ISOLATION, SCREENING AND NUTRITIONAL OPTIMIZATION FOR THE PRODUCTION OF BACTERIAL PHYTASE

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Abstract:
The present work emphases on isolation and characterization of phytase producing bacteria form litter and soil, which shows maximum activity at 40^{0}C, the body temperature of poultry and should retain the activity with minimum loss at 80^{0}C, the feed pelleting temperature. The supplementation of poultry feed with such phytase can increases the broiler performance measured in terms of body weight. A culture enrichment technique was used to isolate phytase-producing microorganisms. 120 different bacterial isolates were obtained from 69 soil and litter samples collected from various poultry, goat and cattle farms. They were screened on wheat extract and phytase screening medium. 90 isolates exhibited zone of hydrolysis around colonies on wheat extract medium. Among these isolates, 25 isolates showed considerable zone of hydrolysis around colonies on phytase screening medium. All the 25 isolates were further screened on the basis of enzyme production. Seven isolates showing high phytase activity were further screened on the basis of their phytase activity at 40^{0}C and 80^{0}C.Isolate SP-46 was selected for further studies and was identified as *Bacillus sp* SP-46.Various nutritional parameters like carbon source, nitrogen source, and substrate concentration were optimized for increased phytase production. The process parameters affecting phytase production were optimized by one at a time approach viz. inoculum size, incubation time, temperature, pH. Cultural conditions optimized by this study helped to enhance the phytase production by 1.89 fold.

Keywords: Phytase, Phytate, Zone of hydrolysis, effect of temperature.

INTRODUCTION:

Phosphorous is one of the major feed ingredients and is supplied to animal in required amount through raw material and added phosphates. Most animal diets are primarily composed of plant-based ingredients. In plants, phosphorous is present in different forms such as attached to organic molecules like phospholipids and proteins but most is present as part of the phytic acid molecule. The 50-80% of phosphorous is bound in phytate which cannot be broken down by endogenous enzymes in poultry (Joseph and Raj 2007; Bae et al.1999). As a consequence, phosphorous from vegetable sources is poorly digested and cannot meet nutritional requirement of poultry. Phosphorous from plant sources must be hydrolysed with phytase in order to become available to broiler chicks as inositols and inorganic phosphates which are readily absorbed in digestive tract (Shamna et al.2012). Phytase (myo-inositol hexakis phosphate phosphohydrolase) catalyses the hydrolysis of phytate (myo-inositol hexakis phosphate) to inorganic monophosphate and lower myo-inositol phosphates and in some cases to free myo-inositol.

More accurately 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 signalling (Wodzinski and Ullah 1996).

Conversely it is considered as an antinutrient factor which needs to be degraded by phytase enzyme in food/feed. Phytic acid interacts with protein forming phytate-protein complex by binding to plant protein. Phytic acid decreases their solubility and digestibility hence reduces their nutritive value. Phytic acid effectively binds to different mineral ions, forming insoluble phytate mineral complex in the intestinal tract and prevent mineral absorption (Selle et al, 2000; Cowieson et al 2006). This reduces the bioavailability of essential minerals. In addition to complex formation with minerals and proteins phytic acid interact with enzymes such as Trypsin, Pepsin, D-amylase, D-glactosidase resulting in a decrease in the activity of these important digestive enzymes. (Deshpande and Cheryan 1984 Kerovuo J. 2000) thus reduces the digestibility.

Ruminants digest phytic acid through action of Phytases produced by their anaerobic ruminal micro flora. However monogastric animals such as swine, poultry and human are not capable of metabolizing phytate phosphorous because of the lack of digestive enzyme hydrolysing the substrate and therefore inorganic phosphate is added to their feed to fulfil the phosphorous requirement. Further the undigested phosphorous is excreted out in manure (Leytem et al.2006) poses a serious phosphorous pollution problem. It contributes to the eutrophication of surface water in area of intensive livestock production (Selle et al.2006).

Due to this scenario phytase enzyme is required to overcome aforementioned problems as food and feed additive. Therefore Phytase has become an important enzyme and is the object extensive research. By working efficiently on the substrate in the prevailing conditions, supplemental Phytase could diminish the antinutritive effect of phytic acid and reduce the cost by removing or reducing the need for supplemental inorganic phosphate. In addition Phytase would be an environment friendly product reducing the amount of phosphorous entering the environment (Wodzinski and ullah 1996; Kerovuo J. 2000).

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

A thermotolarent phytase could have potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The exploitation of phytases in the pulp and paper process could be eco-friendly and would assist in the development of cleaner technologies (Liu B.L. et al.1998).

In the present investigation also, the ability of locally isolated bacterial strain from poultry litter to produce extracellular phytase in a surface liquid fermentation was evaluated and their production conditions were optimized.

MATERIALS AND METHODS:

Sample collection:

Sixty nine 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), were collected from various locality of Aurangabad district.

Isolation of phytase producing bacteria:

One gram of sample was suspended in 100 ml of wheat bran extract medium and incubated at 40°C for 48 hrs. with constant shaking at 120 rpm. 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. The pH was adjusted to 6.0 using 1N HCl and autoclaved at 121°C for 15 minutes (Powar and Jagannathan, 1982). After wards the enriched sample was spread onto wheat bran extract agar plates. The inoculated plates were incubated at 40°C for 24 hrs. After incubation, the plates were observed for the growth of colonies and the clear zones of hydrolysis around them. Each colony with clear zones of hydrolysis was picked up and maintained on wheat bran extract medium till further use.

Screening for best phytase producing isolate:

Qualitative Screening of Phytase Producing Bacterial Strains:

Phytase activity of the isolated strains was screened on wheat bran extract agar media and observing their surrounding clear halo (Chunshan et al.2001; Mittal *et al.*2011). The halo (Z) and colony (C) diameters were measured after 24 hrs of incubation at 40°C. These isolates were further screened by plating the isolates on PSM (Phytase Screening Medium) (L. Vijaysai et al.2011). The media of pH 6 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) (Chunshan et al.2001).

Quantitative Screening of Phytase Producing Microorganisms:

Isolates showing zone of hydrolysis on PSM plates were further screened on the basis of phytase activity in liquid PSM medium. The isolated strains were inoculated in 20 ml of PSM and incubated at 40°C for 24 hrs. 2ml of culture centrifuged at 10000 rpm for 10 min at 4°C, the clear supernatant was used as the crude enzyme and used for the phytase activity assay. For phytase assay, 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°C (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.

Isolates showing high phytase activity were further studied for effect of temperature on phytase activity at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C.Among all the bacterial isolates analyzed, the isolate (SP 46) exhibiting highest phytase activity was selected for further studies.

Identification of isolate SP-46:

Isolate SP-46 was identified according to the identification scheme in Bergey's manual of Systematic 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 and 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,H_2S production (Aneja K.R. 2005)and Sugar fermentation tests.

Optimization of cultural condition for phytase production:

In order to achieve the maximum phytase production optimization of cultural and nutritional parameters were carried out. Effect of various parameters viz. incubation time inoculums age, inoculums size, pH, carbon source, nitrogen source.

Effect of Incubation time:

The fermentation medium in seven different flasks was inoculated with 2% of 24hr old culture and incubated at 40°C for 24, 48, 72, 96, 120, 144, and 168 hrs. After incubation the crude enzyme samples were extracted and assayed for phytase activities.

Effect of inoculums Size:

The different inoculums concentrations like 2, 4, 6, 8, 10, and 12% of 24 hrs old culture with (6.8 X 10^7CFU) were used to inoculated in the fermentation medium and incubated at 40°C for 24hrs. After incubation the crude enzyme samples were extracted and assayed for phytase activities.

Effect of temperature:

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The effect of temperature on phytase production was studied; the fermentation medium was incubated at different temperature from 30°C to 90°C with 10°C interval. After incubation the crude enzyme samples were extracted and assayed for phytase activities.

Optimization of pH :

Optimization pH of fermentation medium for phytase production was carried out by adjusting pH of fermentation medium to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 in different flasks using 1N NaOH and 1N HCl. Each flask was inoculated with 4% of 24 hrs old culture and incubated at 40°C for 24 hrs. After incubation the crude enzyme samples were extracted and assayed for phytase activities.

Optimization of carbon and nitrogen source:

The fermentation medium was added with different carbon source like glucose, lactose, maltose, mannitol, sucrose and starch in 1% concentration and nitrogen source $NH_4(SO_4)_2$, $NaNO_3$, KNO_3, NH_4NO_3, NH_4Cl, peptone, yeast extract, casein, glycine, urea in 0.5% concentration. Such medium was inoculated with 24 hr old culture and incubated at 40°C for 24 hrs. After incubation the crude enzyme samples were extracted and assayed for phytase activities.

Optimization of substrate concentration:

Optimization of substrate concentration was carried out by preparing the fermentation medium containing various concentration of sodium phytate as substrate in range of 0.1- 0.8 %. Such medium was inoculated with 4% of 24 hrs old culture and incubated at 40°C for 24 hrs. After incubation the crude enzyme samples were extracted and assayed for phytase activities.

RESULTS AND DISCUSSION:

All the soil and litter samples were collected from the poultry and cattle farms (Table 1). Samples were used for enrichment of phytase producing bacteria such enriched samples then plated on wheat extract mineral media for isolation. Total 120 different isolates were obtained among these isolates 90 isolates shows zone of clearance on wheat extract mineral media. These isolates were qualitatively screened on PSM on the basis of zone of clearance. Further quantitative screening of 30 isolates showing zone of clearance on PSM studied for phytase activity in liquid PSM medium. As the need of the present work is to isolate a bacterium having the maximum phytase activity at 40°C, the body temperature of the poultry (Bolzani R, et al.1979) and retain the activity with minimum loss at 80°C, the feed pelleting temperature. Seven isolates showing highest phytase activity were further screened on the basis of effect of temperature on activity (Fig.1). Bacterial isolate SP-46 shows the highest enzyme activity 379 FTU at 40°C and 383 FTU at 80°C.

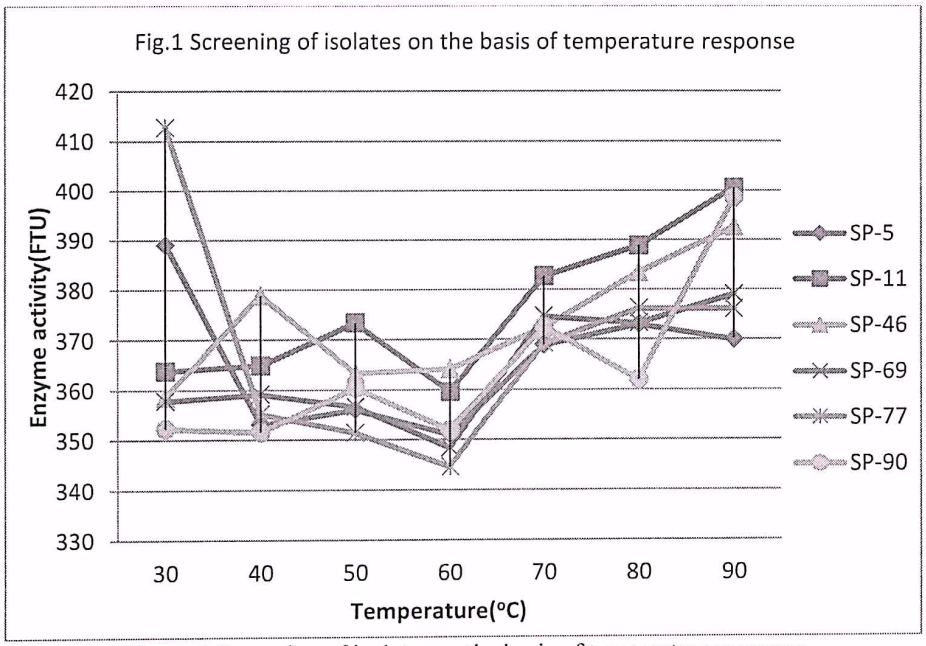

Figure.1 Screening of isolates on the basis of temperature response

Based on the morphological and biochemical characteristics using the criteria of Bergey's Manual of Determinative Bacteriology the selected SP-46 organism was keyed to the genus *Bacillus sp.* and designated as *Bacillus sp.*SP-46. The morphological and biochemical characteristics of isolate SP-46 were tabulated in Table 2.

Bacillus sp. SP-46 cultural conditions optimized for maximum production of phytase carried out in that effect of various parameters viz. Incubation time, incubation temperature, inoculums size, pH, 'C' source, 'N' source.

Incubation time affect the production of phytase (fig.2) The maximum production of phytase was observed after 24 hrs of incubation with maximum activity of 386 FTU. Though the maximum enzyme activity was observed at 24 hr incubation, the enzyme production by SP-46 was quite stable between 72-144 hrs it corresponds to sporulation. The enzyme activity decreased after 24 hrs it might be due to the depletion of nutrient (D J Mukeshkumar et al.2012). Similar results were obtained by other workers for *Pseudomonas aeuginosa*(B. Saeirekha et al.2012).

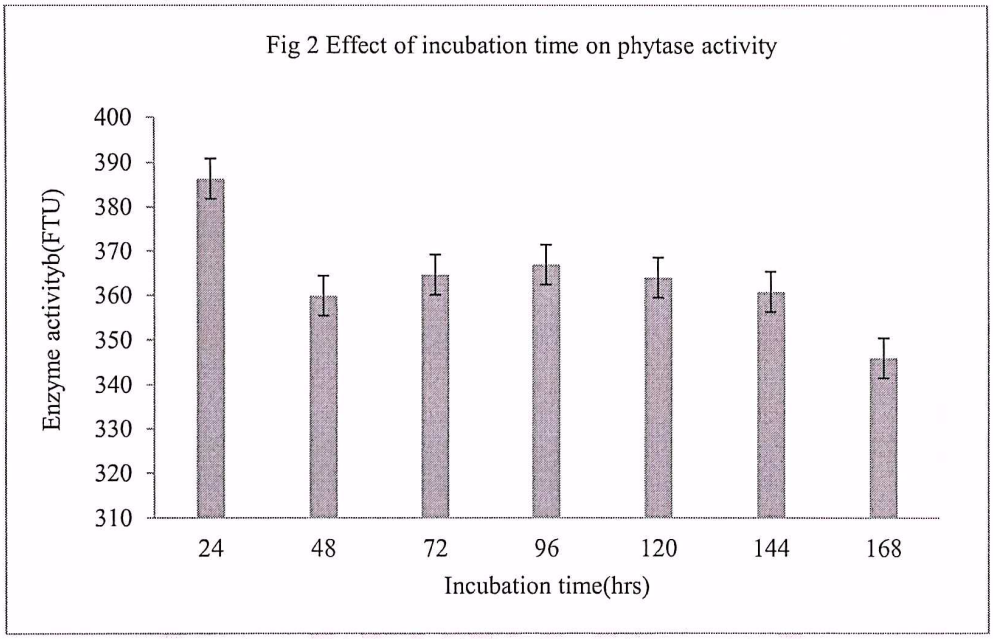

Figure 2 Effect of incubation time on phytase activity

The data obtained for effect of inoculum size on phytase production showed that (fig:3) inoculum in 4% size of production media was effective for best production of phytase, it shows considerable increase in production as compared to initial inoculum again it was comparably low when compared with the optimized inoculum size(10%) of other workers for Bacillus (Shreedevi and Reddy 2012).However this indicates that maximum enzyme production obtained at lower inoculum size in the present study.

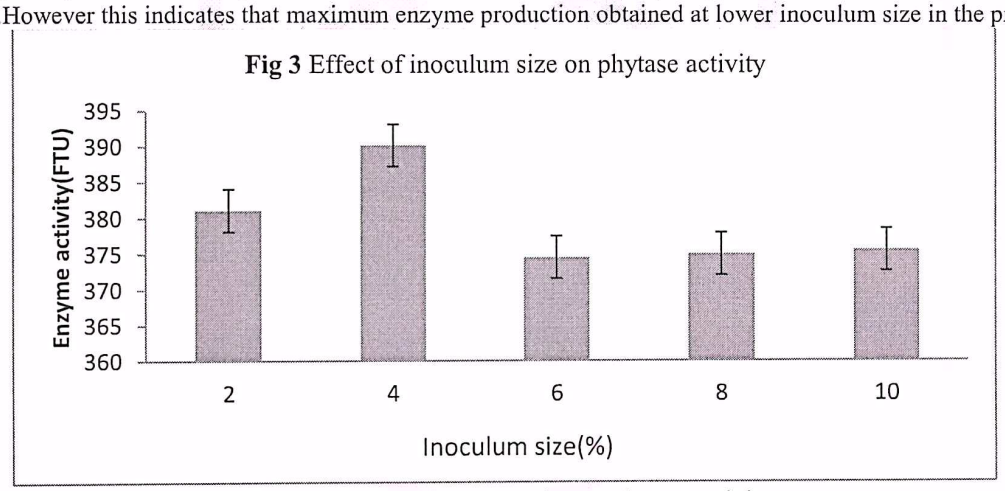

Figure 3 Effect of inoculum size on phytase activity

To determine optimum temperature for phytase production using *Bacillus sp.*SP-46 the isolate was incubated at different temperature from 30°C to 90°C with 10°C interval, Isolate *Bacillus sp.*SP-46 show maximum phytase activity at 40°C and retained it at 80°C which is desirable as the present study leads to application of the enzyme as poultry feed additive and the body temperature of the poultry is 40°C, the poultry feed pelleting temperature 80°C. Depending upon the type and source of organism diverges optimum temperature can be observed. *Pseudomonas aeruginosa* carried out maximum phytase production at 37°C (B. Sasirekha et al.2012).*Nocardia Sp.* Showed highest production at 40°C(Bajaj B.K. and Wani M.A 2011).

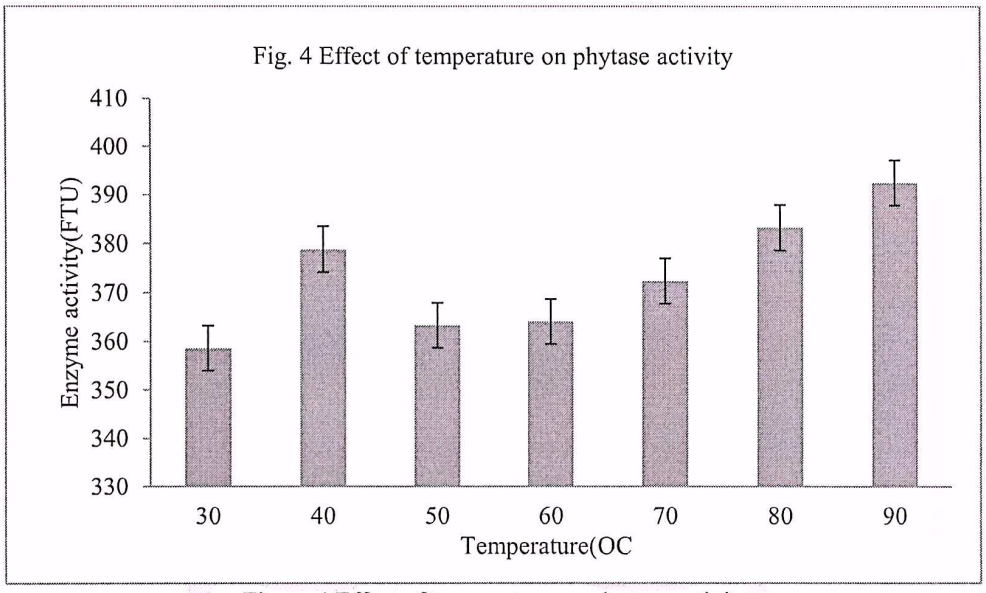

Figure 4 Effect of temperature on phytase activity

Effect of pH on phytase production shown in fig.5. The results reveals that best production of phytase at pH 7, with phytase activity of 372(FTU) the enzyme activity was dropped as pH of the medium increased.it might be due to the change in ionic environment of the active site of the enzyme which decrease the enzyme substrate affinity. As the physiological pH of poultry digestive tract is about 5, the enzyme shows 98% activity at 5.5 which advantageous to be fortify the poultry feed.

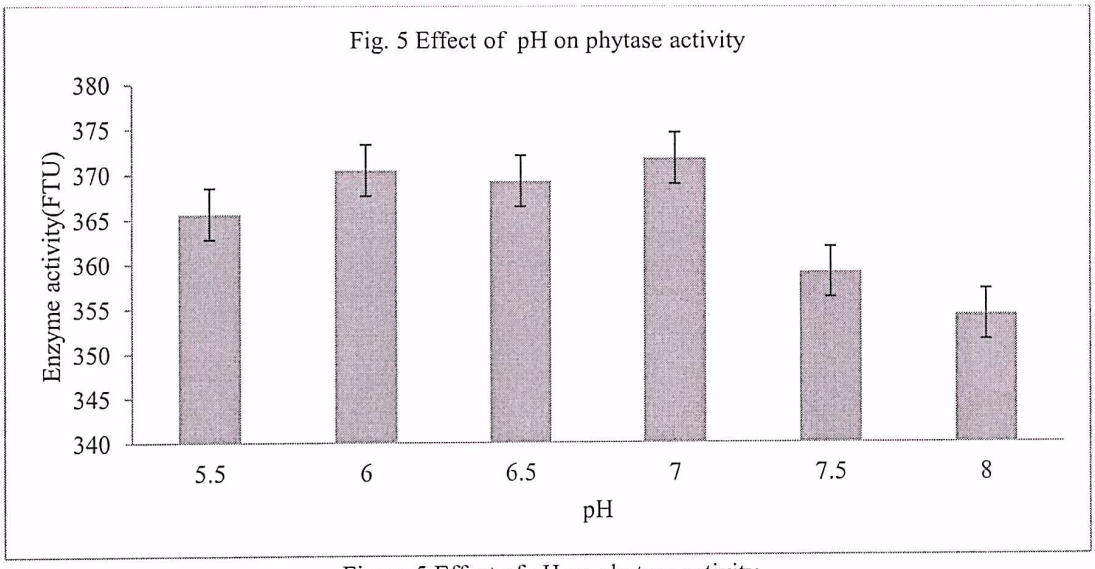

Figure 5 Effect of pH on phytase activity

To study the effect of supplementation of external carbon source various sugars as 'C' source was used. The results showed that Mannitol significantly induced phytase production. (fig.6).Effective carbon source for highest production of phytase depend on the production strain and the cultural conditions. Fructose has been reported for *B. megaterium* (Dhiraj Kumar et al,2013), glucose reported for *B. subtilis, P. aeruginosa* by other coworkers(B sasirekha 2012; D J Mukesh kumar 2012)

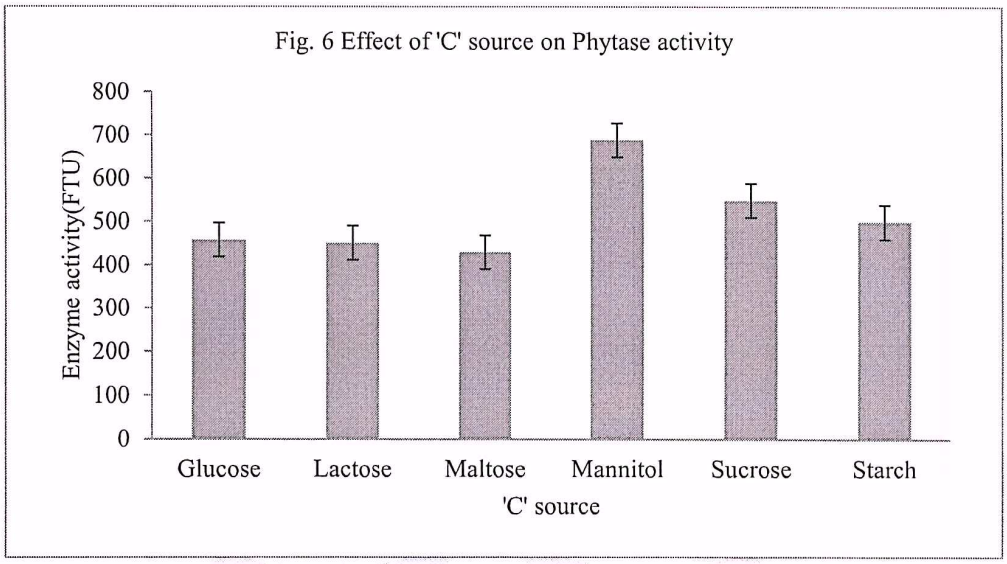

Figure 6 Effect of 'C' source on Phytase activity

Effect of various nitrogen sources were also studied by adding different nitrogen sources to the fermentation medium. The best activity was observed with $NH_4(SO4)_2$ (fig.7) similar results were reported for *B. megaterium* (Dhiraj Kumar et al.2013), *S. cerevisiae* (Man-Jin In et al.2008).Various inorganic nitrogen sources and organic nitrogen sources also have extensively used for phytase production. (Nandkumar at el.2013; Shreedevi and Reddy 2012)

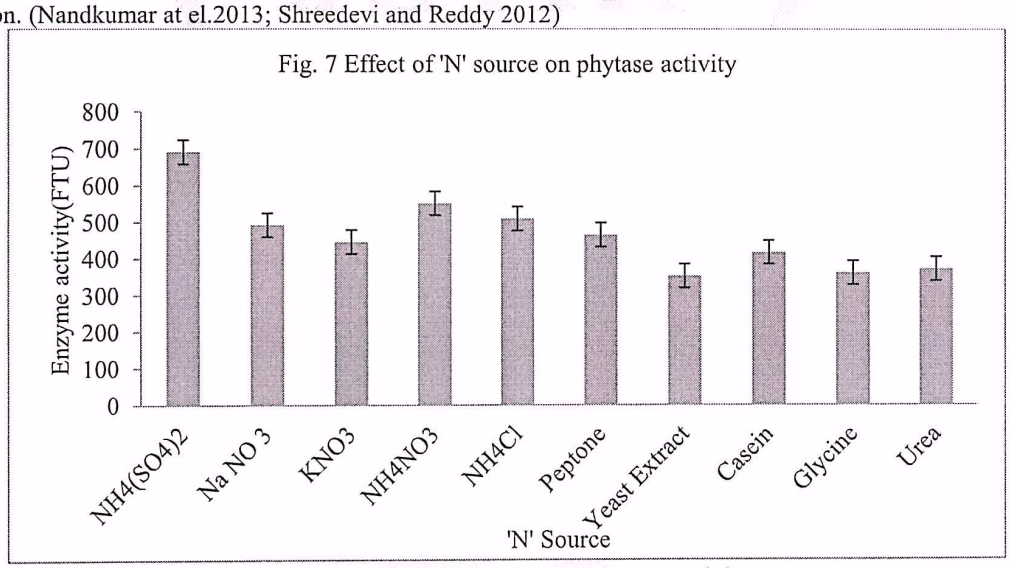

Figure 7 Effect of 'N' source on phytase activity

The results of effect of substrate, sodium phytate concentration on phytase production are shown in fig.8. It revealed that 0.7 % concentration of sodium phytate was the best for maximum production of phytase by *Bacillus sp. SP-46*. The lower concentration shows increase in phytase production whereas further increase in substrate concentration above 0.7% showed decrease in enzyme activity probably because of saturation effect. However the optimum substrate concentration varies according to the organism.0.001% was reported for *S.cerevisiase*(Man-Jin In et al.2008).

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Fig. 8 Optimization of substrate concentration

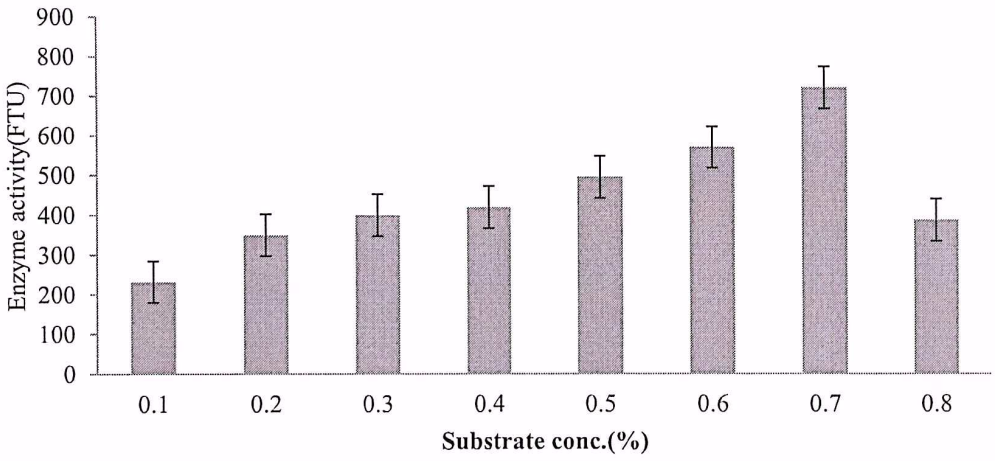

Figure 8 Optimization of substrate concentration

CONCLUSION:

The present work give emphasis on isolation of thermotolerant phytase producing bacteria from soil and litter samples collected from poultry and cattle farms. Result of present work suggest that the bacterial isolate *Bacillus sp.*SP-46 have efficient in production of phytase which have efficiency of production of phytase which shows its maximum activity at body temperature of broiler chicken and retain that activity even at the feed pelleting temperature. *Bacillus sp.* SP-46 shown is best activity at pH.7, temperature 40°C with inoculum size of 4% and incubation time of 24 hrs. Supplementation Mannitol as carbon source, $(NH_4)_2SO_4$ as nitrogen source and sodium phytate in 0.7% were found to be excellent phytase production. Thus cultural conditions optimized by this study helped enhance the phytase production by 1.89 fold. These bacterial phytases thus can be used as feed additive to increase the phosphorus availability to the poultry and reduce the need of addition of inorganic phosphorus to feed and environmental pollution.

Table1 List of samples and site of collection

Sr.no	Sample code	Site of sample collection
1.	PL 1	Khadkeshwara Hatcheries, Bokud Jalgaon
2.	PS 1	Khadkeshwara Hatcheries, Bokud Jalgaon
3.	PL 2	Intensive Poultry Development Block, Beed
4.	PS 2	Intensive Poultry Development Block, Beed
5.	PL 3-6	Central Hatchery, Padegaon, Aurangabad
6.	PS 3-4	Central Hatchery, Padegaon, Aurangabad
7.	GL 1-2	Punya Shlok Ahilyaadevi Mendhi V sheli Farm, Padegaon, Aurangabad
8.	GS 1	Punya Shlok Ahilyaadevi Mendhi V sheli Farm, Padegaon, Aurangabad
9.	CL 1-3	Cattle farm, Padegaon
10.	CS 1	Cattle Farm, Padegaon
11.	PL 9-10	Intensive Poultry Development Block, Osmanabad
12.	GL 3-5	Sheli v Mendhi farm, Osmanabad
13.	GS 2	Sheli v Mendhi farm, Osmanabad
14.	CS 2-7	Frozen Seamen Laboratory, Harsul
15.	CL 4-13	Frozen Seamen Laboratory, Harsul
16.	PS 5-12	Intensive Poultry Development Block, Parbhani
17.	PL 7-8	Intensive Poultry Development Block, Parbhani
18.	PL 11-12	Intensive Poultry Development Block, Parbhani
19.	CL 14-17	Cattle farm, Jalgaon
20.	CL 18-22	Bull Mother Farm, Hingoli
21.	CL 23-24	Cattle farm, Khultabad
22.	CL 25-31	Cattle farm, Aurangabad

PL- Poultry litter; PS- Poultry farm soil; GL- Goat litter; GS- Goat farm soil; CL- Cattle litter;

CS- Cattle farm soil

Table 2 Morphological and biochemical characteristics of isolate SP-46

Sr. No.	Characteristics	Result
A.	**Morphological:**	
	a. Colony	
	i. Shape	Irregular
	ii. Size	10 mm
	iii. Colour	Off White
	iv. Margin	Irregular
	v. Opacity	Opaque
	vi. Consistancy	Butyrous
	b. Bacterial Cell	
	i. Shape	Rod
	ii. Gram Character	Gram Positive
	iii. Motility	Motile
	iv. Spore	Spore former
	v. Capsule	-
B.	**Biochemical:**	
	a. Indol Test	-
	b. Methyl Red test	-
	c. Voges Proskauer Test	-
	d. Citrate utilization Test	-
	e. Catalase Test	+
	f. Oxidase Test	-
	g. Urease Test	-
	h. Starch Hydrolysis	+
	i. Gelatin Hydrolysis	+
	j. Nitrate reduction test	+
	k. H_2S production Test	-
	l. Sugar Fermentation Tests:	
	i. Glucose	A+, G -
	ii. Sucrose	A+, G -
	iii. Lactose	A+, G -
	iv. Maltose	A+, G -
	v. Mannitol	A+, G -
	vi. Xylose	A-, G -

[+: Positive; - : Negative; A+: Acid Production; A-: No acid production: G+: Gas production; G- : No gas production.]

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