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Isolation, optimization and production of two novel bacterial phytases from *Aeromonas* spp. using rice bran

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ABSTRACT

Phytic acid (*myo*-inositol hexakis phosphate, phytate) is the major storage form of phosphorus in cereal, oil and legume. Phytase, a specific group of phosphatase hydrolizes phytic acid to myo-inositol and phosphoric acid. A potent phytase producing bacteria was isolated from soil where beans (a leguminous plant rich in phytate) was grown through phytase screening media with rice bran as sole carbon source. The isolated organism was identified through microscopical and biochemical analysis which was authenticated using Bergey's Manual of Bacteriology as *Aeromonas* spp. The potency of the organism to produce phytase was also tested using a production media having a waste product of rice industry that is rice bran and the organism was found to be highly potent. The production conditions for high quantity of phytase were also done for different inoculums size, pH, temperature, carbon source, nitrogen sources. The results indicated production of two different phytases one active in acidic pH and one in basic pH. Future studies will be on bulk production, purification, characterization and its biotechnological application.

KEYWORDS

Phytase;
Low cost rice bran;
Animal biotechnology;
Two novel phytases.

INTRODUCTION

Phytases [*myo*-inositol(1,2,3,4,5,6) hexakisphosphate phosphohydrolases] have been identified in plants, microorganisms, and in some animal tissues[1] They represent a subgroup of phosphatases which are capable of initiating the stepwise dephosphorylation of phytate [*myo*-inositol(1,2,3,4,5,6) hexakisphosphate], the most abundant inositol phosphate in nature. This classification is irrespective of their *in vivo* function, which remains usually unknown. Based on the catalytic mechanism, phytases can be referred to as histidine acid phytases, b- -propeller phytases, cysteine phytases or purple acid phytases[2,3] Depending on their pH optima, phytases have been divided into acid and alkaline phytases and based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated into 3-phytases (E.C. 3.1.3.8), 6-phytases (E.C. 3.1.3.26) and 5-phytases (E.C. 3.1.3.72).

Till today, phytases have been mainly, if not solely, used as animal feed additive in diets largely for swine

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and poultry, and to some extent for fish. The first commercial phytase products were launched into market in 1991. Meanwhile, the market volume is in the range of 150 million euro[4]. Numerous animal studies have shown the effectiveness of supplemental microbial phytase in improving the utilisation of phosphate from phytate[5-10]. Therefore, including adequate amounts of phytase in the diets for simple-stomached animals reduces the need for orthophosphate supplementation of the feed. As a result, the environment is protected from pollution with excessive manure phosphorus runoffs because the faecal phosphate excretion of the animals is reduced by up to 50 %.

Considering all these above parameters, this investigation will focus on isolating a potent bacterial species for the production of phytase using rice bran as the sole carbon source. Once the organism is isolated, it will be identified and the culture conditions will be optimized for different parameters such as pH, temperature, carbon source and nitrogen source. Our future study will also focus on characterization of the enzyme and purification of the same.

MATERIALS AND METHODS

(Note: all the expts were carried out in triplicates and the results were stastically significant)

Soil samples

Soil samples were collected aseptically in polythene bags from legume growing regions of places of places in and around Bangalore. Places of intense animal husbandry especially poultry was also considered for the sample collection. All the samples were used for isolation and stored in the standard conditions.

Isolation of the organism

1 g of soil samples was suspended in 10 ml of 0.85% saline solution and 100µl of this suspension were plated onto phytase screening medium (PSM) prepared according to the method of Bae *et al*, (3 g glucose, 1 g tryptone, 1 g calcium phytate, 0.3 g CaCl2, 0.5 g MgSO4·7H2O, 0.04g, MnCl2·4H2O, 0.0025 g FeSO4·7H2O, 15 g agar powder per liter). Plates were incubated at 37°C for 24 hrs. The isolates isolated during the primary screening of phytase producers were inoculated into phytase screening liquid (PSL) medium and incubated at 37°C for 24 hrs. After cultivation, the isolates were selected by phytase assay with calcium phytate as the substrate.

Enzyme and protein assays

Phytase activity was assayed by the method of Fiske and Subbarow[10]. Culture supernatants were collected via centrifugation (10,000 rpm, 15 min). The reaction mixture contained 600µL of 0.1 M Tris·HCl buffer (pH 7.0), 0.2% (w/v) sodium phytate, and 500 µL of enzyme solution. After incubation at 37°C for 10 min, the reaction was stopped with the addition of 500 µL of 5% (w/v) trichloroacetic acid (TCA) and 500 µL color reagent mixture (four volumes of 5.5% (v/v) H2SO4 containing 6.25% (w/v) $(NH_4)_6Mo_7O_{24}$ and one volume of 2.7% (w/v) FeSO4· 7H2O solution). The hydrolysate of sodium phytate was measured as the increase in A600 using a UV-visible spectrophotometer. Monopotassium phosphate was used as a standard for the phytase assay. Protein contents were determined by the method, of Lowry *et al*.[11], using bovine serum albumin as a standard at 725 nm.

Identification of the organism

The isolated organism was identified using microscopical examination and also biochemical analysis. The results obtained were analysed using Bergey's Manual of Determinative Bacteriology to identify till the genus level tentatively. Microscopical examination included simple Gram's staining under oil immersion objective. The biochemical analysis was done through biochemical tests selected from the Bergey's Manual of Determinative Bacteriology after having the results from the microscopical analysis. The biochemical tests included Oxidase test (N,N,N,N-Tetramethyl-p-phenylenediamine Dihydrochloride 0.60 g, Stabilizing Agent 0.02 g Dimethyl Sulfoxide (DMSO) 100.0 mL), Catalase test (conc H_2O_2), Glucose Fermentation(GFB- Peptone 10g/l, Glucose 5g/l, NaCl 5g/l pH 7.0).

Optimization

The culture conditions for the production of phytase were optimized for different parameters which included,

a) Inoculum size

The Phytase Production Medium (PPM) (Rice bran

5.0 g/l, Glucose 15g/l, NH_4NO_3 1.5 g/l, KCl 0.5 g/l, $FeSO_4$ 0.1 g/l, $MgSO_4$ 0.5 g/l, MnSO4 0.5g/l) was inoculated with different volume of fresh broth culture of the isolated and identified bacteria ranging from 1ml to 5ml and the inoculated flasks were incubated at 37^0 c for 24 hrs. Assay of phytase post incubation was done and results were recorded.

b) pH

The same PPM was used for the optimization of pH. The pH of the media was set from pH 1.0 to pH 9.5 using 0.1N HCl and 0.1N NaOH, and was inoculated with 5ml fresh broth culture of the isolated bacteria in each flask. The flasks were incubated at 37^0 c for 24 hours. Post incubation assay for phytase was done and the results were tabulated.

c) Temperature

Phytase Production Medium (PPM), was inoculated with 5ml fresh broth culture of the isolated bacteria in different flasks and these flasks were incubated in different temperatures ranging from 0^0 c to 55^0 c for 24 hours at two different pH of 5.5 and 8.5. Assay for phytase post incubation was done and the results were recorded.

d) Carbon source

Phytase Production Medium (PPM), was inoculated with 5ml fresh broth culture of the isolated bacteria in three different flasks containing rice bran, glucose and calcium phytate as sole carbon source. The flasks were incubated at 37^0 c for 24 hrs at two different pH of 5.5 and 8.5 and after incubation phytase assay was done.

e) Nitrogen source

Phytase Production Medium (PPM), was inoculated with 5ml fresh broth culture of the isolated bacteria in three different flasks containing ammonium nitrate, sodium nitrate and potassium nitrate as nitrogen source. The flasks were incubated at 37^0 c for 24 hrs at two different pH of 5.5 and 8.5 and post incubation phytase assay was done.

RESULTS AND DISCUSSSION

Isolation and identification of the organism

About 15 bacterial isolates were obtained on the phytase screening medium (PSM). Only one potent organism was obtained after screening in the phytase screening liquid (PSL). Based on the microscopical and biochemical analysis using Bergey's Manual of Determinative Bacteriology the isolate was identified as *Aeromonas spp* tentatively.

B. Sasirekha (2012) et al and Lotis Escobin-Mopera (2012) et al report phytases produced from *Psuedomonas aeruginosa* and *Klebsiella pnuemoniae*,respectively. Till date no reports in literature state isolating any *Aeromonas spp* for phytase production. Our study reports the first and two distinct novel phytases from *Aeromonas spp*.

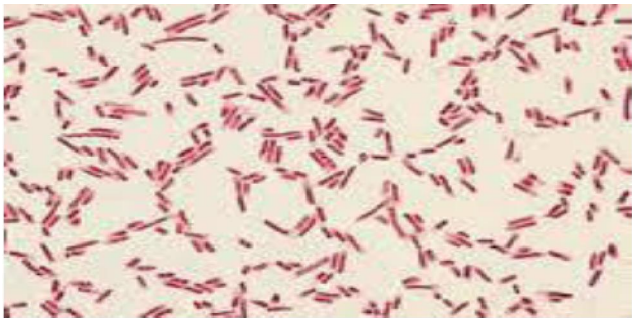

Figure 1 : Gram's Staining of isolated *Aeromonas* spp

TABLE 1: Results of Biochemical analysis

BIOCHEMICAL TEST	RESULT
Gram's Staining	Gram Negative
Morphology	Bacillus
Catalase	Positive
Oxidase	Positive
Glucose fermentation	Positive

Optimization

a) Inoculum size

The phytase activity with respect to inoculum size ranging from 1ml to 5ml per 100 ml of the Phytase Production Medium (PPL) was seen high in 5ml containing PPL indicating a standard 5% inoculum for the production of adequate amounts of phytase.

As stated by Ahmad.T et al[9] the inoculum size plays a vital role in the production of phytase from both bacteria and fungi. Optimization of the inoculum size becomes an essential criterion in order to apply our phytase for industrial production and also the industries demand a lower inoculum size for enzyme fermentation processes. As per Nampoothiri KM (2009) maximum in-

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oculum size being 30% for industrial fermentation, our inoculum size was optimized to just 5% which is way lower and advantageous for industrial application of our phytase.

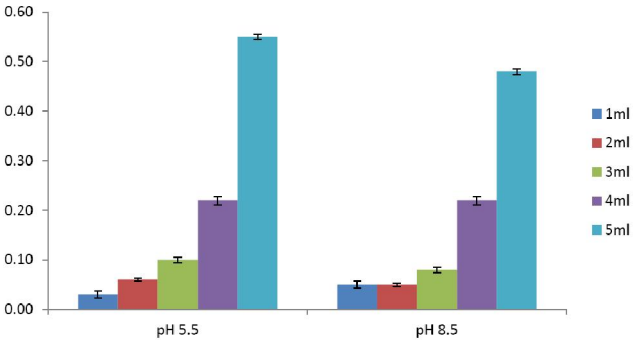

Figure 2 : Results for optimization of inoculum size indicating 5ml/100 ml of PPL to have the highest phytase activity

b) pH

The results obtained with respect to pH optimization indicated that *Aeromonas* spp produced two different phytases, one at pH 5.5 and the other at pH 8.5. This showed that the organism produced two isoforms of phytases one being active in acidic pH and the other in basic pH. The future characterization of the purified enzyme will confirm the above statement.

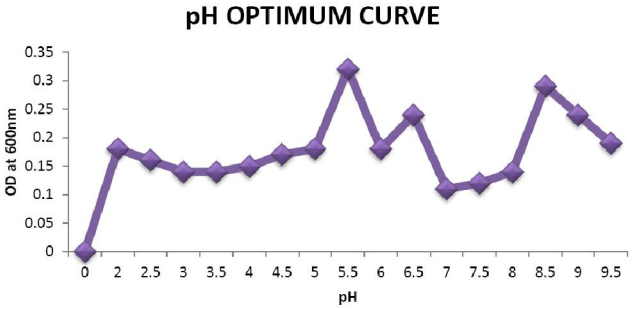

Figure 3 : pH optimization curve for phytase from *Aeromonas* spp

Jareonkitmongkol S (1997) report the optimum pH for production of phytase from *Aerobacter aerogenes* and *Klebseilla pnuemoniea* to be at 7. This goes in par with our study as we were able to produce phytase in two different pH of which one being acidic (5.5) and the other being basic(8.5). Obtaining a production pH on both acidic and basic scale also adds on to the positive aspects of our phytase from *Aeromonas spp.*

c) Temperature

The results of assay of phytase after incubation in different temperatures ranging from 0^0C to 55^0C in two

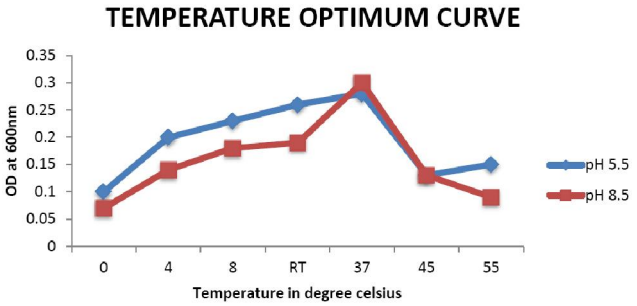

Figure 4 : Temperature optimization curve of phytase from *Aeromonas* spp

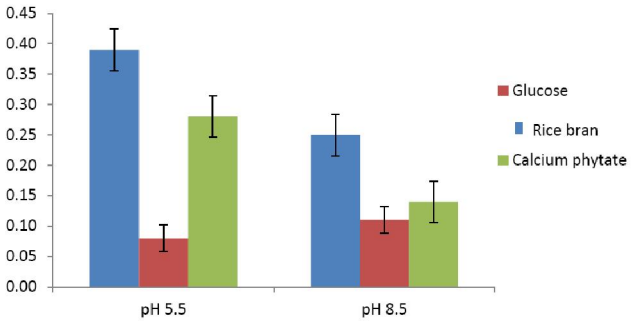

Figure 5 : Carbon source optimization curve of phytase from *Aeromonas* spp

different pH of 5.5 and 8.5 indicated the production in temperature of 37^0C in both the pH.

Ohya M (1998) report production phytase from *Bacillus spp* at the temperature of 55^0C and Midori Ohtani (2012) report the same enzyme production from *Klebsiella spp* at the temperature of 60^0c. Production of phytase at 37^0C adds on an advantage for the biotechnological application as our bacteria *Aeromonas spp* is a mesophile having a growth optimum at 37^0C as well as the production temperature to be at 37^0C as well.

d) Carbon source

The production and activity of phytase studied with respect to three different carbon sources (rice bran, glucose and calcium phytate) at two different pH of 5.5

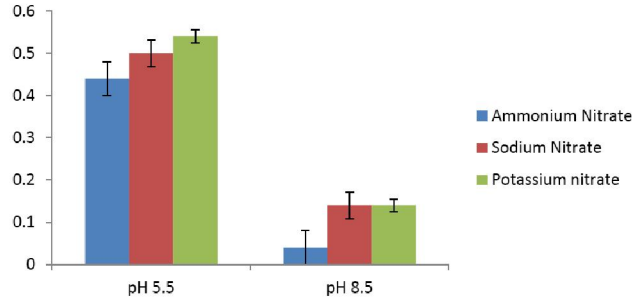

Figure 6 : Nitrogen source optimization curve for phytase from *Aeromonas* spp

and 8.5 indicated high activity with respect to rice bran over calcium phytate of which the latter being a specific substrate. Vithaya Meevootisom (2012), B. Sasirekha (2012) et al and Lotis Escobin-Mopera (2012) use specific and commercially available carbon sources (sodium phytate and calcium phytate) for their studies of phytase from their respective organisms. But, our study aims to use rice bran which is a cheap and waste product of rice processing industry as the sole carbon source in the Phytase production liquid (PPL) for production of enzyme of interest. The optimization results of carbon source substantiate the use of cheap natural substrate over a costly specific substrate for the production of phytase from *Aeromonas* spp.

e) Nitrogen source

The results of production of phytase using three different nitrogen sources (ammonium nitrate, sodium nitrate and potassium nitrate) supplemented in the PPM evidently showed high activity with respect to potassium nitrate at two different pH of 5.5 and 8.5.

CONCLUSION

As an extensive search continues to find a promising novel phytase producing organism, but the isolated Aeromonas spp from bacteria can successfully and effectively produced two different phytases which are highly potent for industrial application. The optimum production pH of phytase from *Aeromonas spp* is been evident in both acidic and basic scale and also the temperature being 37^0C which is a common temperature for increase in biomass and also enzyme production adds to the potency of both our isolated organism and enzyme for industrial application. The use of cheap and natural substrate (rice bran) which has nearly 80% of phytic acid content in it also adds on to the positive aspects of the phytases from *Aeromonas spp*.

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