Stimulatory effect of medium components on phytase production by *Aspergillus niger* and biotechnological application as a poultry feed additive

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Abstract

The present study was conducted for the phytase production using solid-state fermentation (SSF) by *Aspergillus niger*. Optimization of various medium components was carried out for better production of phytase. Maximum enzyme activity (265.12±5.51 IU/g) was obtained, when the fungus was grown in the optimized culture medium containing glucose (1%), NH_4NO_3 (0.5%), $FeSO_4.7H_2O$ (0.1%), KCl (0.1%), and $MgSO_4.7H_2O$(0.1%) at 35 °C after 5 days of incubation time with pH 6. Then phytase was supplemented as poultry feed additive and given to broiler chickens during a feeding trial of 5 weeks. For this purpose, the birds of the control group (T0) were fed on a basal diet without external phytase, whereas, the birds of the experimental group (T3) were given basal feed + 3000 IU phytase per Kg diet. The results exhibited that there was an improvement in the body weight gain (BWG) of chicks i.e. 1903 g - 2090 g for the control group (T0) and experimental group (T3), respectively. The current study thus indicated the affectivity of phytase as a supplement in the broilers diet for better growth performance and recommended its use as an efficient additive in poultry feed formulation.

Keywords: Feed additive; medium components; optimization; phytase; solid state fermentation.

1. Introduction

Phosphorous (P) is an important mineral for growth performance and bone formation of broilers. It also supports energy conversion, egg production, and nervous system development (Li *et al*., 2020). A low amount of phosphorous is available in plant-based diets used for broilers because a large quantity of P is in the form of phytic acid, an organic P source. This phytic acid phosphorous is not usually accessible to the birds fed on plant-based diets due to insufficiency or lack of endogenous enzyme activity (Marchal *et al*., 2021), and is excreted as manure without being digested (Kumar *et al*., 2012; Lalpanmawia *et al*., 2014) and absorbed in the digestive tract

of monogastric animals. Due to the anti-nutritional effect, phytate has a negative effect on the digestion of other nutrients and thus reduces the poultry growth performance (Woyengo *et al*., 2013; Morgan *et al*., 2016).

To fulfill the requirements of phosphorous for the birds, monocalcium phosphate (MCP) or dicalcium phosphate (DCP) in the form of inorganic phosphorous may be supplemented to the poultry feed. But, in this way, the cost of feed and the amount of P released in the form of excreta will be increased causing environmental pollution (Saleh *et al*., 2021). To deal with such problems, supplementation of phytase in the bird's feed is a suitable solution (Lalpanmawia *et al*., 2014).

Phytase, a phosphatase enzyme, can hydrolyze phytic acid (Inositol hexakisphosphate) to inositol, inorganic phosphate, and potentially chelated minerals which are readily absorbed by monogastric animals e.g. poultry and fish (Ajith *et al*., 2018). The main objective of phytase supplementation in animal diet is to (i) use the inorganic P source and other important minerals already present in the plant-based feed (ii) improve the availability of myo-inositol (iii) counter the anti-nutritional effect of phytate (iv) conserve non-renewable sources of phosphorus due to decreasing requirement of its addition in the animal feed (v) minimize the environmental excretion of phosphate (Mahmood *et al*., 2021; Thorsen *et al*., 2021).

Submerged fermentation (SmF) or solid-state fermentation (SSF) can be used for the production of many biocatalysts such as phytase. However, it has been reported that SSF is the best method for the extracellular enzymes production by fungi (Cakmak & Aydogdu, 2021).

Phytase has a very profound role in the animal feed industry as an animals diet supplement, where it improves the digestion and assimilation of phosphorous and a few other inadequately available nutrients including copper, manganese, iron, and zinc in the monogastric animals digestive system (Munir & Maqsood, 2013; Vasudevan *et al*., 2017; Mahmood *et al*., 2021). Phytase plays important role in increasing the body weight gain (BWG) and growth performance of these animals. Phytases also decrease the amount of phosphorous in the animals excrement, which can otherwise cause environmental pollution. Phytases as an animal feed additive can thus be used as a substitute for expensive di-calcium phosphate and reduce the cost of animals feed (Dahiya, 2016; Jatuwong *et al*., 2020).

The present study was carried out to optimize the parameters for phytase production, and then to utilize the phytase as poultry feed additive to check its effect on the growth performance of broilers.

2. Materials and methods

2.1. Chemicals and biochemicals

In this study, the chemicals used were purchased from Sigma-Aldrich (USA), and Merck (Germany) and of analytical grade, however, the biological media were procured from Oxoid (UK).

2.2. Microorganism

A fungal strain i.e. *Aspergillus niger* was obtained from the Microbiology Laboratory, PCSIR Laboratories Complex, Lahore, Pakistan. The strain was maintained on potato dextrose agar (PDA) slants and used further for enzyme production.

2.3. Phytase production

Aspergillus niger was employed for phytase production in solid-state fermentation process. Growth medium (Rice polish, (% w/w) 0.1 $FeSO_4.7H_2O$, 0.1 KCl, 0.1 $MgSO_4.7H_2O$, 0.5 NH_4NO_3) was taken in an Erlenmeyer flask (250 ml) and an appropriate volume of distilled water (pH 5.5) was added in it. The culture medium was sterilized in an autoclave for 15 minutes at 121°C. Then, under aseptic conditions, 10% (v/w) inoculums of *Aspergillus niger* were amended in the fermentation medium after cooling at room temperature and incubation was done at 35°C for 5 days in an incubator (Mahmood *et al*., 2021).

At the end of the incubation period, 50 ml of 0.2 M citrate buffer (pH 5.5) was poured into all SSF media (including control) and shaken in a water bath for 1.5 hours. The content was firstly filtered through sterilized muslin cloth and then centrifugation was performed at 4°C for 15 min at 10,000 rpm in a centrifuge machine. The obtained filtrates were used as a crude source of enzyme for measuring the enzyme activities.

The activity of phytase was measured according to a slightly modified procedure as used by McKie & McCleary (2016) by calculating the amount of inorganic phosphorous liberated from the phytic acid solution.

2.4. Screening and optimization of different medium components for enhanced phytase production

2.4.1. Effect of different carbon and nitrogen sources on phytase production

Different carbon sources e.g. 1% (w/w) glucose, maltose, fructose, glycerol, sucrose, starch, and nitrogen sources i.e. 0.5% (w/w) urea, yeast extract, tryptone, malt extract, peptone, $(NH_4)_2SO_4$, CH_3COONH_4, NH_4NO_3, $NaNO_3$, and NH_4Cl were studied for their effect on enhanced phytase production.

2.4.2. Effect of various concentrations of medium components on phytase production

Influence of different concentrations of medium ingredients i.e. NH_4NO_3, KCl, $MgSO_4.7H_2O$, and $FeSO_4.7H_2O$ on phytase production was studied and the suitable concentration level of each ingredient was determined. The concentration of each component was used in the range of 0.025% to 0.15% (w/w), except for NH_4NO_3 with concentration varying from 0.25% to 1.5% (w/w). All experiments were conducted in triplicate by changing the concentration of one medium component at a time but keeping the concentration of others constant.

2.5. Biotechnological application of phytase as a poultry feed supplement

Basal feed containing phytase as an additive was given to experimental birds group compared to control group (without external phytase) and effect of phytase on the growth performance of broilers was investigated (a preliminary experimental work was revealed in an early published study).

2.5.1. Birds, housing, and experimental design

For this purpose, a feeding trial of 5-week for broiler chicken was performed with four dietary treatment groups i.e. T0, T1, T2, and T3, as shown in Table 1. One day old, 40 broiler chicks (Cobb) were purchased from the Punjab chicks hatchery, Lahore, Pakistan. The chicks were at random separated into four groups such as T0 (control group), T1, T2 & T3 (experimental groups) containing 10 birds each based on given feed (Table 1).

Table 1. Feed supplementation groups of experimental birds

Groups	Supplementation (Phytase @ IU/Kg basal feed)	No. of chicks
T0	0.00 (basal feed without phytase)	10
T1	1000	10
T2	2000	10
T3	3000	10

The feeding experiment of broiler chicks was conducted in a clean room at PCSIR Laboratories Complex, Lahore. A digital thermometer was used to monitor the temperature and humidity. The temperature was retained at the beginning of the experiment at 33°C and then progressively decreased to 24-25°C by lowering 3°C each week. The relative humidity of 50-60% and room temperature 24-25°C were maintained during the remaining period of the experiment. Throughout the experimental period of 35 days, the light was continuously provided. Fresh water and feed were given to the birds of each group ad libitum. Feed intake (FI), Body weight gains (BWG), and feed conversion ratio (FCR) of broilers was determined at the weekly interval. The animal experimental procedure was done according to approved protocols of the Animal Ethics Committee, Govt. College University, Lahore.

FCR was calculated by applying the following formula:

$$\text{Food conversion ratio (g/g)} = \frac{\text{Total Feed intake (g)}}{\text{Total weight gain (g)}}$$

2.5.2. Formulation of diets for the growth of broiler chickens

Different components of basal diets given to control and experimental groups of chicken birds are shown in Table 2. Phytase was mixed in liquid form to the feed and given to experimental birds.

Table 2. Composition of experimental diets (g/100 g) for chicks

Ingredient composition (%)	Starter feed (0-3 weeks)	Finisher feed (4-5 weeks)
Maize	60.2	65.65
Soybean meal	30	24
Corn gluten meal	2.5	3.0
Soybean oil	2.8	3.0
Limestone	1.2	1.2
Dicalcium phosphate (DCP)	1.7	1.6
Vitamin mineral mix	1	1
Salt	0.3	0.3
Lysine	0.15	0.15
Methionine	0.15	0.1

2.6. Statistical analysis

A completely randomized design (CRD) was set up to conduct all experiments with three replicates. Analysis of variance (ANOVA) of all parameters was computed using COSTAT computer package (CoHort Software, 2003, Monterey, California).

3. Results

During the present research work, optimization of different medium components was carried out for maximum phytase production from *Aspergillus niger*. The obtained phytase was then used as poultry feed additive.

3.1. Effect of different carbon sources on phytase production

The fermentation medium was amended separately with various carbon sources i.e. (1% w/w) fructose, maltose, glucose, sucrose, starch, and glycerol. The results indicated that different carbon sources showed a different effect on phytase production, whereas, glucose gave maximum phytase yield such as 213.29±3.92 IU/g compared to the control (without any external carbon source) i.e. 198.66±2.59 IU/g, as shown in Figure 1.

3.2. Effect of various nitrogen sources on phytase production

Different nitrogen sources i.e. 0.5% (w/w) peptone, urea, tryptone, yeast extract, malt extract, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, CH_3COONH_4 and $NaNO_3$ were employed in growth medium to obtain higher yield of phytase. It was exhibited by the results that enhanced production of phytase (241.79±5.84 IU/g) was obtained when NH_4NO_3 was supplemented in a culture medium (Figure 2) and $NaNO_3$ produced the second-highest phytase yield (240.28±3.59IU/g).

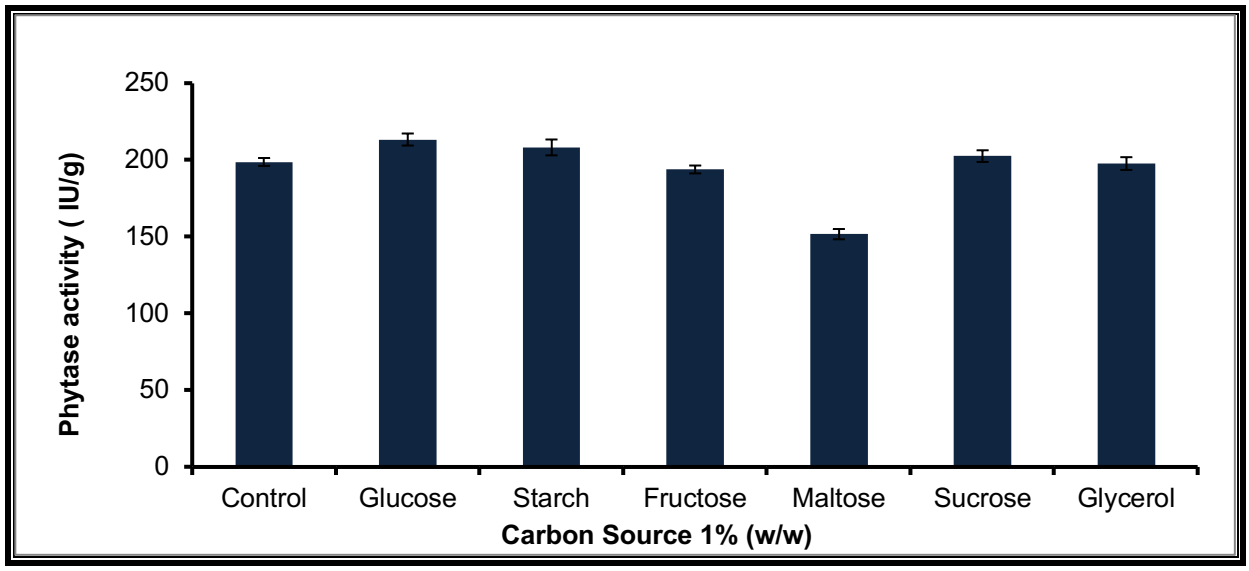

Fig. 1. Effect of different carbon sources on phytase production. Bars represent standard errors.

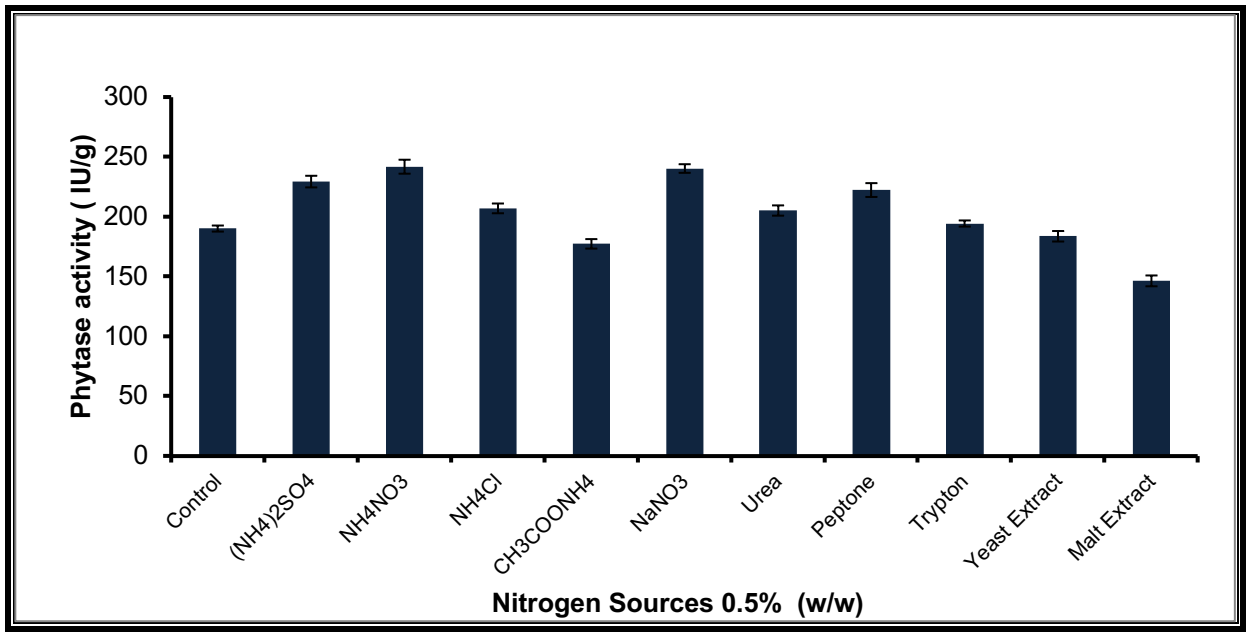

Fig. 2. Effect of various nitrogen sources on phytase production. Bars represent standard errors.

3.3. Effect of different concentrations of NH_4NO_3

Various conc. of NH_4NO_3 ranging from 0.25% to 1.5% (w/w) were applied in the culture medium over the control (without any external source of NH_4NO_3) to determine the optimum concentration for the maximum yield of phytase. The highest phytase production (252.28±5.29 IU/g) was observed at 0.5% NH_4NO_3 (Figure 3). A decrease in enzyme production was noted at lower or higher concentrations of NH_4NO_3 other than the optimum.

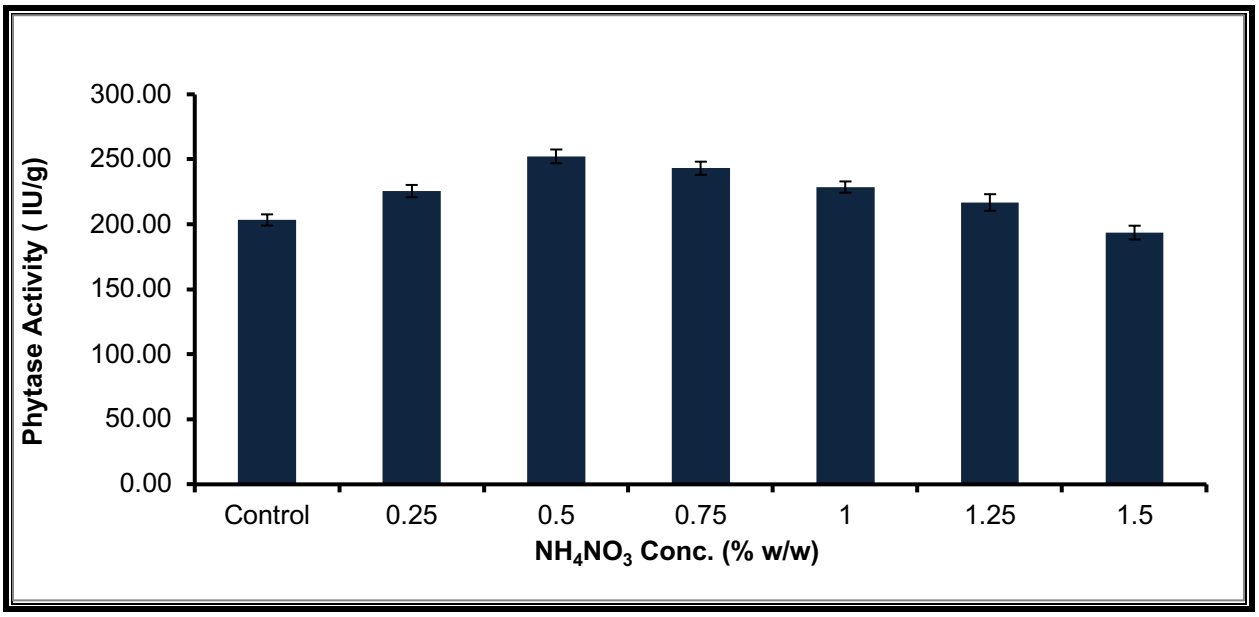

Fig. 3. Effect of various conc. of NH_4NO_3 on phytase production. Standard errors are shown.

3.4. Effect of different concentrations of KCl

The influence of various amounts i.e. 0.025% to 0.15% (w/w) of KCl in the growth medium was studied for the optimum yield of phytase. The growth of the fungus is slightly supported due to the presence of KCl in the culture medium as indicated by the increase in enzyme production. It was reported that 0.1% (w/w) KCl conc. gave the maximum production of phytase (263.23±5.12 IU/g) as shown in Figure 4.

3.5. Effect of different concentrations of $MgSO_4.7H_2O$

Various quantities of $MgSO_4.7H_2O$ such as 0.025% to 0.15% (w/w) were utilized in the growth medium for increased synthesis of phytase. The results revealed that phytase production was maximum (259.78±5.23IU/g) in the presence of 0.1% (w/w) $MgSO_4.7H_2O$, as represented in Figure 5.

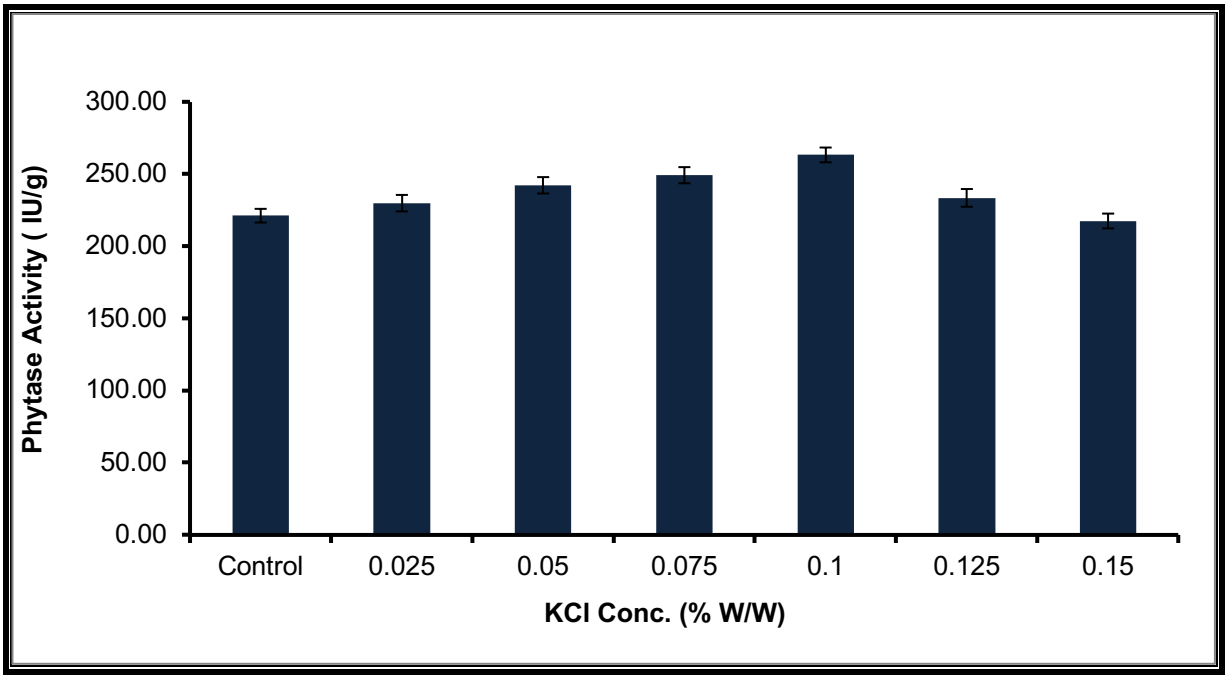

Fig. 4. Effect of various conc. of KCl on phytase production. Standard errors are exhibited.

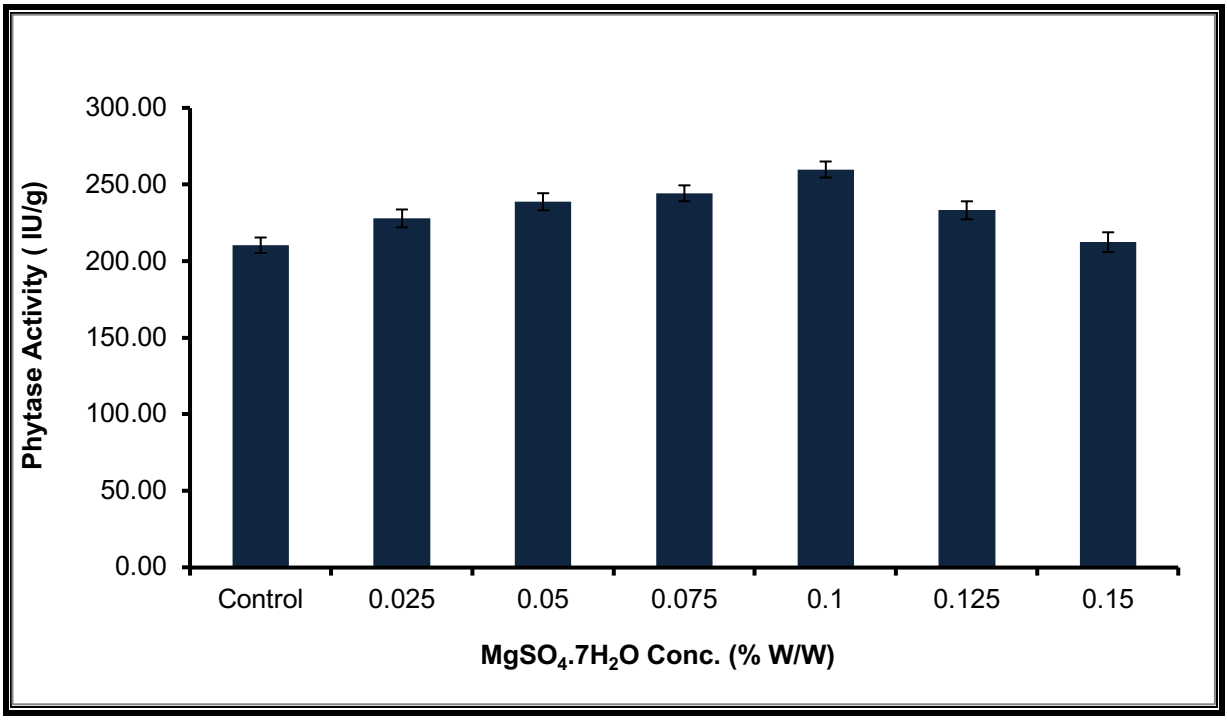

Fig. 5. Effect of various conc. of $MgSO_4.7H_2O$ on phytase production. Standard errors are given.

3.6. Effect of different $FeSO_4.7H_2O$ concentrations

The influence of Fe++ ions on phytase yield was investigated by amending the cultivation medium with different concentrations (0.025% to 0.15% w/w) of $FeSO_4.7H_2O$. Among all the concentrations tested, 0.1% (w/w) $FeSO_4.7H_2O$ gave better phytase production i.e. 265.12±5.51 IU/g (Figure 6).

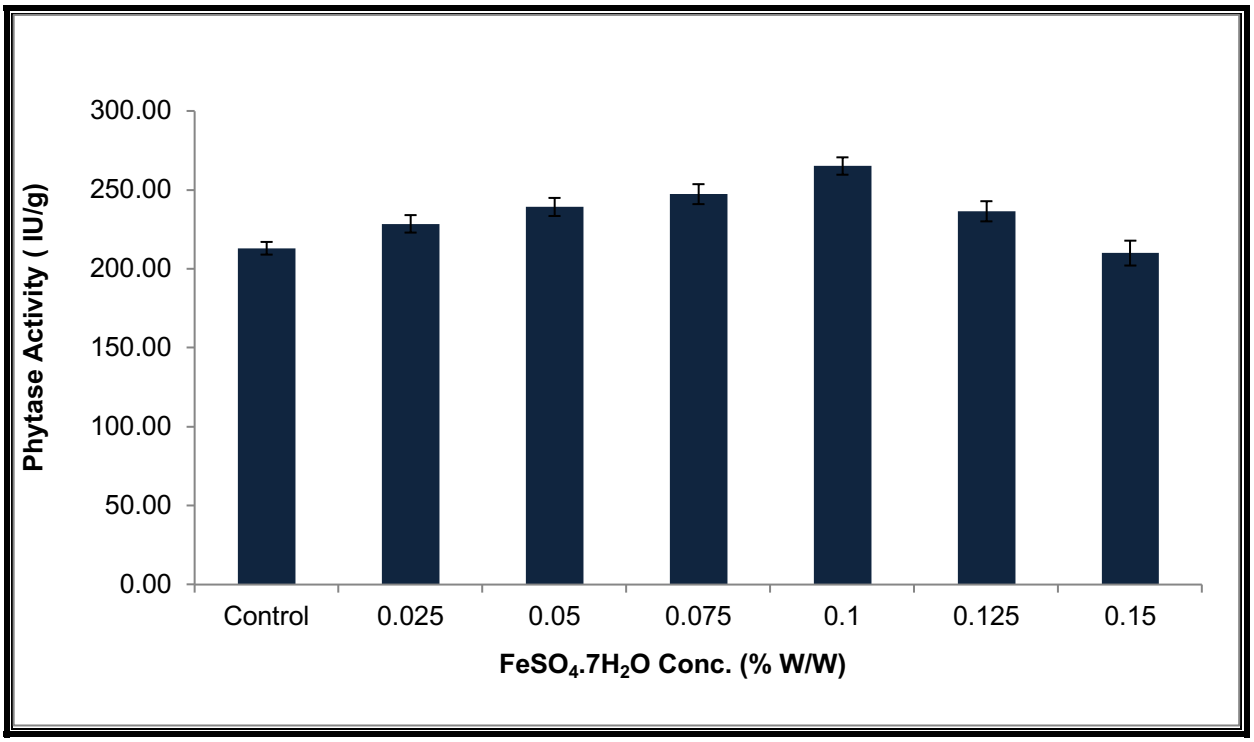

Fig. 6. Effect of various conc. of $FeSO_4.7H_2O$ on phytase production. Standard errors are indicated.

3.7. Impact of phytase on the growth performance of broiler chickens

Feed intake (FI) and BWG (Body weight gain) for broiler chicks were determined after every week. Then feed conversion ratio (FCR) was estimated as described in Table 3 & 4. The results showed that during the first three weeks of the experiment, the chicks kept in group T3 fed on starter feed exhibited the highest body weight gain (905 g) and feed intake (1240 g), whereas the highest food conversion ratio (1.40 g/g) was with group T0 in comparison to other groups (Table 3). After 3rd week, birds were grown on a finisher diet for the next two weeks. After five-week of the feeding trial, the results indicated that maximum feed intake (3240 g) and body weight gain (2090 g) was shown by the group (T3) of experimental birds compared to others (Table 4).

Table 3. Growth performance characteristics of broiler chickens after 0-3 weeks

Groups	Bodyweight gain (g/bird)	Feed intake (g/bird)	Feed conversion ratio (g/g)
T0	780±2.95	1092±3.18	1.40±0.0055
T1	840±4.51	1138±5.93	1.35±0.0046
T2	869±3.65	1190±4.89	1.37±0.0033
T3	905±5.55	1240±6.77	1.37±0.0023

Table 4. Growth performance characteristics of broiler chickens after 0-5 weeks

Groups	(Bodyweight gain) g/bird	(Feed intake) g/bird	(Feed conversion ratio) g/g
T0	1903±6.85	3114±12.81	1.64±0.0027
T1	1970±7.57	3160±12.13	1.60±0.0023
T2	2028±8.21	3200±13.03	1.58±0.0026
T3	2090±10.07	3240±15.31	1.55±0.0040

Each value in the table 3 & 4 is an average of ten replicates, ± indicates standard error T0 = Control group (Basal feed without phytase), T1, T2, T3 = Experimental groups (Basal feed with 1000 IU, 2000 IU, 3000 IU phytase per Kg diet)

4. Discussion

For the improved phytase production from *Aspergillus niger*, selection and optimization of different medium components were conducted under solid-state fermentation. The phytase enzyme produced during the present study was then applied as a supplement in the diets of broiler chickens.

The production of any cultivation is affected by medium composition and process parameters. The earlier studies indicated that the yield of phytase was affected by many physico-chemical parameters i.e. composition of culture medium, nitrogen and carbon sources, different minerals, the type of microorganism, growth of the cell, cultivation methods, size of inoculums, incubation period, temperature and pH of the medium (Suleimenova *et al*., 2016).

During the present study different carbon (1% w/w) and nitrogen (0.5% w/w) sources, were tested for higher phytase production. The results revealed that 0.5% (w/w) NH_4NO_3 produced the best phytase yield (252.28±5.29 IU/g) as shown in Figure 3. Many researchers had obtained similar results that NH_4NO_3 was proved as best nitrogen source and showed the highest production of phytase by *Rhizopus sp*. And *Aspergillus niger* (Sandhya *et al*., 2015; Suresh & Radha, 2016; Tian & Yuan, 2016). Figure 1 showed that glucose 1% (w/w), among different carbon sources, gave a maximum phytase yield (213.29±3.92IU/g). Glucose was investigated as

an appropriate carbon source for enhanced phytase production during many earlier fermentation studies (Buddhiwant *et al*., 2016; Qasim *et al*., 2017).

Different inorganic salts ($MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$) were also added in culture medium in order to test their effect on phytase production. Figure 4, 5 & 6 showed that the production of phytase is improved due to utilization of these salts. An experiment was conducted by Mahmood *et al*. (2021), in which a better amount of phytase was produced by *Aspergillus niger*, when fermentation studies were carried out in a medium comprising rice polish, 0.5% NH_4NO_3, 1% Glucose, 0.1% each of KCl, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ using solid-state fermentation.

Biotechnological application of phytase enzyme was performed as poultry feed additive and the effect of phytase was investigated on the growth of broilers. For this purpose, one control group (T0) without phytase in the basal feed and three experimental groups (T1, T2, T3) with varying concentrations of phytase in the basal feed were made (Table 1). It was revealed by the results that the average body weight of chicks related to experimental groups was higher compared to the control group and it was highest for experimental group T3 (with phytase @ 3000 IU/Kg diet) compared to the other three groups during the 5 weeks of chicks growth (Table 3 & 4).

Ajith *et al*. (2018) experimented with the submission of phytase as poultry feed additive and the growth characteristics of broilers were monitored. The study indicated that better growth performance for broilers was achieved by supplementing their feed with 500 FTU/kg phytase and 0.8% calcium in the diet, with decreased phosphorous excretion in the surrounding environment.

For the biotechnological application of phytase, the effect of phytase was studied on the growth performance of broilers by pre-treating corn-soya diets with microbial phytase. The results showed that there was an increase of the availability of 60% inorganic phosphorus when phytase obtained from microorganisms was provided to the broilers fed on diets containing less phosphorous. There was a decrease of 50% phosphorous in the chickens manure. The results also indicated the 5.8-13.2% increase in body weights of chickens, after 21 days of phytase supplementation in their diets (Jatuwong *et al*., 2020).

5. Conclusion

In the current study, the production of phytase by *Aspergillus niger* was investigated. The optimization of different medium ingredients was carried out using solid-state fermentation. The optimized conditions were determined as glucose (1%), KCl (0.1%), NH_4NO_3 (0.5%), $FeSO_4.7H2O$ (0.1%) and $MgSO_4.7H_2O$ (0.1%). Under optimized culture conditions, the maximum production of phytase was recorded as 265.12±5.51 IU/g. It was further reported that the incorporation of phytase in poultry diets enhances the body weight gain (BWG) of broilers (2090 g) belonging to the experimental group (T3) in comparison to the control group (T0) (1903 g). The biotechnological studies of phytase as poultry feed additive indicated its beneficial effect

on the growth performance of poultry birds. Collectively, all these results may justify the possibility of the production of phytase by *Aspergillus niger* and its utilization as a supplement in poultry diet formulations.

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