Bioprospecting Actinomycetes For Production Of Thermotolerant Phytase

Suchita Bharambe-Chaudhari and Swati Peshwe

Abstract: Phytate [myo-inositol(1,2,3,4,5,6) hexakisphosphate] is the main storage form of phosphorus (P) in many plants which is the key food ingredient for animals but, this phytate-bound P is not available to monogastric animals as they lack the endogenous enzyme required to hydrolyze, and make the available phytate phosphorous. For this reason the availability and digestibility of phytate phosphorous is very low in these animals. Phytase (myo-inositol hexakisphosphate phosphohydrolase) catalyses sequential hydrolysis of phosphate ester bond of phytate and releases a usable form of inorganic phosphorus. Fortification of animal feed with phytase is an effective way to increase the availability of phytate bound phosphorus. It also reduces the anti-nutritional properties of phytic acid and eutrophication caused by excretion of phytic acid. The aim of the present work is to isolate thermotolerant phytase producing actinomycetes. So that feed supplementation of phytase produced by these organism can increases the body performance measured in terms of body weight. Sixty two different actinomycetal isolates were isolated from sixty nine soil and litter samples collected from various poultry, goat and cattle farms. These isolates were further screened for phytase activity in liquid Phytase Screening Medium(PSM). Isolates showing high phytase activity further studied for effect of temperature for phytase activity at 30^0C, 40^0C, 50^0C, 60^0C, 70^0C, 80^0C, 90^0C.

Key words: Actinomycetes, Feed fortification, Phosphorus ,Phytate, Phytase, , Poultry, thermotolerant,.

1. INTRODUCTION:

Phytate (inostol-phosphoric acid/myo- inostol hexakis phosphate/Phytic acid) is the foremost storage form of phosphorus in plants. It may contribute to the phosphorus supplementation for the monogastric animals like poultry, pigs but due to lack of native phytate degrading enzyme these animals unable to utilize phytate as phosphorus source hence this phosphorus requirement fulfilled by supplementation of inorganic form of phosphate. Such unutilized phosphorus when excreted out it leads to the ecological problem like eutrophication. Along with this the phytate form complexes with protein and inhibit digestive enzymes such as trypsin, pepsin, amylase, tyrosidase. (Boling S D et al, 1986). Phytate is mixed potassium-, magnesium- and calcium salt of phytic acid that is present as a chelate in cereals, leguminous and oil seeds. Besides serve as phosphorous store phytic acid also shows antioxidant property, antineoplastic property (Dvorakova 1998). Phytic acid also have important role in ripening, germination and signaling (Wodzinski and Ullah 1996). So pretreatment of the phytate rich feed is the need to hydrolyze the phytate into less phosphorylated form .Its will result in phytate phosphorus availability to monogastric animals and also reduces the chance of eutrophication like problem. Phytases (myoinositol-hexakisphosphate phosphohydrolase; EC 3.1.3.8 and 3.1.3.26) hydrolysis of myo-inositol hexakisphosphate) to free inorganic phosphate , to yield lower myo-inositol phosphate esters and, in some cases, free myo-inositol, making phosphorus available for bioabsorption (Irvine GCJ and Cosgrove DJ, 1972) Thus phytase can be the solution for the adverse effect of phytate. The primary objective of the research is screening phytase producing organism which will yield an enzyme to meet the demand of thermotolarent phytase and its production.

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It is also aimed to design an efficient and cost effective methodology for production of phytase for its efficient use on need for effective mineral utilization by broiler chicks.

2. MATERIALS AND METHODS:

2.1 Collection of Sample:

Seventy eight samples, twelve poultry farm soil samples (PS 1-12), twelve poultry litter samples (PL 1- 12), seven cattle shed soil samples (CS 1-7), thirty one cattle litter samples (CL 1-12), two goat shed soil samples (GS 1-2), five cattle litter samples (CL 1-5), seven garden soil sample(IG1-7) from various locality of Aurangabad district and soil sample(N 1-9, k 1-12) from Narayangaon and Kathmandu, Nepal respectively were collected.

2.2 Culture Media

Actinomycetes isolation from the collected samples was carried out on starch casein agar and primary screening of actinomycetes was done using wheat extract mineral agar and further quantitative screening was carried out using liquid phytase screening media.

2.3 Isolation and screening of phytase actinomycetes:

2.3.1 Calcium carbonate treatment of soil samples:

The collected samples were dried at 40^oC for 2 hours using hot air oven. Pretreatment of sample carried out with calcium carbonate (1:0.1 w/w). Calcium carbonatesample mixture was transferred to a sterile petri plate with moist environment for 8 days (Gurug et al, 2009; Zoe Yi Ng and Selvaraj Amsaveni, 2012).

2.3.2 Isolation of actinomycetes:

Each of the pretreated sample was taken in one gram quantity and mixed with 9 ml of physiological saline. Serial dilution of the pretreated sample was carried out upto 10^{-6}, 0.1 ml of 10^{-2}-$10^{-6}$was plated on starch casein agar medium and plates were incubated at 40^oC for 8 days. After incubation actinomycetes colonies were identified morphologically and subcultured on starch casein agar. (Monisha Khanna, Renu Solanki and Rup Lal, 2011).

2.3.3 Screening of phytase producing actinomycetes:

Qualitative screening of Phytase Producing actinomycetes:
Isolated actinomycetes then screened on mineral medium containing wheat extract as phytic acid source. The media consisted of 0.04% $(NH_4)_2$ SO_4, 0.02% $MgSO_4.7H_2O$, 0.1% Casein, 0.05% KH_2PO_4, 0.04% K_2HPO_4 dissolved in wheat bran extract. autoclaved at 121^oC for 15 minutes (Powar and Jagannathan, 1982) (Chunshan et al.2001; Mittal et al.2011).The inoculated plates were incubated at 40oC for 8 days. Actinomycetal colonies with zone of hydrolysis were selected and maintained on mineral medium with wheat extract medium.

Quantitative Screening of Phytase Producing actinomycetes:
Isolates showing clear zone on mineral medium containing wheat extract were screened secondarily on the basis of phytase activity in in liquid phytase screened medium(PSM). The media consisted of Glucose 1.5 %, $(NH_4)_2$ SO_4, 0.5%, KCl 0.05%, $MgSO_4.7H_2O$, 0.01%, NaCl0.01%, $CaCl_2.2H_2O$, 0.01%, $FeSO_4.7H_2O$, 0.001%, $MnSO_4.7H_2O$, 0.001%, Na-Phytate 0.5 % (Sigma), pH 6. (Chunshan et al.2001). at 40oC for 5 days. The culture was centrifuged at 10000 rpm for 10 min at 4°C, the clear supernatant was used as the crude enzyme for phytase activity assay. For phytase assay procedure , 150 µl of crude enzyme was incubated with 600 µl of 0.2% w/v sodium phytate solution in acetate buffer (0.1 M, pH 5.5) for 30 min at 40^oC (Bajaj and Wani 2011). The reaction was stopped by adding 750 µl of 5% trichloroacetic acid Solution. The phosphate released during the reaction was estimated (Bajaj and Wani 2011; Fiske and Subbarrow 1925) spectrophotometrically. One unit of phytase (FTU) is defined as the amount of enzyme that liberates 1 µmol of phosphate per min under the assay conditions.

2.4 Effect of temperature on phytase activity on Actinomycetal isolates:
Isolates showing high phytase activity were further studied for effect of temperature on phytase activity at 30^0C, 40^0C, 50^0C, 60^0C, 70^0C, 80^0C, 90^0C.To select the actinomycetes which shows maximum activity at 40^0C and retain the activity at 80^o C.

2.5 Identification of Actinomycetal isolate asp-2:
Genera of purified isolates asp-2 was identified based on morphological comparisons to the existing description of known genera as given in Bergey's Manual of Determinative Bacteriology (Holt, J. G .et al.,1994) Preliminary identification of the isolate was carried out by morphological characterization like colony characters, Gram's Staining, motility, spore staining, capsule staining, biochemical characterization like Indol test, Methyl red test, Voges- Proskauer test, citrate utilization test, catalase test, oxidase test, urease test, starch hydrolysis, gelatin hydrolysis, nitrate reduction test,H2S production (Aneja K.R.,2005)and Sugar fermentation tests.

2.6 Optimization of cultural condition:

2.6.1 Effect of inoculums age:
Effect of inoculums age was studied by inoculating 2% spore suspension of asp-2 of different age (2 days to 13 day old culture and incubated at 40^0C for 5 days. and the crude enzyme samples were collected for phytase activity assay.

2.6.2 Effect of Incubation time:
Fermentation medium was inoculated with 2 % of 4 day old asp-2 spore suspension inoculum and incubated at 40^0 C for 1-13 day. After incubation crude enzyme samples were analysed for phytase activity assay.

2.6.3 Effect of Incubation size:
Different volume of viz.2, 4, 6, 8, 10 % of 5 day old asp-2 spore suspension (87x 10^{-7}CFU) were used to inoculate the production medium and incubated at 40^0C for 6 days. After incubation the crude enzyme samples were extracted and assayed for phytase activity.

2.6.4 Effect of pH:
Effect of pH on phytase production on asp-2 was studied by adjusting the pH of production medium to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 in different flask with 1N NaOH and 1N HCl. All the flask were inoculated with 6 % of 5 day asp-2 spore suspension and incubated at 40^0C for 6 days. After incubation crude enzyme samples were extracted doe phytase activity assay.

2.6.5 Effect of carbon source:
In 1% concentration different carbon source like lactose, glucose, maltose, mannitol, sucrose were added to the fermentation medium. Such medium was inoculated with 6% 5 day old spore suspension and incubated at 40^0C for 6 days. After incubation crude enzyme samples were extracted doe phytase activity assay.

2.6.6 Effect of nitrogen source:
The fermentation was added with different nitrogen source source NaNO3, NH4(SO4)2, KNO3, NH4NO3, NH4Cl, peptone, yeast extract, casein, urea in 0.5% concentration and inoculated with 6% 5day old spore suspension and incubated at 40^0C for 6 days. After incubation crude enzyme samples were extracted doe phytase activity assay.

2.6.7 Effect of substrate concentration:
Optimization of substrate concentration for phytase production by asp-2 was carried out by preparing the fermentation medium with various concentration of substrate (Sodium phytate) like 0.1 – 0.8%. The medium was inoculated with 6% of 5 day old spore suspension and incubated at 40^0C for 6 days. After incubation crude enzyme samples were extracted doe phytase activity assay.

3. RESULT AND DISCUSSION:
For this work collection of soil samples was done from various poultry, cattle and goat farms and garden. Mostly the research work on phytase have been carried out with bacteria (Singh and Satyanarayana, 2008) and fungi (Gulati et al. 2007) but much less work with actinomycetes, the present work focus on phytase production with

actinomycetes. The samples were dried at 40^{0}C and treated with calcium carbonate for stimulating isolation of actinomycetes. From all the calcium carbonate treated samples total 62 isolates were isolated on starch casein agar amongst these 37 isolates were shows zone of clearance on wheat extract mineral media. All of the 37 isolates then screened quantitatively in liquid phytase screening medium. Isolate asp-2 shows the highest activity 1902 FTU at 40^{0}C and retain 98% of this activity at 80^{0}C was selected for further studies Since the need of present work to produce phytase with maximum activity at 40^{0}C , body temperature of broiler and retain the activity with minimum loss at 80^{0}C, feed pelleting temp. The isolates showing highest activity in liquid PSM further screened on the basis of the highest phytase activity at 40^{0}C and retain the activity with minimum loss at 80^{0}C(Fig.1).

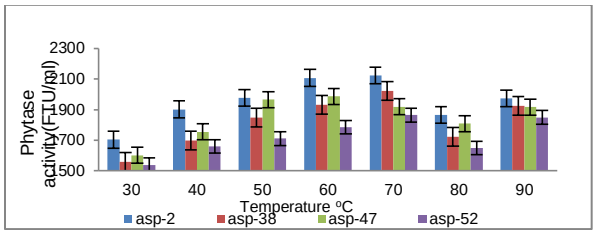

***Fig.1**. Screening of actinomycetes on the basis of temperature*

Morphological and Biochemical characteristic studies of asp-2 using the criteria of Bergeys manual of Determinative Bacteriology allude to Streptomyces sp. The morphological and biochemical characteristics of Streptomyces sp. asp-2 were mentioned in table no.1.

***Table 1:** Morphological and biochemical characteristics of asp-2*

Sr.on	Characteristics	Result
1	Morphological:	
	Colony	
	i. Shape	Irregular
	ii. Colony color	White
	iii. Arial mycelium	White
	iv.Substrate mycelium	Off white
	v. Elevation	Raised
	vi. Appearance	Powdery
	vii. Gram Character	Gram Positive
	viii. Motility	-
2	Biochemical:	
	Indol Test	
	Methyl Red test	+
	VogesProskauer Test	
	Citrate utilization Test	+
	Catalase Test	+
	Oxidase Test	+
	Urease Test	+
	Starch Hydrolysis	+
	Gelatin Hydrolysis	+
	Nitrate reduction test	-
	H2S production Test	+
	Sugar Utilization	
	Glucose	++
	Sucrose	+
	Maltose	+
	Mannitol	+
	Lactose	+
	Xylose	+

Optimization of various parameters like inoculum age, incubation time, inoculums size, pH, 'C', source, 'N' source was done for maximum production of phytase.

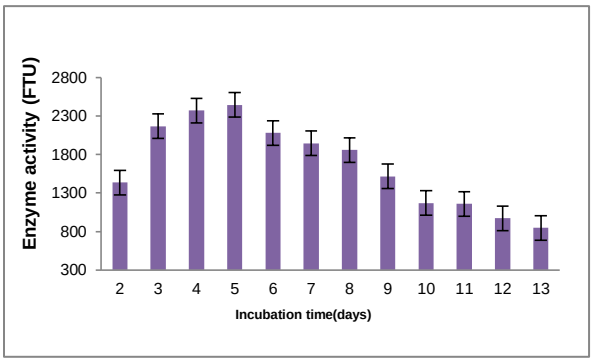

***Fig. 3**. Effect of incubation time on phytase activity*

The effect of incubation time on production of phytase was studied(Fig.3). It was observed that for maximum production of phytase incubation time of 5 days needed. Increasing the fermentation form 2 days gradual increase in phytase production was observed upto 5 days but extending the fermentation time beyond 5 days showed gradual decrease in phytase activity, it might be due to accumulation of molecule which inhibits the activity. In other studies actinomycetes showed phytase production more than 5 days (Nasrabadi et al. 2012) whereas bacteria showing maximum activity at 3 days (S.Sreedevi and B. Reddy, 2012; Hosseinkhani et al, 2009).

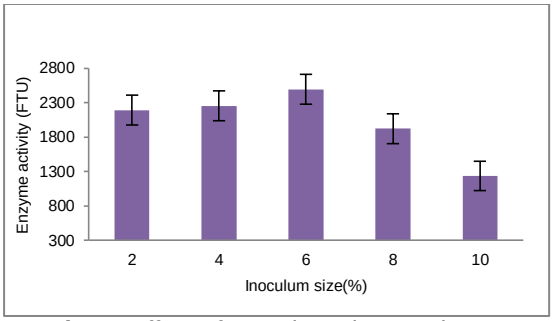

***Fig.4**. Effect of Inoculum size on phytase activity*

When the effect of inoculum size on phytase production was studied it was observed that the actinomycetal isolate showing maximum production with 6% of inoculum but the production with 2 and 4% is also have considerable potential and increase in inoculum size not showing decrease in production(Fig.4).

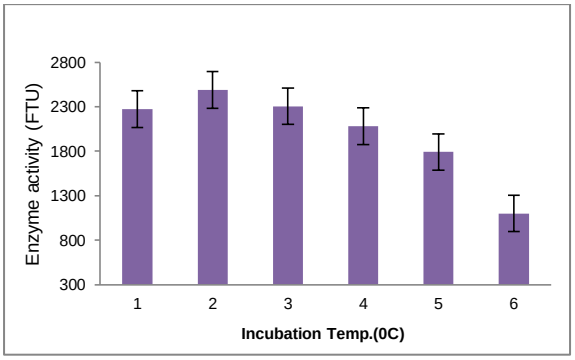

***Fig. 5**. Effect of incubation temperature on phytase activity*

Optimum temperature for phytase production with Streptomyces sp.asp-2 was investigated in temperature range of 30^0C - 80^0C and it was observed that at 40^0C asp-2 showed maximum production of the enzyme (Fig.5) . Similar results were obtained with Nocardia sp. (Bajaj and Wani, 2011). Bacillus sp.C43 carried out the maximum phytase production at 45^0 C. (S.Sreedevi and B. Reddy, 1012).

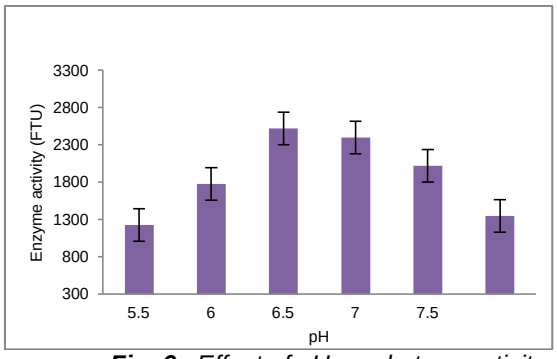

***Fig. 6**. Effect of pH on phytase activity*

To determine optimum pH for phytase production the isolate was inoculated in production medium with pH (Fig.6). Maximum enzyme production was in production medium with pH 6.5, however on both side of optimum pH decrease in production was observed. The reports similar results were obtained with A. vancoresmycina S-12 (D.Bandopadhyay, et al. 2016). Maximum phytase production with M. jalaludinii with pH 6.8- 7.2 (Lan et al, 2002).

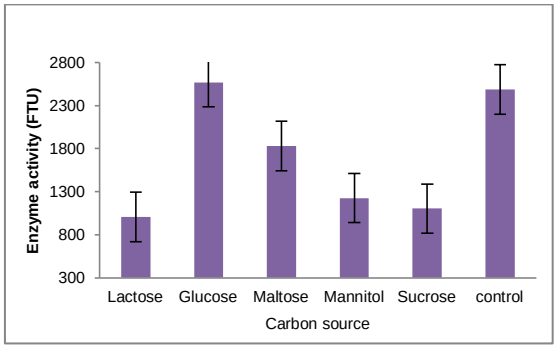

***Fig. 7**. Effect of Carbon source on phytase activity*

To study the effect of carbon source on phytase production glucose in the medium was replaced by 1% concentration of various sugars and it was observed that maximum production of phytase carried out with glucose(Fig. 7). Similar results were observed by other aothors that phytase production was enhanced by glucose (Hussin et al, 2011) but other authors also reported that phytase production was enhanced by glucose plus sucrose(Hossseinkhani et al, 2009).

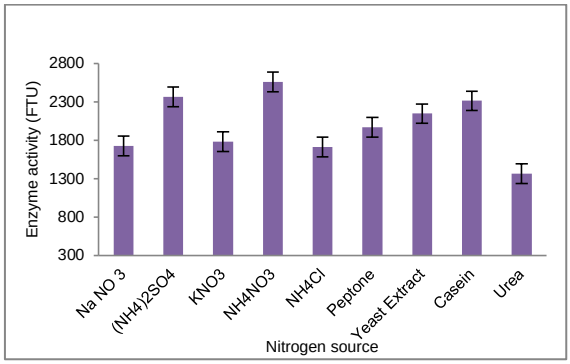

***Fig. 8**. Effect of nitrogen source on phytase activity*

The effect of various nitrogen sources was studied and NH4NO3 was observed to be the best source for maximum phytase production(Fig.8). Similar results reported with bacteria that shows NH4NO3 is best nitrogen source for production of enzyme (S.Sreedevi and B. Reddy, 1012) and also with fungi(Suresh and Radha, 2016) . It was also observed that yeast extract enhanced the phytase production by actinomycetes(Bajaj and Wani, 2011).

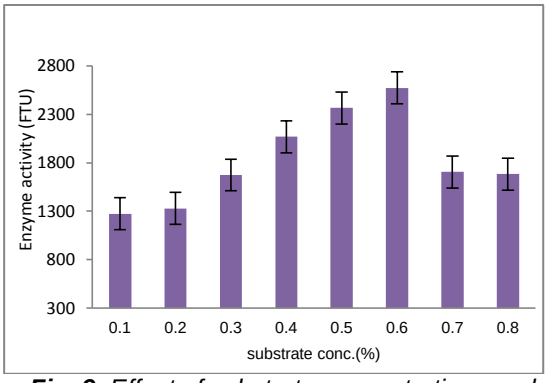

***Fig. 9**. Effect of substrate concentration on phytase activity*

The effect of concentration of substrate, sodium phytate on production was studied(Fig.9) .It was investigated that 0.6 % 0f sodium phytate showed significant increase in phytase production by Streptomyces asp-2 whereas Streptomyces luteogriseus R10 showed significant production with 1% concentration of sodium phytate(Aly, MM et al, 2015).

4. CONCLUSION:

The present work focused on screening on thermotolerant phytase actinomycetes. Actinomycetal isolate Streptomycetes sp. asp-2 was screened out as efficient phytase producer which shows maximum phytase activity at 40^0C and retains the activity at 80^0C with minimum loss. The best activity of Streptomycetes sp. asp-2 observed at 5 day age, pH 6.5, temperature 40^0C with inoculum 6% when incubated for 5 days. 1% glucose as 'C' source, NH4NO3 as 'N' source with 0.6% of inorganic substrate sodium phytate. This optimization experiment increases the phytase production by Streptomycetes sp. asp-2 by 1.35 fold. This phytase from Streptomycetes sp. asp-2 feed additive which can withstand the feed processing temperate.

5. ACKNOWLEDGMENTS:

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