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Exploiting Aspergillus terreus for the Production of Thermotolerant Phytase

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ABSTRACT Animal feed is primarily composed of plant parts that contain phytic acid, *Myo*-inositol hexakisphosphate. It serves as a phosphorus source for animals. However, it also chelates the divalent and trivalent essential minerals making them unavailable to the animals. To overcome this antinutritive effect it is proposed to fortify the animal feed with phytase to hydrolyze the phytic acid. The present study is aimed at the production of thermotolerant phytase so that it can withstand the higher temperature employed for the animal feed pelleting. The present study aimed to isolate thermotolerant phytase-producing microorganisms, economize, and optimize enzyme production using agro-waste as a source of phytate. The thermotolerant phytase producing *Aspergillus terreus fsp-4* employed in the present study exhibited the highest activity at 40°C which is the body temperature of poultry as well as 80°C, the temperature employed in feed pelleting.

INTRODUCTION

The oilseeds, legumes, and cereals; serve as a source of nutrients for animals also the major source of phosphorous in the form of phytic acid (Han et al.1987; Bae et al.1999; Chang et al. 2004; Joseph and Paulraj 2007; Jatuwong et al. 2020).

Phytic acid contributes about 60-85percent of total phosphorus in plant seeds (Cosgrove 1966; Graf 1983; Reddy et al. 1989). Phytic acid is chemically m*yo*-inositol 1, 2, 3, 4, 5, 6 hexakis-dihydrogen phosphate. It is a simple carbohydrate where each carbon binds with phosphate groups (Shamsuddin 2002).

Monogastric animals like pigs, poultry, fish are unable to consume the phytate phosphorus from the animal feed because they lack gastrointestinal phytases. Phytates are antinutritive as they chelate important divalent cations making them unavailable to the animals (Reddy et al. 1989). Over a wide pH range, phytic acid can interact with proteins and form a protein-phytate complex which reduces their availability to proteases resulting in inefficient digestion (Kumar et al. 2010).

Therefore, the animal feed must be fortified with inorganic phosphorus to fulfill their nutritional demand, which increases feed cast and also add to phosphorus pollution (Naves et al. 2012; Dersjant-Li et al. 2015). To alleviate this problem the animal feed is fortified with phytic acid hydrolyzing enzyme phytase that involves in sequential hydrolysis of phytic acid into lower *myo*-inositol phosphates and inorganic monophosphate, free *myo*-inositol, thus eliminating the anti-nutritional effect of phytic acid (Vohra and Styanarayana 2003).

Phytase activity has been reported in various microorganisms viz. bacteria, fungi, yeast, actinomycetes, and in plants as well as in animal tissue.

Objective

The present study was aimed to isolate efficient fungi for the production of thermotolerant phytase which has maximum activity at 40°C (body temperature of broiler chicken) with retention of activity at 80°C (the feed pelleting temperature). Optimization of nutritional and physical parameters for efficient phytase production by the selected proficient fungal isolate. Economic production of phytase using agro-residues for its application as a poultry feed additive.

MATERIAL AND METHODS

Sample Collection

Seventy-nine soil and litter samples were collected in sterile polyethene bags using sterile spatula from poultry shed, cattle shed, goat shed, and garden. Collected samples were dried

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at 40°C for 2 hrs in a hot air oven and stored at room temperature.

Isolation of Fungi for Phytase Production

One gram of each sample was diluted serially. 10^{-4}-10^{-10}dilutions of each sample were spread on potato dextrose agar plates, and incubated for 5-7 days at 40°C. All the morphologically different colonies were maintained as pure culture and stored at 4°C for further use.

Qualitative Screening for the Potential Phytase Producing Fungal Isolate

All the fungal isolates isolated from samples were spot inoculated on WEM (Wheat extract mineral Medium) consisting of 0.04% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$, 0.1% Casein, 0.05% KH_2PO_4, 0.04% K_2HPO_4 in wheat bran extract and autoclaved at 121°C for 15 minutes (Powar and Jagannathan, 1982) (Chunshan et al. 2001; Mittal et al. 2011). The plates were incubated at 40°C for 5-7 days, the colonies showing zone of hydrolysis were primarily selected as phytase producers. Phytase production was confirmed by growing them on PSM (Phytase Screening Medium) consisting Glucose 1.5 %, $(NH_4)_2SO_4$, 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 5 (Chunshan et al. 2001) at 40°C for 5-7 days. The phytase-producing colonies were identified by the phytate-specific staining method. The plates were flooded with 2% (w/v) aqueous cobalt chloride for 5 min at room temperature. It was discarded and again the plates were flooded with the fresh solution containing a 1:1 volume of 6.25% (w/v) aqueous solution of ammonium molybdate and 0.42% (w/v) aqueous ammonium metavandate, allowed to react and discarded after 5 minutes. The plates were observed for the presence of a zone of hydrolysis (Bae et al. 1999 and Vijaysai et al. 2011). Isolates with a zone of hydrolysis were selected as phytase producers and were preserved on WEM slants for further studies.

Quantitative Screening for the Potential Phytase Producing Fungal Isolate

Fungal isolates producing phytase were further screened based on quantitative phytase production. The isolates were inoculated in PSM with 2% of inoculum size and incubated at 40°C, 72 hrs. The cell-free crude enzyme was obtained using Whatman filter paper no.1 followed by centrifugation at 4°C, 10000 rpm for 10 min.

Phytase Assay

The reaction mixture consisted of one hundred (100) ìl of the crude enzyme with 600 µl of 0.2% w/v sodium phytate in acetate buffer (0.1 M, pH 5.5) as a substrate for 30 min at 40°C (Bajaj and Wani 2011). 750 µl of 5% trichloroacetic acid solution was added to stop the reaction. Spectrophotometric estimation of the phosphate was carried out at 700nm according to Fiske and Subbarow method (Bajaj and Wani 2011; Fiske and Subbarrow 1925). One unit of phytase (FTU) is defined as the amount of enzyme that liberates 1 µmol of phosphate per min under the assay conditions.

Temperature Based Screening of Fungal Isolate

Seven fungal isolates with the highest phytase activity were considered for temperature-based screening at 30°C -90°C with 10°C interval. The two most efficient fungi showing the highest activity at 40°C and retained the activity at 80°C were selected for further studies.

Screening of Agroresidues as Inexpensive Phytate Source for Phytase Production

Agroresidues are the by-product either produced at the time of harvest in the field or produced during the processing of the crop. These agro residues can be the source of proteins, carbohydrates; minerals, etc thus can be employed as a crude substrate for the production of various bioactive compounds. It is inexpensive abundantly available which reduces the production cost and leads to bioconversion of the agrowaste to the useful product too. Twenty-five different agro residues were collected from the local market. All the agro residues were dried and ground into 30 mesh size particles. The agro residues collected for the present study are enlisted in Table 1.

Table 1: Agro residues screened for phytase production

S. No.	*Agro residue*	*Abbreviation*	*Botanical name*
1.	*Tur* with cotelydon	T	*Cajanus cajan*
2.	*Tur* hull	TH	*Cajanus cajan*
3.	*Bajra* bran	B	*Pennisetum glaucum*
4.	*Rape seed* meal	RH	*Brassica napus*
5.	Deoiled soya meal	S	*Glycine max*
6.	*Corn* meal	CM	*Zea mays*
7.	*Cotton* seed oil cake	CC	*Gossypium hirsutum*
8.	Deoiled *rice* bran	RB	*Oryza sativa*
9.	*Linseed* flour	L	*Oryza sativa*
10.	*Parijat* seeds	P	*Nyctanthes arbor-tristis*
11.	Lemon peel	LP	*Citrus limon*
12.	Orange peel	OP	*Citrus sinensis*
13.	*Sunflower* hull	SH	*Helianthus annuus*
14.	Karanj cake	KC	*Millettia pinnata*
15.	*Mung* bran	MB	*Vigna radiata*
16.	*Black gram* bran	BGB	*Vigna mungo*
17.	Deoiled neem cake	NC	*Azadirachta indica*
18.	*Groundnut* cake	GC	*Ararchis hypogaea*
19.	*Jowar* bran	JB	*Sorghum vulgare*
20.	*Sesame* flour	SF	*Sesamum indicum*
21.	*Sugar cane* baggasse	SC	*Sacharum officinarum*
22.	*Nachani* bran	NCN	*Eleusine coracana*
23.	*Corn* comb	C	*Zea mays*
24.	*Kardai* cake	Kr	*Carthamus tinctorius*
25.	*Coconut* meal	COM	*Cocos nucifera*

Screening For Selection of Efficient Combination of Microbial Isolate and Agroresidues

All the agro residues were screened for the production of fungal phytase. Each of the 25 agro residues were mixed in 2% concentration with basal mineral medium containing 0.1% NH_4NO_3, 0.05% $MgSO_4$,$7H_2O$, 0.05% KCl, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 5.5 (Mittal et al. 2012) and autoclaved at 121°C for 20 min. The medium was inoculated with 4% (v/v) each of fungal isolate (fsp-4, fsp-16 with CFU 176 x 10^8) and incubated at 40°C for 72 hrs. The best combination of agro residue and fungus showing maximum phytase production at 40°C and retained its production efficiency even at 80°C was maintained on WEM slants and selected for further studies.

Identification of Efficient Phytase Producer

The efficient phytase-producing fungi fsp-4 was identified by means of microscopic analysis and ITS gene sequencing and phylogenetic analysis.

Location of Phytase Enzyme in the Fungi

For locating verify whether the enzyme was intracellular or extracellular the fungal biomass was obtained on filtration of the fungal broth after incubation. The fungal biomass was then suspended in 15 ml cold acetate buffer of pH 5.5. The fungal suspension was sonicated (11 s cycles of overall 5 min with 2 min resting in an ice bath). After sonication, centrifugation of the homogenate culture was carried out for 15 minutes at 6500 rpm at 4°C. The phytase activity was determined using the cell-free extract (Klimek- Ochab et al. 2011).

Comparative Study of Fermentation Technique for Phytase Production

The production of fungal phytase was carried out using three different fermentation procedures viz. solid-substrate, liquid submerged, and liquid surface fermentation.

Solid-State Fermentation (SSF)

Solid substrate fermentation was performed by moistening 5 gm of selected agro residue with 10 ml of basal mineral medium containing 0.1% NH_4NO_3, 0.05% $MgSO_4$, $7H_2O$, 0.05% KCl, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 5.5. The substrate was autoclaved (121°C for 20 min.) in a 250 ml flask allowing sufficient surface area for growth. The sterilized solid-state fermentation medium was inoculated with 4% of spore suspension (CFU 168 x 10^7) of potent fungal strain fsp-4 and incubated at 40°C for 72 hrs.

For extraction of phytase, the fermented substrate was mixed with 10 ml of sterile distilled water agitated at 150 rpm for 1 hr. The crude enzyme was obtained by filtration and centrifugation to remove the suspended solids, at 10000 rpm for 15min at 4°C. Phytase activity and soluble protein were analyzed (Esakkiraj et al. 2010; Papagianni et al. 2000).

Submerged Fermentation (SmF)

Submerged fermentation was performed in a 250 ml flask containing 100 ml of a mineral medium comprising of 2% agro residue. 4% of spore suspension (CFU 168 x 10^7) of fungal isolate fsp-

4, was inoculated and the flask was incubated at 40°C for 72 hrs. After incubation the fermented broth was filtered, followed by centrifugation at 10000 rpm for 15 minutes. The phytase activity of the crude enzyme was determined.

Optimization of Process Parameters for Increased Phytase Production

The cultural conditions for the production of phytase were optimized by varying one variable at a time (OVAT). The medium used consisted of Rice Bran, 2%; NH_4NO_3,0.1 %; $MgSO_4$,$7H_2O$, 0.05%; KCl, 0.05%; $FeSO_4$, 0.001%; $MnSO_4$, 0.001%; pH 5.5 At each step of the experiment, phytase activity was assayed and the biomass was determined in terms of dry weight. The process variables used as shown in Table 2.

Inoculum (Spore Suspension) Preparation

The potential isolate *A. terreus strain fsp-4* was inoculated on slants of WEM medium and incubated at 40°C for 72 hrs. After incubation, the spores were aseptically scrapped in 10 ml of sterile saline and stored at 4°C for further use.

RESULTS AND DISCUSSION

Enrichment, Isolation, and Screening of Phytase Producing Fungi

On enrichment and isolation total of 49 fungal isolates were obtained. Based on the zone of hydrolysis on the WEM medium, 34 fungal isolates were selected. The zone of hydrolysis on WEM might be due to the organic acids produced by microorganisms therefore the phytase activity was further confirmed on PSM medium. The isolates showing the ability to produce phytase were subjected to secondary (quantitative) screening using liquid PSM. The phosphate released by phytase-mediated hydrolysis was estimated by the improved Fiske and Subbarao method (Bajaj and Wani 2011; Shimizu 1992). Seven fungal (fsp-2, fsp-4, fsp-13, fsp-16, fsp-30, fsp-32, fsp-37) showing highest phytase activity were selected for temperature-based screening for thermotolerant phytase production (Table 3).

Amongst these two fungal isolates (fsp-4, fsp-16) were selected on the basis of higher phytase activity at 40°C and retention of the enzyme activity at 80°C, for further studies. The selected isolates exhibiting zone of hydrolysis at 40°C are shown in Figure 1.

Table 2: Various process variables optimized as OVAT

S. No.	*Optimized condition*	*Variation*	*Other conditions*
1.	Inoculum age	Inoculum age 2 days to 12 days	4% inoculum, 6 days incubation at 40°C
2.	Incubation Time	1 day to 12 days	4% inoculum, 7day old culture, 6 days incubation at 40°C
3.	Inoculum Size	2, 4, 6, 8, 10, 12, 14% (v/v)	7day old culture, 6 days incubation at 40°C
4.	Substrate concentration (Rice Bran*)*	2, 4, 6, 8, 10, 12 and 14% (w/v)	2% inoculum, 7day old culture, 6 days incubation at 40°C
5.	Optimization of pH	pH 3.5 to pH 7.0	2% inoculum, 6% rice bran, 7day old culture, 6 days incubation at 40°C
6.	Incubation temperature	30-70°C	2% inoculum, 6% rice bran, pH 4.5, 7day old culture, 6 days incubation at 40°C
7.	Nitrogen source	$NH_4(SO_4)_2$, $NaNO_3$, KNO_3, NH_4NO_3, NH_4Cl, Peptone, Yeast Extract, Casein, Glycine and Urea in 0.1% concentration.	2% inoculum, 6% rice bran, pH 4.5, 7day old culture, 6 days incubation at 40°C
8.	Carbon source	Sucrose, Maltose, Lactose, Glucose, Starch, Fructose, Galactose, Arabinose, Raffinose, Xylose at 0.1 concentration	2% inoculum, 6% rice bran, pH 4.5, 7day old culture, 0.1 % NH4Cl, 6 days incubation at 40°C
9.	Metal ions	5mM, 10mM, and 15 mM of $CaCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$, NaCl, $CuSO_4$, $ZnCl_2$, $MnCl_2$ and $CdCl_2$.	2% inoculum, 6% rice bran, pH 4.5, 7day old culture, 0.1 % NH4Cl, 0.1% Fructose,6 days incubation at 40°C

Table 3: Screening of fungal isolates on the basis of temperature

Fungal isolate	*Phytase activity (FTU) at*						
	30°C	*40°C*	*50°C*	*60°C*	*70°C*	*80°C*	*90°C*
fsp-2	3357	3339	3447	3455	3426	3568	2458
fsp-4	3740	3735	4313	4417	4010	4039	3333
fsp-13	3529	3542	3645	3787	3708	3939	3756
fsp-16	4144	3622	3557	3729	4007	4226	4097
fsp-30	3548	3727	3722	3621	3730	3763	3916
fsp-32	3131	3174	3217	3346	3251	3309	3413
fsp-37	3333	3727	3609	3333	3711	3703	3333

Source: Current study

Fig. 1. Selected efficient phytase producing fungi
Source: Authors

The temperature-based screening strategy used in the present studies is unique considering the body temperature of the poultry and the temperature employed for the feed pelleting as reported by Bharambe-Chaudhari and Peshwe (2019) for screening of bacterial phytase producers.

Mittal et al. (2011) reported similar isolation of acido-thermophilic extracellular phytase for application in poultry feed.

In similar studies, soil from poultry, cattle shade, and pulses field was also screened by Singh et al. (2013). They isolated 32 microbial colonies on phytase specific agar medium at 28°C. They further screened the isolates qualitatively and quantitatively using phytase specific medium. Various soil, grain, and fruit samples were used by Betancur et al. (2012) for isolation and screening of phytase-producing fungi belonging to diverse genera.

Screening of Agroresidues as a Source of Phytate

To make the enzyme production economical, agro-residues were employed as a source of phytate. The potential fungal isolates were further screened for phytase production using the phytate present in agro residues. It was observed that the agro-residues supported phytase production. It is economic as the pure phytate drastically increases the expenditure of enzyme production. The agro-residues are a cost-effective source of phytate as compared to the commercially used pure phytate for the production of the enzyme.

All the locally available agro residues were exploited for phytase production by fsp-4, fsp-16. Amongst all the combinations of agro residues and selected fungi, fsp-4 showed 103 FTU at 40°C and 133 FTU phytase activity at 80°C. Increased temperature may result in more activation of the enzyme as it is observed in some combinations, however, there was less activity at 40°C. On the basis of results shown in Table 4; fsp-4 showed 100 percent retention of its activity at 80°C with rice bran hence it was selected for further studies.

Nampoothiri et al. (2004) reported thermostable phytase production by *Thermoascus aurantiacus* using wheat bran extract with maximum phytase activity of 152.36 Uml^{-1} after 72 hrs of incubation at 45°C. Bárbara et al. (2019) studied phytase production by *Acremonium zeae B* and *Kuluyveromyces marxianus* using soybean meal, rice bran, cornmeal, wheat bran as crude substrates. The fungi *Acremonium zeae B showed* 0.05Uml^{-1} *and Kuluyveromyces marxianus* showed 0.06 Uml^{-1} phytase activity. isolate fsp-4 showed 100 percent retention of activity at 80°C, it was selected for further studies. Deepika et al. (2018) reported that a 1:1 mixture of coconut oil cake and wheat bran supported the highest 43.56 U/gds phytase production.

Table 4: Phytase activity of selected isolates with various agro residues

Agro-residue	Enzyme activity (FTU)			
	Fsp-4		*Fsp-16*	
	40 °C	*80 °C*	*40°C*	*80 °C*
T	38	78	61	122
TH	20	84	2	91
B	97	47	91	56
R	66	35	0	61
S	31	9	29	37
CM	71	60	11	24
CC	4	9	26	47
RB	**103**	**133**	20	100
L	57	37	64	53
P	117	46	79	107
LP	18	84	42	38
OP	11	55	11	35
SH	11	40	4	17
KC	4	193	43	139
MB	38	261	24	120
BGB	38	196	29	156
NC	50	121	31	108
GC	78	313	9	186
JB	92	86	6	12
SF	57	68	42	9
SC	188	58	196	26
NCN	112	82	35	18
C	87	107	109	48
Kr	24	49	4	62
COM	66	57	12	62

Source: Current study

Studies by Tanruean et al. (2021) also reveal that rice bran supported the highest phytase production by *Thermoasus aurantiacus* strain SL16W.

Identification of Efficient Phytase Fungal Isolate

Macroscopic and Microscopic Characteristics

Beige to cinnamon-colored colonies, with 4-5 cm diameter showing yellow pigmentation on the reverse of the colony on 5-6 days incubation at 40°C was observed on WEM medium. The scanning electron microscopy reveals that the fungal isolate has septate mycelium with a compact conidial arrangement (Fig. 2).

Identification of Efficient Phytase Producer by Molecular Characterization

PCR amplification of the ITS region of fsp-4 was carried out. Analysis of the sequence ob-

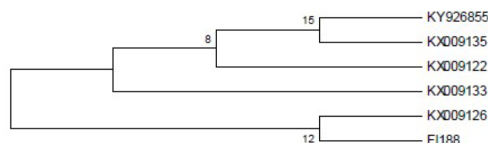

Fig. 2. SEM image of fsp-4
Source: Authors

tained by BLAST was compared with the GeneBank database at BLASTN site at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). The ITS region gene sequence of fungal isolate fsp-4 exhibited 99 percent resemblance to *Aspergillus terreus* KY926855.1. The five hits of homologous sequences of isolate fsp-4 were obtained using NCBI nucleotide sequence database cluster algorithm was used to draw a phylogenetic tree. Further, the phylogenetic tree was generated using MEGA 6.0software (Fig.3).

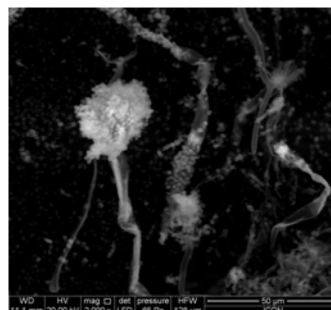

Fig. 3. Phylogenetic tree for the isolate (FI188: Sample)
Source: Authors

Location of Phytase Enzyme in the Isolates

The *A. terreus strain fsp-4* was studied to evaluate whether the enzyme is intracellular or extracellular. The study reveals that extracellular phytase activity is 8.28-fold more than that of intracellular phytase activity (Table 5). Ghareib et al. (1988) studied an intracellular phytase enzyme from *Macrophomina phaseolina* showing activity of 5.60 U. Shah and Trivedi (2012) dem-

Table 5: Location of phytase enzyme

Location of enzyme	*Phytase activity (FTU)*
Intracellular	46
Extracellular	381

Source: Current study

onstrated extracellular phytase production from *Aspergillus timari* with138 U.

Comparative Study of Fermentation Techniques for Phytase Production

Various environmental factors viz. cultural conditions like temperature, pH, substrate concentration, and water activity of the medium, inoculum size can affect the production of the enzyme. Comparative evaluation for a suitable method for high production of phytase by *A. terreus* strain fsp-4 divulges that Liquid surface fermentation supports good production (Table 6). Das and Ghosh (2012) reported the highest phytase production with SmF using *Aspergillus niger* and *A. ficuum*. Similarly, Nampoothiri et al. (2004) reported submerged fermentation most suitable for *Thermoascus aurantiacus* mediated thermostable phytase production.

Table 6: Comparision of fermentation techniques for phytase production

Fermentation technique	*Phytase activity (FTU)*
SSF	195
SF	385
SmF	280

Source: Current study

Optimization of Process Parameters Using One-Variable-At-A-Time (OVAT) Approach

Optimization of Inoculum Age

The growth and production of metabolic products by the microorganism are known to be affected by its inoculum age for fermentation (Ebune et al. 1995). Effect of inoculum age on enzyme production with *A. terreus strain fsp-4* showed maximum enzyme production with seven-day old inoculum. There was a gradual increase in enzyme production from day three to seven as a result of an increase in age and utilization of nutrients for growth and metabolic activities (Table 7). Similarly, the decrease in enzyme production after the seventh day may be due to reduced metabolic activities and depletion of nutrients. Maximum biomass (0.33gm dry weight /100ml) and enzyme production (503 FTU) was obtained with seven-day-old inoculum. Statistical analysis of optimization of inoculum age was carried out using Minitab software revealed that the experiment is in agreement with significance at 95 percent confidence level as the p-value is 0.000.

Table 7: Optimization of inoculum age

S. No.	*Inoculum age (Days)*	*Phyase activity (FTU)*	*Dry weight of fungal mass (gm/100ml)*
1	3	20	0.26
2	4	27	0.21
3	5	33	0.15
4	6	40	0.27
5	7	47	0.33
6	8	53	0.3
7	9	60	0.26
8	10	67	0.25
9	11	73	0.25
10	12	80	0.2

Singh and Satyanarayana (2012) obtained similar results with *Sporotrichum thermophile.* They reported that six day old spore suspension showed the highest phytase production. Shivanna and Venkateswaran (2014) studied phytase production by *A. niger* CFR335 and *A. ficuum*SGA 01and revealed that one and ten day old spore suspension of both the microorganism showed minimum production whereas six day old spore suspension showed the highest enzyme production.

Optimization of Incubation Time

The enzyme production was maximum (585 FTU) with six day incubation period with a gradual increase in biomass (Table 8). Apparently, the production of enzymes was related to the growth of the microorganism, thus the enzyme activity was reported significantly low at the first two days of fermentation. While extending the incubation time also showed a decrease in enzyme activity might be due to depletion of nutrients and/or proteolytic degradation of enzyme. The statistical analysis for optimization of incubation time showed p-value is 0.000, the experiment was significant at 95 percent confidence level.

Tanruean et al. (2021) reported that *Thermoascus aurantiacus* strain SL16W showed maximum phytase production at 12 days of fermentation.

Table: 8 Optimization of incubation time

S. No.	*Inoculum time*	*Phyase activity (FTU)*	*Dry weight of fungal mass (gm/100ml)*
1	1	39	0.02
2	2	156	0.16
3	3	395	0.3
4	4	430	0.31
5	5	460	0.31
6	6	585	0.41
7	7	523	0.42
8	8	505	0.43
9	9	490	0.5
10	10	445	0.54
11	11	419	0.52
12	12	392	0.5

Source: Current study

Alves et al. (2016) reported similar results, using *Muscodor sp.* which showed the maximum phytase production on the sixth day (144 hrs) of incubation with enzyme activity of 26.51U/ml. Research reports of Lee et al. (2005) states five days of incubation results in maximum phytase production using *Aspergillus sp. L117* with a parallel increase in biomass. Yoon et al. (1996) studied phytase production using *Enterobacter sp. 4* and reported that maximum production can be achieved on incubation for 3 days at 37°C. Sreedevi and Reddy (2012) mentioned a similar report of 3 days of incubation for maximum production of phytase by *Bacillus sp*. C43at 37°C.

Optimization of Inoculum Size

Inoculum size has a significant impact on the production of metabolites. A low level of inoculum might not be adequate to achieve the required growth of the fungal isolate for utilization of available nutrient substrate and production of respective metabolite (enzyme). Similarly higher level of inoculum may lead to rapid utilization of nutrients while increased biomass production leads to depletion of nutrients resulting in decreased enzyme production (Roopesh et al. 2006). In the present work, *A.terreus strain fsp-4* showed maximum (433 FTU) production of phytase at inoculum size of 2 percent while the increased concentration of inoculum showed a decrease in phytase released by the microorganism (Table 9). The statistical analysis revealed p-value is less than 0.05, it could be stated that the experiment was significant at 95 percent confidence level. Awad et al. (2011) reported similar results with 2 percent inoculum volume with *Penicillium funiculosum* NR467 giving maximum enzyme production of 74 U/g. Initially increase in inoculum size enhances the enzyme production to achieve the maximum (up to 2ml) however further increase in inoculum size exerts an inhibitory effect on enzyme production.

Table 9: Optimization of inoculum size

S. No.	*Inoculum time (%)*	*Phyase activity (FTU)*	*Dry weight of fungal mass (gm/100ml)*
1	2	433	0.32
2	4	404	0.34
3	6	407	0.25
4	8	405	0.26
5	10	400	0.2
6	12	393	0.18
7	14	401	0.19

Source: Current study

Effect of Substrate Concentration

It was observed that 6 percent rice bran as a source of phytate supported maximum phytase production (531FTU) by *A. terreus strain fsp-4;* however, an increase in substrate concentration adversely affect the production of an enzyme (Table 10). This might be due to the saturation phenomenon of the enzyme. The paired t- test revealed that the experiment was significant at 95 percent confidence level with a p-value < 0.05.

Similarly, Jain and Singh (2016) reported 3 percent rice bran concentration supported the highest phytase production by *Bacillus subtilis subsp. subtilis* JJBS250 followed by a decrease in production with an increase in substrate concentration

Awad et al. (2014) reported corn bran along with corn cob 1:1 ratio improved production of phytase by *Penicillium purpurogenum.*

Optimization of pH

Maximum enzyme production (788 FTU) was obtained at pH 4.5 with corresponding maximum biomass (Table 11). The phytase production, as well as biomass production, gradually decreased on either side of pH 4.5. This might be

Table 10: Optimization of substrate concentration

S. No.	Substrate concentration (gram%)	Phyase activity (FTU)	Dry weight of fungal mass (gm/100ml)
1	2	427	0.3
2	4	518	0.3
3	6	531	0.34
4	8	487	0.26
5	10	456	0.21
6	12	337	0.18
7	14	298	0.12

Source: Current study

due to the acidic environment required by the fungus for its metabolic activity. The experimental model was observed to be significant as the *p*-value was < 0.05, that is, above 95 percent confidence level. Comparative studies on the effect of pH on commercial phytases, two phytases from *E. coli*, one from *A.niger* and one from *P. lycii* phytase in the pH range of 2.0 to 8.5 was carried out by Tran et al. (2010). They reported that phytases from both the variants of *E coli* showed the highest activity at 2.5 furthermore have comparable activities at pH 3.5 and 4.5. Also reported phytase from *A.niger* exhibited maximum activity while the phytase from *P. lycii* exhibited maximum activity at pH 4.5.Tran et al. (2011).

Table 11: Optimization of pH

S. No.	pH	Phyase activity (FTU)	Dry weight of fungal mass (gm/100ml)
1	3.5	507	0.23
2	4	518	0.3
3	4.5	788	0.45
4	5	563	0.32
5	5.5	558	0.25
6	6	460	0.24
7	6.5	418	0.13
8	7	354	0.12

Source: Current study

Optimization of Incubation Temperature

A. terreus strain fsp-4 demonstrated the highest phytase production at 40°C, and a gradual decrease in phytase production at 50°C, 60°C, 70°C (Table 12). Various studies revealed that fungal strains showed the highest production of phytase over a wide range of temperatures 27-50°C.

Suliasih et al. (2022) reported the highest phytase production by *Bacillus altitudinis* at 35°C. Thakur et al. (2017) reported maximum production of phytase by *Aspergillus fumigatus* at 40°C as compared to other temperatures in the range of 25°C to 50°C. Optimization studies on the production of phytase by isolating *Bacillus* CH3-1 were carried out by Khianngam et al. (2017). They reported that the production of phytase (20.59 U/ml) was obtained at the optimum temperature of 45°C.

The studies by Salmon et al. (2016) revealed that *Ganoderma sp.* MR-56 showed the highest phytase production (12.8U/ml) at 30°C in a medium containing soybean molasses at pH 6.0. They also reported low phytase production below and above this temperature.

Statistical analysis of the experiment was carried out using the paired t-test was applied. From the t-test, the *p*-value was determined that be <0.05 signifying the experiment.

Table 12: Optimization of temperature

S. No.	Temperature (°C) (gram%)	Phyase activity (FTU)	Dry weight of fungal mass (gm/100ml)
1	30	390	0.32
2	40	568	0.48
3	50	514	0.23
4	60	463	0.19
5	70	464	0.13

Source: Current study

Optimization of Nitrogen Source

Nitrogen has a crucial role in the synthesis of nitrogen contain-ing cellular substances, such as amino acids, purines, DNA, and RNA. It indicates that it is one of the vital components for cell growth and its metabolic activities. Thus nitrogen sources in fermentation were essential nutrients for phytase production, consequently, in the fermentation medium, the influence of different nitrogen sources was studied on the phytase production by *A.terreus strain fsp-4*. The results are as shown in Table 13. It was observed that ammonium chloride as nitrogen source showed highest (609 FTU), followed by

ammonium sulfate, ammonium nitrate comparatively urea, peptone, sodium nitrate, potassium nitrate, casein, glycine, and yeast extract were insignificant for phytase production as well as biomass production.

The results reveal that the fungal isolate prefers an inorganic nitrogen source for enzyme production as compared to organic nitrogen sources. The ammonium salts supported enzyme production as well as biomass production. In the case of sodium nitrate, although biomass production is significant, phytase production was not observed.

Similar results were obtained by Suresh and Radha (2015) they studied different nitrogen sources for the production of phytase by *Rhizopus oligosporus* MTCC 556. They used a 1 percent w/v concentration of ammonium chloride, ammonium sulfate, ammonium nitrate, sodium nitrate, yeast extract, and peptone for this study and reported that comparatively, inorganic nitrogen sources have a significant effect on phytase production. They reported that ammonium sulfate followed by ammonium nitrate, ammonium chloride, sodium nitrate, peptone, yeast extract. Statistical analysis for optimization of nitrogen source was carried out it revealed p-value <0.05.

Table 13: Optimization of nitrogen source

S. No.	*Nitrogen source*	*Phyase activity (FTU)*	*Dry weight of fungal mass (gm/100ml)*
1	$NH_4(SO_4)_2$	555	0.5
2	$NaNO_3$	19	0.45
3	KNO_3	10	0.1
4	NH_4NO_3	521	0.5
5	NH_4Cl	609	0.52
6	Peptone	93	0.12
7	Yeast Extract	7	0.15
8	Casein	9	0.08
9	Glycine	15	0.1
10	Urea	177	0.14

Source: Current study

In et al., (2008) evaluated the effect of various nitrogen sources for the production of phytase using *Saccharomyces cerevisiae* CY and reported that the highest activity (52.66mU/mg) was obtained with ammonium sulfate followed by peptone > yeast extract > potassium nitrate > sodium nitrate supplemented media

Optimization of Carbon Source

Although in production medium agro residue also served as a carbon source for phytase production by *A. terreus strain fsp-4,* various additional carbon sources viz. sucrose, maltose, lactose, glucose, starch, fructose, galactose, arabinose, raffinose, and xylose were used along with the agro residue to achieve enhanced phytase production. The results showed that amongst the various carbon sources studied fructose> lactose> glucose> starch> arabinose> maltose> sucrose> Galactose> Raffinose> xylose was meagerly significant over control (Table 14). Though the biomass of the fungal propagule was developed highest in medium supplemented with glucose and fructose compared to control however the carbon sources did not enhance the enzyme production. It indicates that the agro residue used is itself sufficient to be a carbon source for the production of phytase by *A.terreus strain fsp-4.*

Table 14: Optimization of carbon source

S. No.	*Carbon source*	*Phyase activity (FTU)*	*Dry weight of fungal mass (gm/100ml)*
1	Control	578	0.45
2	Sucrose	423	0.51
3	Maltose	435	0.49
4	Lactose	568	0.5
5	Glucose	562	0.66
6	Starch	557	0.53
7	Fructose	578	0.67
8	Galactose	387	0.29
9	Arabinose	524	0.45
10	Raffinose	346	0.21
11	Xylose	262	0.11

Source: Current study

Esakkiraj et al. (2010) studied nine carbon sources for evaluating their competence to enhance the production of phytase with *Pseudomonas* AP-MSU 2. They reported that fructose showed about a three-fold increase in phytase production with respect to control. Moreover, other carbon sources too significantly improved phytase production over control. Chitturi and Jeevana (2016) studied the effect of various carbon sources in 15 concentrations for phytase production by *Aspergillus niger* and reported that glucose served as an efficient car-

bon source to achieve the highest activity than other carbon sources under study.

The statistical analysis of optimization of carbon source for phytase production was carried out by paired t-test. It was observed that the experimental model of carbon source optimization, significant at a 95 percent confidence level as the p-value was <0.05.

Effect of Metal Ions

Various metal ions were used to study their effect on phytase production by *A.terreus strain fsp-4*. They were added in varying concentrations (5mM, 10mM, 15mM) in the production medium in the form of metal ion salt. 5mM concentration of Mn^{2+}, Mg^{2+} showed the most significant effect on phytase production, but the increased concentration showed inhibitory activity. 10 mM concentration of Na^{+} showed a significant effect. However, Zn^{2+}, Cu^{2+}, Cd^{2+} have an inhibitory effect on phytase production (Table 15). Sreedevi and Reddy (2012) reported a significant effect of 5 mM Ca^{2+} on phytase production by *Bacillus sp.C43* whereas 5 mM Mn^{2+} was observed to have a comparable effect with control. They report that the increased response of enzyme in production medium due to metal ion maybe because of its potency to improve permeabilization of the enzyme else induction of enzyme. However, the inhibitory effect of metal ions may be due to their inhibitory effect on growth, inhibition, or inactivation of the enzyme.

CONCLUSION

The potential fungal isolate obtained in the present study was found to be thermotolerant and produces phytase which is stable at poultry body temperature as well as feed pelleting temperature. The optimization of nutritional and process parameters by the conventional one variable at-a-time approach improved the production of phytase. However, the combined effect of the parameters for enhanced phytase production using statistical models needs to be studied further. The temperature-based approach used in screening the potential isolate is unique in itself to apply the phytase in poultry feed. The agro residues are used as a source of phytate for enzyme production making the process highly economical.

RECOMMENDATIONS

The fortification of animal feed with phytase proves to alleviate the antinutritive effect of phytate on monogastric animals. Phytase used in animal diet formulation can increase the digestibility of nutrients and higher feed utilization by animals thereby increasing the poultry production. The agro residues, the waste from agricultural practices, are a worthy and attractive source of nutrients for the production of enzyme and makes the production cost-effective. Thus, the strategy employed in the present studies will be appropriate to improve poultry

Table 15: Effect of metal ions on phytase production

S. No.	*Metal ion*	*Phytase activity (FTU) at 5 mM*	*Phytase activity (FTU) at 10m*	*Phytase activity (FTU) at 15 mM*	*Dry mass of fungal mass (gm/100ml) 5mM*	*Dry mass of fungal mass (gm/100ml) 10mM*	*Dry mass of fungal mass (gm/ 100ml)15 mM*
1	Control	1050			0.55	0.37	0.4
2	$CaCl_2$	816	1034	988	0.28	0.4	0.38
3	$Mg Cl_2$	1339	1103	1032	0.29	0.45	0.4
4	$CoCl_2$	454	377	562	0.1	0.32	0.29
5	$NiCl_2$	1144	1133	829	0.13	0.34	0.12
6	NaCl	1078	1289	1205	0.38	0.44	0.38
7	$CuSO_4$	206	349	107	0.23	0.39	0.11
8	$ZnCl_2$	508	258	53	0.2	0.2	0.09
9	$MnCl_2$	1443	970	875	0.45	0.3	0.32
10	$CdCl_2$	1031	353	408	0.23	0.2	0.25

Source: Current study

production as well as minimize the associated problem of eutrophication.

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