Review Article

Optimization of culture conditions for phytase producing *Coprinopsis cinerea* using rice bran extract as substrate

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ABSTRACT

Phosphorus is an essential element for vital life processes but is not readily available due to its presence in bound forms. Phytate, the main reservoir for phosphorus in soil and plant materials is not easily digested in monogastric animals due to lack the enzyme phytase which catalyse the hydrolysis of phytase to release phosphorus and other nutrients. This study was aimed at optimization of culture conditions for phytase producing *Coprinopsis cinerea AmutBmutpab* 1-1 isolated from poultry dumpsite in Minna, Niger State. Phytase producing microorganisms were isolated from soil sample collected from a poultry dumpsite in Randan Ruwa, Minna, Niger State, Nigeria. The best phytase producing strains was identified through molecular characterization. Fermentation conditions (incubation time, temperature, carbon source and pH) were optimized for enhanced phytase production. The results showed that *Coprinopsis cinerea AmutBmutpab* 1-1 the best phytase producer with a phytase activity of 642 U/mL at optimum conditions of 3 days of incubation, 3% glucose concentration, pH of 6.5 and temperature of 32.5°C. The result of this study showed that rice bran is a good alternative for microbial phytase production.

Keywords: Phytase, phytate, hydrolysis, solubility, optimization.

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INTRODUCTION

Phytase is a phosphatase enzyme which catalyse the removal of phosphate groups from phytate, a compound recognized as the dominant storage form of phosphorus in both soil and plant tissues [1].

Phytate comprises six phosphate esters of inositol, referred to as IP1 through IP6, with IP6 being the most prevalent, comprising between 83% and 100% of total inositol phosphates [2]. Phytate is characterized by a high charge density, enabling strong interactions with soil minerals.The presence of multiple hydroxyls and oxo groups gives phytate a strong chelating capacity, allowing it to form complexes with mineral cations [3].

These groups also enable phytate and its deprotonated forms to participate in extensive hydrogen bonding, which contributes to their solubility and acidity in water-based environments [2,3].

Organic acids mobilize phytate through three main mechanisms. First, they can displace phosphate by ligand exchange, substituting it with carboxylate groups [4] Second, carboxylates can solubilize metal ions like Fe and Al, which act as phosphorus binding sites. Lastly, they can break down organic matter that complexes with phosphorus via Fe/Al bridges, thereby releasing phosphate from these organo-metallic complexes [3]. Phytate solubilization is further enhanced through metal chelation, which helps free phosphorus from metal-phytate complexes and prevents the microbial degradation of organic acids by forming stable complexes with soil particles [5]. Although microbes are generally effective at degrading organic acids, this process is significantly slowed when these acids are adsorbed onto soil surfaces.

Under various physicochemical conditions, the phosphate ester bonds in phytate are notably resistant to breakdown [6]. The molecule's negative charge facilitates interactions with soil metals, leading to the formation of metal-phytate complexes that significantly influence its solubility [7]. Regarding pH, solubility tends to be greater at pH 5.0 than at pH 7.5. Most metal-phytate complexes, except for those involving aluminum, resist acidic hydrolysis and remain stable under conditions of high temperature and pressure, though calcium phytate is an exception. Across all pH levels, calcium can interact with phytate to form either soluble compounds like Ca_1- and Ca_2-phytate or insoluble forms such as Ca_3-phytate [8].

First identified in 1907, phytases have since been recognized as one of the top ten innovations in agricultural science over the past century [9]. These enzymes, belonging to the phosphatase family, are capable of stepwise removal of phosphate groups from the phytate molecule [10]. Phytases are classified based on their source (plant, microbial, or animal), their optimal pH (acidic or alkaline), and their mode of catalytic action. Despite differences in reaction mechanisms, four main groups of phytases have been identified, each with distinct structural and functional traits: β-propeller phytases (BPPs), protein tyrosine phosphatase-like phytases (PTP-like), purple acid phosphatases (PAPs), and histidine acid phosphatases (HAPhy) [11].

Some phytases are intracellular and may not contribute directly to external phytate degradation, implying that total phytase activity in soil or manure doesn't always reflect the level of phosphorus availability to plants [12]. However, increased phytase activity in the rhizosphere has been associated with improved plant growth under low-phosphorus conditions [3]. This enzymatic activity often reflects the metabolic demands of microbial communities in the soil.

Initially identified in rice bran phytase was known for its participation in the formation of various phosphatidyl inositol derivatives, either as end products or intermediates[13],[14]. Some plant phytases can also be identified as alkaline or purple acid phosphatases [15]. Plant phytase are most active during seed germination as they help to release

phosphorus to meet the plant's nutritional requirements [16].

Another importance of phytases in plants in the prevention of heavy metals translocation such as in the case of *Pteris vittata* PvPHY1, a new root-specific phytase expressed in tobacco which was found to improve growth and P availability by 10%–50% [17]. The optimum temperature range for plant phytase is between 40 to 60°C, however, higher temperatures between 70 – 90°C are associated with phytase inactivation. At temperatures above 80°C such as during pellet formation, plant derived phytases are subject to inactivation [18]. Lack of a sustainable method of enzyme production has been the leading problem associated with the sythesis of plant phytases [16]. Additionally, synthesizing phytase from plants is time-consuming, complicated, and costly and therefore not economically advantageous [19]. By using an optimized substrate, growth conditions, and manufacturing techniques, microorganisms may be employed for mass production. Due to this reason, the generation of microbial phytase is considered the most preferred method [20].

MATERIALS AND METHODS

Study Area

This study was conducted at the Federal University of Technology located at 9°31'15"N to 9°32'30"N latitude and 6°26'15"E to 6°28'00"E longitude, Gidan kwano Bosso, Minna Niger state. the poultry farm is situated at Randan Ruwa in Bosso Local Government Area of Niger State, Nigeria, at coordinates 9.65°North and 6.54°East near the main town of Bosso, which is located at 9°39'12"N and 6°30'58"E. The rice mill is located at Chanchaga (9°36'50"N and 6°33'25"E), Minna

Collection of Soil Samples

Soil sample was collected from poultry farm dumpsite in Randan Ruwa area of Bosso, using a clean spatula at a depth of 5-10 cm into a sterile sample container. The soil sample was transported to the Microbiology Laboratory, Federal University of Technology, Minna. Rice bran was collected rice mill in Chanchaga, Minna in a sterile polyethylene bag and was transported to the Microbiology laboratory FUT Minna.

Pretreatment of Rice Bran

The rice bran was washed with clean water and sun dried after which it was milled into powder using a blending machine[21].

Stabilization of Rice Bran and Lipid Extraction

Hundred (100) grams of bran powder was placed for 1 min in an oven at 40°C. Then, the sample was mixed homogenously and placed in the oven again for another 1 min and then was cooled to room temperature (28ºC $\pm$2). Total lipid was extracted from rice bran samples by adding 400 mL of hexane to the rice bran powder and allowed to stay for 24 h. This was followed by evaporation of rice bran to dryness after which was stored at 28°C until further analysis [22].

Preparation of rice bran extract

One hundred grams (100 g) of rice bran was soaked in 500 mL of distilled water containing 30 mL of 37% HCl. The mixture

was allowed to stand at room temperature (28ºC ± 2) for 2 days after which it was filtered. The pH of the filtrate was adjusted to 6.5 and heated at 80°C in a water bath for 30 minutes and then filtered. The solution was neutralized and refiltered [23]

Determination of Phytate Content of Rice Bran Extract

Phytic acid content in the extract was quantified by spectrophotometry as described by Sahini and Mutegoa [23]. One milliliter (1 mL) of ferric (III) chloride solution was added to 0.5 mL rice bran extract. The solution was heated for 30 min in a boiling water bath. After being cooled to room temperature (28º C±2). The solution was centrifuged for 30 min at 4500 rpm. Then, 1 mL of the supernatant was transferred to another test tube and mixed with 1mL of 2,2'-bipyridine. The absorbance of the reaction mixture was measured at 519 nm against distilled water. The absorbance zwas with standard phytic acid solutions.

Isolation of Microorganisms from Soil Sample

One (1) gram of the soil samples was suspended in 9 mL of distilled water and was serially diluted up to 10^{-7} dilution factor. With the help of a sterile pippete, 0.1 mL each of 10^{-4} and 10^{-6}dilutions was introduced in petri-dishes containing nutrient agar (NA) and Sabouraud dextrose agar (SDA) respectively (spread plate). These were incubated at 37°C for 24 h and 28°C for 5 days respectively after which the plates were observed for growth. All morphologically distinct colonies were purified by subculturing and will be kept in (NA) and SDA slants at 4°C .

For the bacterial isolates, the colonial morphology (size, shape, colour, smell, elevation) of the isolates were observed and the isolates were stained to obtain the Gram's reaction followed by biochemical characterization. The fungal isolate were characterized based on colors of conidia, texture, and sporulation after which they were compared to known taxa for identification [24].

Qualitative Screening of microoganisms for Phytase Production

The qualitative screening was carried out on phytase screening medium according to the method of Qasim *et al*. [24] with little modifications. The phytase screening medium was composed of, ammonium nitrate (0.2%), potassium chloride (0.05%), magnesium sulfate (0.05 %), manganese sulfate (0.03%), ferrous sulfate (0.001%) , agar agar (20g) in 250 mL rice bran extract at pH 6.5. The phytase screening medium (PSM) was inoculated with bacterial and fungal isolates and incubated at 37 °C for 24 hours and 28°C for 3 days respectively Positive isolates were detected through presence of zone of hydrolysis around colony. Isolates which shows zone of hydrolysis on phytase screening agar were confirmed by subculturing on fresh PSM.

Quantitative screening of microoranisms for phytase production

The bacterial and fungal isolates that showed clear zone on PSM were quantitatively screened for the production of phytase by submerged

fermentation. The isolates were cultured in 250 mL Erlenmeyer flasks containing 100 mL phytase screening broth (PSB). An inoculum density of (10^7 spore/mL) was used to assay for the fungal isolates and then incubated at 30°C for 5 days and 150 rpm shaking incubator while a bacterial inoculum of 10^6 (0.5 McFarland standard) was used and incubated at 37°C. The bacterial and fungal cells were separated from the culture broth by centrifugation at 6000 rpm/min and the supernatant obtained was used for phytase assay [25].

Phytase activity was determined using the modified procedure of Mahmood et al. [26]. In this method, 1% (w/v) phytic acid solution and enzyme extract 0.2 mL each was taken in a test tube and incubated at 37°C for 15 min. Then, 0.4 mL of 15% tricholoroacetic acid (TCA) was added to stop the reaction. The above mixture was then incubated at 50°C for 15 min after adding colour reagent (ammonium molybdate). A blank containing 0.2 mL of citrate buffer (0.2 M, pH 5.5) instead of 0.2 mL enzyme extract was run in parallel. A spectrophotometer was used to determine the absorbance of the reaction mixture at 655 nm against blank. One phytase unit is defined as the amount of the enzyme that releases 1µM of inorganic phosphorus per millimeter permin under the standard assay conditions.

Molecular Characterization of Best Phytase Producing Isolate

The isolate with the highest phytase activity was identified through molecular characterization as follows:

Amplification of 18s rRNA genes

Amplification of 18S rRNA gene was carried out in the extracted DNA using Taq Mix (2x) (Master Mix) and universal primers ITS1 (forward) and ITS4 (reverse), targeting the 18S rRNA gene [25].

Gel Electrophoresis:

To verify the success of PCR amplification and check for the presence of 18S rRNA amplicon, agarose gel electrophoresis was performed. A 1.5% agarose gel was prepared. The gel was placed in a gel tank containing X1 TAE buffer. Four (4) uL of the PCR products were carefully loaded into the wells, and the electrophoresis was conducted at 100V, 400 A for 20 mins until the DNA bands migrated sufficiently. The gel was viewed under UV-Transluminator to check for bands [25].

PCR products with the expected size were purified and initially sent for Sanger sequencing using universal primers at Inqaba Biotechnology. The obtained sequencing data were analyzed using bioinformatics tools. Sequences were compared against public databases to identify the fungi. The results were validated based on sequence similarity and taxonomic classification.

Optimization of Fermentation Conditions for Phytase Production

Optimization of fermentation conditions was carried out to determine suitable conditions for optimum yield of phytase. The effect of various process parameters (carbon source, incubation time, pH and incubation temperature) on phytase production were tested as described by Qasim *et al.* [27]. The design for the

optimization studies was generated using the Response surface Methodology (BBK-low, middle and high levels). The interaction effect of fermentation parameters was also determined.

Data analysis

The data obtained was subjected to analysis of variance. The significance of difference will be tested at the significance level p= 0.05. Response surface methodology was used in generating the interaction of fermentation variables on phytase production.

RESULTS

Phytate content and proximate composition of rice bran extract

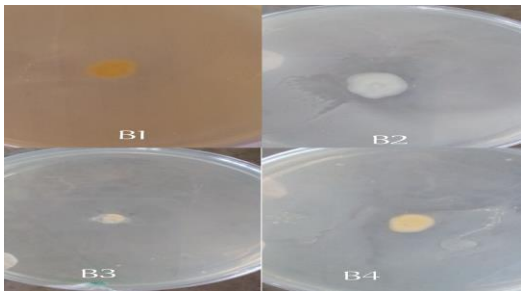
Table 1 shows the phytate content and proximate composition for the prepared rice bran extract. In this study, a phytate content of 22mg/100g of the rice bran used was obtained. The rice bran extract has an ash content of 1.98%, total moisture content was 96.33%, protein was 0.35%, fibre content was 0.06% and carbohydrate content was 1.29%

.Table 1. Phytate content and proximate composition of rice bran extract

Sample	Phytate (mg/mL)	Ash (%)	Moisture (%)	Crude protein (%)	Fat (%)	Carbohydrate (%)
Extract	22.19	1.96	96.35	0.35	0.06	1.28
	22.84	1.97	96.31	0.35	0.06	1.29
Mean value	22.52	1.97	96.33	0.35	0.06	1.29

Isolated and screened bacterial and fungal isolates with Phytase production potential

A total of seven bacteria and three fungi were isolated from soil collected from poultry waste dumpsite. The results for qualitative and quantitative screening of bacteria and fungi isolated from poultry waste dumpsite is represented in Table 2. Four bacteria and two fungi were found to be positive for phytase production. Highest phytase activity was obtained with fungal isolates. Plate 1 represents the screened bacterial and fungal isolates on phytase screening medium showing the zone of hydrolysis.



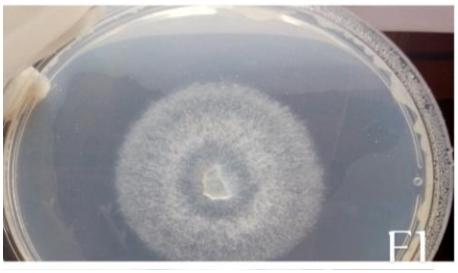


Plate 1: Screened Bacterial and fungal isolates on phytase screening medium B1: *Escherichia* sp, B2:*Bacillus* sp., B3:*Enterobacter* sp., *Pseudomonas* sp. F1: *Coprinopsis cineria*

Table 2: Colony Morphology, Hydrolysis and Phytase activity for Screened Isolates

Isolates/Morphological characteristics	Color	Colony size	Edge	Surface	Texture	Hydrolysis (mm)	Phytase activity(U/mL)
B1	Yellowish	Small	Round	Flat	Creamy	-	32.69
B2	Whitish	Medium	Rough	Flat	Creamy	+	174.00
B3	Orange	Small	Rough	Raised	Dry	-	54.08
B4	Yellowish	Small	Smooth	Flat	Creamy	+	183.14
F1	Whitish	Small	-	Wooly	-	+	564.41

Molecular characteristics of isolates with high phytase activity

Gel Image of PCR products

The gel electrophoresis results for the PCR products obtained from the 18S rRNA gene amplification is represented in figure 1. The lanes 2 the fungal isolate F1 The presence of DNA bands in the lanes confirm the success of the PCR amplification. The ladder lane (lane 1) on the left serves as a size reference.The summary of 18S rRNA sequencing of the fungal isolates is shown in table 3. F1 was identical to *Coprinopsis cinerea* strain AmutBmut pab1-1 (99.78%).

FIGURE 1: Gel Image of PCR products from *Coprinopsis cinerea strain AmutBmut pab1-1*

Table 3: Summary of 18S rRNA sequencing results of the fungal isolates

Sample ID	Sequence bp	Identity of the closest homologs of the sequence	% identity	Accession Number
F1	587	*Coprinopsis cinerea strain AmutBmut pab1-1*	99.78%	OM238134.1

Optimized Fermentation Conditions for Coprinopsis cinerea strain AmutBmut pab1-1

The results of optimization of fermentation conditions for phytase production with *Coprinopsis cinerea* is represented in Table 4. It showed that the highest enzyme activity was achieved at 3 days of incubation, 3% glucose, pH of 6.5 and temperature of 32.5°C. However, an incubation period of 7 days, glucose concentation of 5%, incubation temperature of 32.5 and a pH of 4.75 resulted in a low phytase activity of 20.67 U/mL.

Table 4: optimized fermentation conditions with *Coprinopsis cinerea* strain AmutBmut pab 1-1.

Run	Incubation time (days)	Carbon source(%)	Temperature (°C)	pH	Activity(U/mL)
1	5	5	25	4.75	168.33
2	5	1	32.5	6.5	70.33
3	7	5	32.5	4.75	20.67
4	7	3	25	4.75	41.67
5	5	3	32.5	4.75	120.33
6	5	5	40	4.75	199.67
7	5	3	40	6.5	296.33
8	7	3	32.5	6.5	193.00
9	5	5	32.5	6.5	172.67
10	7	3	32.5	3	147.33
11	7	3	40	4.75	230.00
12	5	1	32.5	3	88.67
13	3	5	32.5	4.75	291.67

14	5	1	25	4.75	35.00
15	3	3	32.5	6.5	642.00
16	5	3	25	3	51.67
17	3	3	40	4.75	38.33
18	3	3	32.5	3	228.67
19	3	1	32.5	4.75	314.67
20	7	1	32.5	4.75	6.67
21	5	3	25	6.5	65.33
22	5	3	32.5	4.75	203.00
23	3	3	25	4.75	59.67
24	5	5	32.5	3	90.33
25	5	1	40	4.75	279.67
26	5	3	40	3	33.00

Table 5 shows the analysis of variance (ANOVA) for the linear model of phytase production. The model's F-value of 1.60 and its associated p-value of 0.2102 indicate that the model is not statistically significant. This implies the variation explained by the model is not large enough to be distinguished from random noise, and the model terms are not useful for predicting the response. Furthermore, the lack-of-fit F-value of 15.59 implies that the lack-of-fit is not statistically significant.

Table 5: Analysis of Variance for Linear Regression Model for Phytase Production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.553E+05	4	38829.57	1.60	0.2102	not significant
A-Incubation time	18486.75	1	18486.75	0.7640	0.3920	
B-Carbon source (gucose)	25791.92	1	25791.92	1.07	0.3136	
C-Temperature	67198.84	1	67198.84	2.78	0.1105	
D-pH	43840.76	1	43840.76	1.81	0.1926	
Residual	5.081E+05	21	24197.09			
Lack of Fit	5.065E+05	20	25325.72	15.59	0.1974	not significant
Pure Error	1624.50	1	1624.50			
Cor Total	6.635E+05	25				

The response surface methodology version 14.0 established the interaction plots between carbon source, pH, and temperature and incubation time as shown below.

The effect of incubation time, temperature, carbon source concentration, and pH on phytase production

Figure 2 shows the effect of incubation time, temperature, carbon source concentration, and pH on phytase. The incubation time and carbon source plots (A and C) show that lower incubation time (3 days) and carbon source concentration (1%) are associated with increase in phytase activity, while higher levels of these factors (7 days and 5% respectively) yielded decrease in enzyme activity. Temperature and pH (plots B and D) on the other hand yielded increase in enzyme activity as their levels increase. Decrease in either of these factors yielded a decrease in enzyme activit

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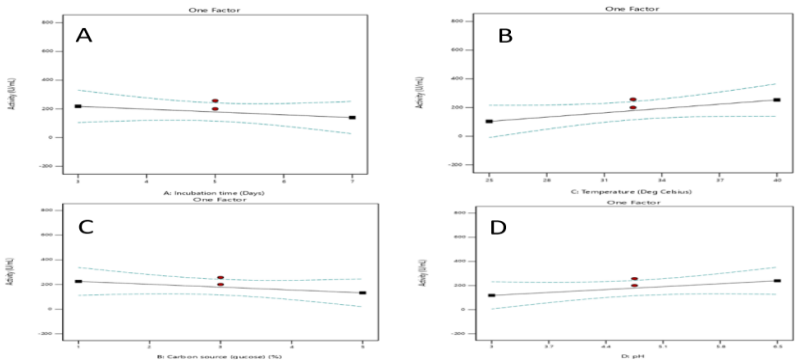


Figure 2: Effect of Incubation time (A), Temperature (B), Carbon source (C), and pH (D) on phytase production.

Effect of carbon source and incubation time on phytase production

Figure 3a is an interaction plot, showing how the effect of carbon source concentration on phytase production depends on the level of incubation time (and vice versa). The plot indicates that a higher carbon source concentration (5%) consistently results in lower enzyme activity compared to a lower concentration (1%), regardless of incubation time. However, the effect of incubation time changes based on carbon source level. At a low carbon source (1%), enzyme activity is highest at 3 days and decreases significantly with longer incubation time (7 days). At a high carbon source (5%), enzyme activity is low at both 3 and 7 days showing a less pronounced decrease over time.

The effect of temperature and incubation time on phytase production

Figure 3b illustrates the interaction between temperature and incubation time on phytase production. The model revealed that the effect of each factor is dependent on the level of the other. The plot shows that a shorter incubation time (3 days) consistently yielded higher activity than a longer time (7 days). However, the influence of temperature changed based on the incubation time.

The effect of pH and incubation time on phytase production

Figure 3c depicts the interaction between pH and incubation time on phytase production. The analysis indicates that the effect of incubation time is dependent on the pH level, and vice versa. The plot reveals that a shorter incubation time (3 days) consistently yielded higher activity than a longer time (7 days). Across the pH range tested. However, the influence of pH changes based on the incubation time.

The effect of temperature and carbon source on phytase production

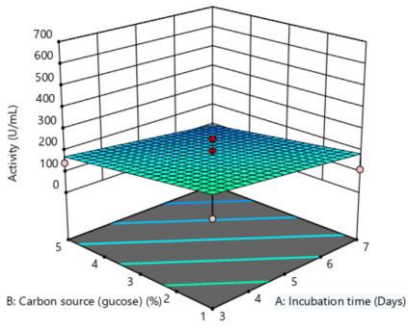
Figure 3d illustrates the interaction between temperature and carbon source concentration on phytase production. The model revealed that the effect of temperature depends on the level of carbon source, and vice versa. The plot indicates that a lower carbon source concentration (1%) consistently resulted in higher phytase activity than a higher concentration (5%) across the temperature range tested. However, the influence of temperature was more pronounced at the lower concentration.

The effect of pH and carbon source concentration on Phytase production

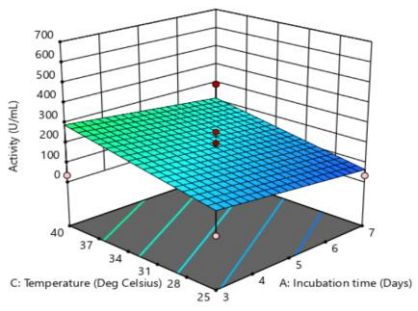
Figure 3e shows the interaction between pH and carbon source concentration on phytase production. The analysis indicates that the effect of each factor is dependent on the level of the other. The plot reveals that a lower carbon source concentration (1%) consistently yielded higher activity than a higher concentration (5%) across the pH range tested. However, the system's response to pH was strongly moderated by the carbon source level.

The effect of pH and temperature on Phytase production

Figure 3f shows the interaction between pH and temperature on phytase production. The analysis indicates that both factors have a positive individual effect on activity, but their effects are interdependent. The plot reveals that the highest activity is achieved in the region of high pH and high temperature. However, the nature of the interaction shows that the positive effect of increasing temperature is strong across all pH levels but is most pronounced at near-optimal pH (6.5).



a.



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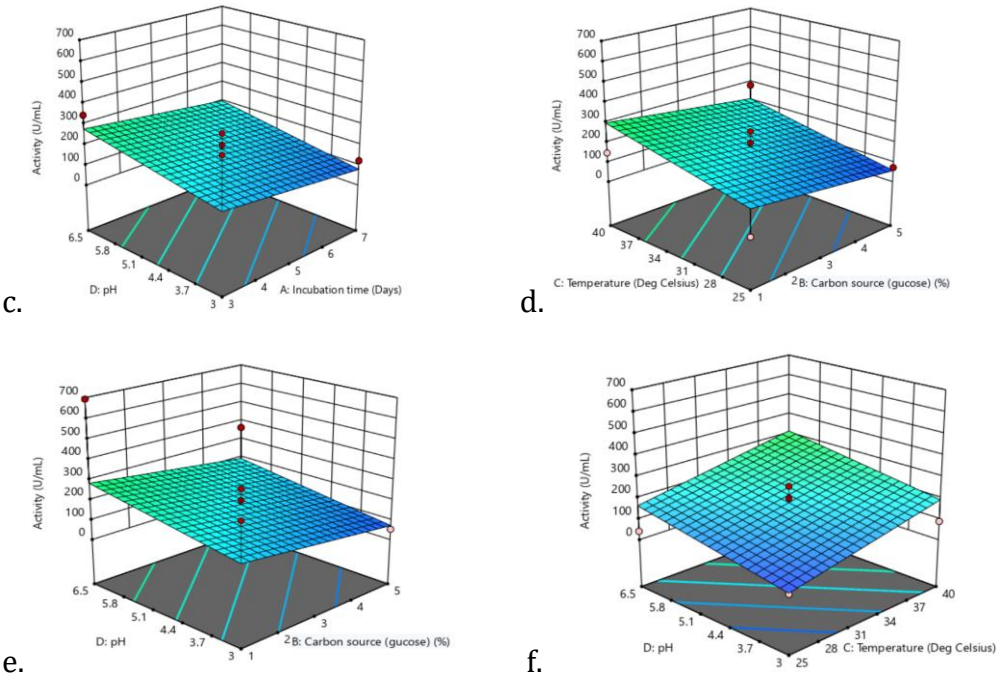

Figure 3 (a: Interaction effect of carbon source and incubation time on phytase production, b: Interaction effect of temperature and incubation time on phytase production, c: Interaction effect of pH and incubation time on phytase production, d: Interaction effect of temperature and carbon source on phytase production, e: Interaction effect of pH and carbon source on phytase production, f: Interaction effect of pH and temperature on phytase production).

DISCUSSION

A suitable medium must be provided for the microoganisms in order to grow and achieve maximum enzyme production [26]. In this study, a high phytase activity was achieved when *Coprinopsis cinerea* was grown in rice bran extract medium. This was due to the presence of phytate and other nutrients in the extract.One of the main sets back encountered in the aspect of phytase application is its production cost. Hence, there is need for the use of cost-effective substrate such as the rice bran extract to achieve maximum enzyme production. Simas *et al.* [28] used rice bran as a carbon and phytate source to produce phytase by *Aspergillus niveus* for supplementation in animal feed.

Most of the nutrients for microbial growth are supplied in the rice bran extract [29]. However, the concentration of carbon was insufficient in the substrate and there is need to make up for these inadequate nutrients. In the present study, the proximate analysis of the rice bran extract used indicated that the total carbohydrate content was 1.29%. This suggest that the rice bran extract has low carbon content. Glucose was used as carbon supplement for phytase production in the current study. Several studies have demonstrated the use of glucose as a simple carbon source in enhancing maximum phytase production [18,30,31]. Different glucose concentrations were tested for their effect on phytase production. *Coprinopsis cinerea AmutBmut pab1-1* gave the highest enzyme activity with a 3% glucose

concentration. Many researchers have reported that an optimum glucose concetration of 3% is required for maximum phytase activity [32, ,26,7].

The effect of incubation time on phytase production was studied to determine the optimum incubation time required for enhanced enzyme production. The highest phytase activity was attained at 3 days of incubation. However, in the study of Jatuwong *et al.* [32], the highest production of phytase from *Pholiota adiposa* was observed after 7 days of incubation. Reports of many researchers have also proven that the optimum incubation time for phytase production from fungi ranges from 3 to 7 days [33,34,8].

To investigate the influence of temperature on phytase production, the inoculated substrates were incubated at different temperatures i.e, 25, 32.5 and 40 respectively. The results obtained from this study showed that phytase with the highest enzyme activity was produced at 32.5. Reports from previous studies have shown that the optimum temperature for fungal phytase production is between 25-40°C [33]. The study of Afolabi and Atunwa [35] reported an optimum temperature of 40°C for phytase production by fungi: *Aspergillus niger* PKruw7, Aspergillus flavusPBDJ7 and *Aspergillus niger* MOJ5b. However, the peak activity of phytase was observed at 60°C in the study of Sadaf *et al.* [35]. This difference may be because of differences in the type of fungi used and differences in optimum fermentation conditions for the isolates.

CONCLUSION

Phytase producing microorganisms were isolated, screened, characterized and identified. Culture conditions were optimized for best enzyme production with rice bran extract as a substrate and the main source of phytate for microbial growth. The rice bran extract used has a phytate content of 22.02mg/100g. The optimized culture conditions were 3 days incubation, 3% glucose concentration, 32.5°C at pH 6.5 for phytase production with *Coprinopsis cinerea*. This study has shown that fungi are more suitable for phytase production and that optimum culture conditions are necessary for best phytase production.

The discussion and conclusion will be questionable since all the factors have no significant influence on the response (activity of phytase). Of what value is the optimization. The graphs may show changes in the response with changes in the factors, whether singly or in combination. It will not still matter because the model was not significant.

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