the glucosamine content using Reissig method. The phytase enzyme extraction was subjected to NH₄SO₄ precipitation, centrifuged and dissolved in acetate buffer (pH 6.0). Sephadex G-25 was used for desalting and the fraction was estimated for phytase activity. PAGE was performed at room temperature and 200V for 2-3 h and protein bands were obtained after silver staining. Gel filtration was done on Sephadex S-200 column equilibrated with acetate buffer (pH 5.5) for the molecular weight estimation. Cytochrome C, BSA, carbonic anhydrase, alcohol dehydrogenase, and β-amylase as standard protein according to Andrew's method. The purification resulted in 69% enzyme recovery and specific activity of 49.83 IU/ml of protein.

Partially purified phytase was characterized after precipitation and desalting of the enzyme. The effect of pH on the enzyme activity was determined by treating with buffers of different pH ranging from 2.0-10.0 at 50 °C. The enzyme was subjected to a range of temperatures to determine the optimum temperature. Enzyme activity was studied by standard assay conditions and the values were compared with a standard devoid of inoculation. The optimum pH was found to be 6.0. The optimum temperature was found to be 55 °C. The effect of metal ions was studied by treating the culture with various metal ions at 50 °C for 30 min at standard assay conditions. It was found that phytase activity was moderately enhanced by Ca²⁺, Fe²⁺, Fe³⁺, Ba²⁺ and Pb²⁺¹⁵. Enteric bacteria were also found to produce phytase and it was purified using techniques based on solubility and chromatographic methods. The best purification strategy was found to be a combination of NH₄SO₄ precipitation, gel filtration and ion exchange chromatographic methods. The amount of protein was found using Bradford method considering BSA as standard. The phytase fractions were separated by SDS-PAGE according to Laemmli method. A single protein band was obtained for both the purified enzyme and the molecular masses were found to be 45kDa and 43kDa for the strains, *Klebsiella* sp. RS4 and *Shigella* sp. W3, respectively¹⁶. Phytase was also produced from lactic acid bacteria isolated from dairy products. Non-lactic acid bacteria were used as a negative control for the *E. coli* isolates from the faecal flora of a healthy donor. The isolates were grown in a modified MRS broth overnight in which the only available phosphate source was 0.1% sodium phytate, centrifuged at 400 rpm, and the supernatant was collected and tested for phytase activity. A complete phytase assay system was used to find the phytase activity in probiotic isolates according to manufacturer's instructions. The phytase activity was found to be much higher in the bacterial strain (like 4.0-5.4 mU), than those by probiotic isolates²⁶.

The phytase enzyme was also produced by the bacteria which inhabited the roots of mangroves. Partial purification was done using (NH₄)₂SO₄ precipitation followed by dialysis carried out at 4 °C. Five isolated strains were incubated with phytase production media in a shaker incubator at

200 rpm at 37 °C for 3-5 days. This fermented broth was subjected to centrifugation at 10,000 rpm for 10 min at 4 °C and the supernatant was collected and was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and saturation. The fraction was incubated overnight and centrifuged at 9000 rpm for 30 min at 4 °C and dissolved in acetate buffer followed by enzyme assay. The enzyme fraction with highest activity was desalted using a dialysis bag and subjected to SDS-PAGE according to modified Laemmli method to check the homogeneity. The purified isolate showed a high enzyme activity of 5.583 U/mg and 72% enzyme recovery with 10.3-fold purification. R_f value was calculated as the ratio of distance traveled by each isolate & distance traveled by the dye front. The molecular weight of the sample was determined from the standard curve obtained by plotting standard protein against R_f values. The molecular weight was estimated to be in the range 16-22 kDa. To study the effect of temperature on enzyme activity, the sample was incubated at 50, 60, 70 and 80 °C and cooled to 4 °C followed by assay. To study the effect of pH on enzyme activity, the sample was treated with buffers ranging from 2.0-9.0 and then assayed. Optimum temperature and pH were found to be 45-55 °C and 5.0, respectively⁸.

Geobacillus stearothermophilus is a thermophilic bacterium that has been found to produce phytase. The strain DM12 was grown on a phytase production media and incubated in a shaker incubator at 200 rpm at 65 °C for 48 h. The phytase medium consisted of 1.5% glucose, 0.5% ammonium sulphate, 0.05% potassium chloride, 0.01% sodium chloride, 0.01% calcium chloride (hydrated), 0.001% ferric sulphate, 0.001% manganese sulphate and 0.5% sodium phytate. The pH was made to 7.0. The culture taken was 16 hours old. After incubation, the culture was subjected to centrifugation for 10000 rpm for 10 min at 4 °C. The supernatant was collected. This is the crude enzyme. This supernatant was then fractionated by stepwise precipitation with ammonium sulfate powders at different saturations. These samples were again given for centrifugation at 12000 rpm for 20 min and the precipitate was collected. It was dissolved in 1 ml of 0.1 M acetic acid buffer and was assayed for enzymatic activities.

The aliquot with the highest amount of phytase activity was given for dialysis overnight against Tris-HCl buffer of pH 7.5 to remove the remaining amount of salt. These samples were then loaded onto a Q-sepharose column of 1.5 × 24 cm and were previously put in equilibrium by 20mM Tris-HCl buffer of pH 7.5 and the column was eluted with a linear gradient of 0.1 M NaCl at a flow rate of 1ml/min. Now, these fractions were checked for phytase activity. This is the purified form of the enzyme. These purified fractions were then subjected to SDS-PAGE to check the homogeneity. The phytase activity was detected by incubation of the gel in a 4mM sodium phytate solution in 0.1 M sodium acetate buffer for 30 min. After washing with water, the phytase bands were detected by immersing the gel in a coloring agent (malachite green) for a period of 1-2 h until the green bands were visible¹.

Different bacterial colonies were obtained after inoculating on nutrient agar media from the soil samples. Their phytase activities were screened by re-plating each of the single colonies on wheat bran extract agar media plates and observing the clearance zones. The most efficient isolates were inoculated into a 10% concentration of wheat bran media. Pre sterilized 0.2% calcium chloride was added just before inoculation. The contents of the flask were mixed and incubated in a shaker incubator at 200 rpm at 37 °C for 72 h. The fermented broth was centrifuged at 6000 rpm for 30 min at 4 °C, and the supernatant collected used to detect the phytase activity checking. The more the amount of phosphate released, the better was the phytase activity²¹.

For the phytase obtained from *Bacillus subtilis* MJA, the supernatant obtained after the centrifugation of the culture was dialyzed and concentrated using a filtration system. It was then transferred to an anion-exchange chromatographic column which was equilibrated with 20 mM Tris-HCl buffer of pH 8. After washing the column, the enzyme was eluted out at a flow rate of 1 ml/min by using a linear gradient from 0 to 100% of 1 M NaCl in 20 mM Tris-HCl buffer of pH 8. The enzyme was collected in fractions of the volume of 5 ml each. These fractions were checked for phytase, and the ones containing phytase activity were pooled together, dialyzed, and concentrated

using the TFF filtration system. The concentrated enzyme was applied to a gel filtration system that had been pre-equilibrated with 50 mM phosphate buffer and 200 mM NaCl at pH 8 and eluted using the same buffer at a flow rate of 1 ml/min. The fractions were collected every 5 min and once again, the fractions with high phytase activity were collected and pooled together. During the purification processes, all the collected and pooled fractions were tested for absorption (wavelength- 280 nm), total protein (wavelength- 595 nm), and phytase activity (wavelength- 355 nm)²¹.

Phytase was produced from *Aeromonas* species. *Aeromonas* sp. culture was first cultivated at 37 °C at 200 rpm and at pH 7 in 2 L of MPB medium for 48 h. The culture broth was then centrifuged, and the supernatant obtained was the crude enzyme solution. These supernatants were then precipitated with the help of 80% saturated ammonium sulfate and resuspended in 10 mM Tris-HCl buffer of pH 8. This was then given for dialysis and the dialyzed fractions were loaded onto a DEAE-Sephacel column and eluted out with 200 mM NaCl. The enzyme activity was assayed using 0.1 M Tris-HCl buffer at pH 7 containing 2 mM sodium phytate at 37 °C for 30 min. The reaction was stopped by adding 250 µl of 10% (w/v) trichloroacetic acid¹³. 100 µl of the enzyme was isolated from *Bacillus licheniformis*, along with 900 µl of the substrate (sodium phytate in 0.2 M Tris-HCl buffer of pH 7.0 supplemented with 1 mM calcium chloride) were incubated together at 55 °C for 30 min. The protein concentration was then measured using Bradford's dye-binding assay method⁶.

The activity of phytase from phosphate-solubilizing bacteria was measured using a modified method of Fiske and Subbarow. After 48 hours of incubation, an exact 150 µl of PSB bacteria (phosphate-solubilizing bacteria) was added with 600 µl of substrate and incubated at 45 °C. 750 µl of 5% trichloroacetic acid was added to stop the reaction. The released inorganic phosphate was measured spectrophotometrically¹⁹. Phytase was obtained from *Lactobacillus plantarum* by ammonium salt precipitation. Once the supernatant is obtained after centrifugation of bacterial broth, it was subjected to ammonium sulfate precipitation. All the precipitates obtained were dissolved in small amounts of 0.1 M Tris-HCl buffer of pH 5.5 and

the phytase activities were checked. The active fractions were given for dialysis against the same buffer. The active fractions were pooled and allowed to stand at 4 °C. The pure form of the enzyme was thus obtained²⁸.

Citric pulp fermentation with *Aspergillus niger* FS3 was found to produce a new kind of phytase. This phytase was purified by cationic-exchange, anionic exchange chromatography, and chromatofocusing. In cationic exchange chromatography, the first step of phytase purification from the crude extract was conducted with SP Sepharose, which was pre-equilibrated with 25 mM glycine-HCl of pH 2.85. The column was then filled with the crude extract with a dilution of 1:3 along with 25 mM glycine-HCl of pH 2.85 and was washed with the same buffer. The enzyme proteins were then eluted out with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Fractions of 10 ml were collected. All the fractions were checked for the amount of phytase activity, and the ones showing the highest phytase activity were taken, pooled, and dialyzed against 50 mM Tris-HCl of pH 7.8. The second step is anionic exchange chromatography. It was conducted using a Mono Q column by using 50 mM Tris-HCl buffer of pH 7.8 for equilibration. The pooled samples obtained in the first step were loaded into the Mono Q column and eluted using a linear gradient of 0 to 0.5 M NaCl concentration.

The eluted-out volume was collected into 50 tubes such that each had 6 ml of the sample. These fractions were checked for phytase activity and the ones showing the phytase activity were pooled. The last step in purification was the chromatofocusing step. The second pooled samples were loaded into a Mono PTM column, and the separation was performed using 25 mM imidazole-HCl of pH 6.2 in a pH of 6.2 to 4.0 inside the column. The elution buffer used was diluted 8 times in 25 mM imidazole-HCl with pH adjusted to 4.0 with HCl and applied over 30 min to elute out the bound proteins based on their isoelectric points. The fractions that were phytase-active were collected, pooled and lyophilized. From SDS-PAGE analysis, the molecular weight of pure phytase was found to be 108 kDa, had an optimum pH of 5.0 to 5.5 and an optimum temperature of 60 °C. It displayed a high affinity towards phytate. It had a high phytate-degrading activity²⁸.

The phytase purification from *Aspergillus tamari* was usually done by DEAE-cellulose column chromatography. The crude phytase extract produced was purified by DEAE-cellulose column chromatography. Alcohol treatment was done prior to column chromatography. The enzyme activity was studied by sodium acetate buffer (pH 5.0) with sodium phytate at 37 °C. The amount of Pi released was measured according to a modified ammonium molybdate method. The amount of enzyme required to release 1 μmol of Pi per min under standard assay conditions was defined as 1 unit of enzyme activity. The phytase purified was found to have an enzyme recovery of 20.3% with 51-fold purification. The pH stability and optimum pH were determined using buffers of different ranges of pH (1.0-10.0). The enzyme was subjected to different temperature ranges.

Phytate degrading enzyme was purified by running FPLC at 25 °C with flow rate 1 ml/min. The enzyme was also precipitated using ammonium sulfate, then suspended in Tris-HCl buffer and dialyzed against the same buffer followed by centrifugation. The dialyzed precipitate was loaded onto the DEAE-Sepharose CL 6B column and the required enzyme fraction was pooled after subsequent fractionation. This fraction was loaded onto a 16/60 Sephacryl S-200 HR column and the resulting fraction was dialyzed against sodium acetate buffer (pH 5.0). This fraction is further applied onto a Mono S HR 5/5 column equilibrating with sodium acetate buffer (pH 5.0) and fraction with phytase-activity was pooled. The enzyme activity was found to be 133 U mg⁻¹.

SDS-electrophoresis was performed according to the Laemmli technique. The molecular weight of the purified protein was found using gel-filtration on 16/60 Sephacryl S-200 HR. Gel filtration technique concluded that the molecular mass of the enzyme was 85,000 ± 2500 Da, while SDS-PAGE accurately estimated the molecular mass to be 85,000 Da¹⁵. Phytase was also obtained from *Saccharomyces cerevisiae*, known as Baker's yeast was shown to express phyA genes that are responsible for the production of phytase enzyme. 1 ml of the yeast cell culture was centrifuged at 5000 rpm for 10 min. The supernatant formed was the crude extracellular phytase enzymes. The phytase activity was assayed by 0.8 ml of sodium phytate

which was pre-incubated 65 °C for 5 min and 0.2 ml of the phytase was added to it. The mixture was kept for incubation at 65 °C for 30 min and afterward, it was stopped by the addition of 1 ml of 50 g/L trichloroacetic acid. These mixtures were then eluted out by column chromatography and the pure form of phytase enzyme was obtained. Yeast cell surfaces have a special quality of handling the phytase enzyme with a highly homogeneous quality even without purification¹⁵.

Citric pulp fermentation with *Aspergillus niger* FS3 was found to produce a new kind of phytase. This phytase was purified by cationic-exchange, anionic exchange chromatography, and chromatofocusing. In cationic exchange chromatography, the first step of phytase purification from the crude extract was conducted with SP Sepharose, which was pre-equilibrated with 25 mM glycine-HCl of pH 2.85. The column was then filled with the crude extract with a dilution of 1:3 along with 25 mM glycine-HCl of pH 2.85 and was washed with the same buffer. The enzyme proteins were then eluted out with a linear gradient of 0 to 0.5 M NaCl in the same buffer. Fractions of 10 ml were collected.

All the fractions were checked for the amount of phytase activity and the ones showing the highest phytase activity were taken, pooled and dialyzed against 50 mM Tris-HCl of pH 7.8. The second step is the anionic exchange chromatography. It was conducted using a Mono Q column by using 50 mM Tris-HCl buffer of pH 7.8 for equilibration. The pooled samples obtained in the first step were loaded into the Mono Q column and eluted using a linear gradient of 0 to 0.5 M NaCl concentration. The eluted-out volume was collected into 50 tubes such that each had 6 ml of the sample. These fractions were checked for phytase activity and the ones showing the phytase activity were pooled. The last step in purification was the chromatofocusing step. The second pooled samples were loaded into a Mono PTM column, and the separation was performed using 25 mM imidazole-HCl of pH 6.2 at a pH of 6.2 to 4.0 inside the column. The elution buffer used was diluted 8 times in 25 mM imidazole-HCl with pH adjusted to 4.0 with HCl and applied over 30 min to elute out the bound proteins based on their isoelectric points. The fractions that were phytase-active were collected, pooled and lyophilized.

From SDS-PAGE analysis, the molecular weight of pure phytase was found to be 108 kDa, had an optimum pH of 5.0 to 5.5, and an optimum temperature of 60 °C. It displayed a high affinity towards phytate. It had a high phytate-degrading activity⁵.

The pI value of phytase enzyme from *Aspergillus fumigatus* was slightly higher than phytase enzymes from other sources as was found from protein sequence analysis. It was also more basic compared to the other protein structures present in the culture broth, purification could be done by a one-step process. The enzyme was subjected to cation-exchange chromatography at pH 5.0 and the phytase was eluted as a single peak at 500 mM NaCl in the step gradient. The purified enzyme was analyzed by SDS-PAGE²⁵.

CONCLUSION: Since there is an increase in demand for phytase production due to the excessive accumulation of phosphates in the environment, many researchers have been carried out to extract the enzyme from different sources. In majority of the experiments conducted, it was observed that micro-organisms were used as the sources for the production of phytase enzyme. The possible reasons for this could be that microorganisms are easily manageable and have a rapid rate of multiplication.

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