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Optimization of Phytase Production from *Rhodotorula mucilaginosa* RG-PK20 Using Agricultural Waste

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ABSTRACT

Some ingredients in poultry feed contain phytic acid, which prevents the absorption of nutrients. Microbial phytase enzymes can help with this problem. However, the phytase-producing gene of the novel yeast *Rhodotorula mucilaginosa* RG-PK20 has been constrained by the high cost of traditional substrates such as sodium phytate. The aim of this study is to evaluate the production of phytase from *R. mucilaginosa* RG-PK20 using a phytic acid source (substrate) from agricultural waste, with in vitro testing in poultry feed. The fermentation process was conducted utilizing a substrate-to-medium-to-yeast culture ratio of 1:1:1 v/v/w over a period of four days at various temperatures (25, 28, and 30 °C) and pH levels (3, 4, 5, and 6, with 7 as a control). Glucose and urea supplements were given when the optimal conditions were established by measuring the phytase content and activity. The molecular weight of the phytase was confirmed by SDS-PAGE analysis, and the ability of the enzyme to hydrolyze phytic acid was evaluated *in vitro*. Corn cobs generated the highest amount of phytase, with a concentration of 25.29 mg/mL and activity of 4.46 U/mL. The *in vitro* tests revealed an 81% reduction in phytic acid levels in poultry feed. These results demonstrate the potential of phytase derived from *R. mucilaginosa* RG-PK20 to reduce phytic acid in poultry feed ingredients.

Keywords: *agricultural waste; phytase; phytic acid; poultry feed; Rhodotorula mucilaginosa*

INTRODUCTION

More than 60% of the ingredients in current feed formulations are derived from plants such as corn, wheat, rice bran, beans, and palm kernel meal, among others. Some ingredients are high in phytic acid (Chen & Xu, 2023; Deak & Johnson, 2007; Nissar *et al*., 2017). Phytic acid acts as an anti-nutritional factor by reducing the bioavailability and absorption of essential minerals such as phosphorous, iron, and zinc in animals, which are crucial for growth (Chondrou *et al*., 2024; Joshi & Satyanarayana, 2017; Kumar *et al*., 2017). As a solution, supplementation of exogenous phytase is needed. Philippi *et al.* (2023) found that additional phytase in poultry feeds enhanced phosphorus digestibility, bone mineralization, and nutrient absorption, as well as increased egg production (Jlali *et al*., 2023; El-Hack *et al*., 2018). Some microbiomes, such as bacteria, fungi, and yeast, can naturally produce phytase in both intracellular and extracellular forms (Joudaki *et al*., 2023; Teigiserova *et al*., 2021).

A previous study showed that yeast reduced phytic acid in corn and increased amino acid content in copra meal (Muniroh *et al*., 2021). *Rhodotorula mucilaginosa* RG-PK20 is a new strain of the *R. mucilaginosa* yeast that has been shown to produce phytase, which is unicellular with pink colonies characterized by high growth rates in various habitats. The phytase gene is present in this yeast, which was isolated from fermented raisins (Seprianto *et al*., 2023a), as shown molecularly by amplification of the phytase gene (± 500 bp) using particularly in silico-designed primers (Seprianto *et al*., 2023b). Previous studies have highlighted the potential of *R. mucilaginosa* RG-PK20, demonstrating its tolerance to acidic pH and bile salts (Chen *et al*., 2022; Tian *et al*., 2022), thereby positioning it as a promising candidate for probiotic applications (Ogunremi *et al*., 2015). Research on *R. mucilaginosa* yeast as a phytase-producing strain in Indonesia has yet to be reported or investigated. The production of phytase by *Rhodotorula* is significantly influenced by both pH and temperature. Furthermore, the yeast has demonstrated the ability to produce phytase, with activity observed at temperatures ranging from 25 °C to 30 °C and a pH of between 4 and 6. However, it is essential to determine the optimal conditions for maximum enzyme production. It has also been reported that phytase from *R. mucilaginosa* can improve mineral mobilization in animal feed, increasing the bioavailability of essential elements, including calcium, iron, and zinc (Pable *et al*., 2014). However,

the substrate in the form of Na-phytate is necessary for synthesizing phytase (Coban & Demirci, 2014); hence, it may not be cost-effective.

This study investigates the use of agricultural by-products, specifically from rice bran, corn cobs, and soybean meal, to replace Na-phytates as a substrate for phytase production. These ingredients are inexpensive and widely available but include considerable phytic acid, which yeast uses to synthesize phytase. Feizollahi *et al*. (2021) reported that the phytic acid concentration in rice bran was 6.55%–8.7% of dry matter. On the other hand, corn grain contains phytic acid up to 80% of the total phosphorus. On a dry matter basis, the content varies from 7.15 to 7.60 g/kg in seed but is significantly higher in cob and germ, with concentrations ranging from 6.0% to 7.0% (Fukuji *et al*., 2008; Khan *et al*., 1991; Pramitha *et al*., 2020; Singh *et al*., 2018). Soybean meal has a lower phytic acid level (1%–2% DM) than the other two ingredients but still accounts for 75% of the phosphorus content (Jain & Singh, 2017). It has been identified as a promising substrate for phytase production. Its widespread availability and cost-effectiveness make it an attractive alternative to other, more expensive or less accessible raw materials (Dailin *et al*., 2019). Soybean meal is primarily an imported commodity and is rich in proteins, carbohydrates, and fats, which are essential nutrients supporting the growth of phytase-producing microorganisms. This nutritional profile enables effective fermentation processes, facilitating phytase production in significant quantities (Adebo *et al*., 2022). Furthermore, the utilization of soybean meal helps to reduce waste from the soybean processing industry, contributing to sustainability by optimising the use of agricultural by-products. In addition, all three include additional elements that can be utilised as yeast growth media, including proteins, lipids, carbohydrates, and other kinds of minerals.

The aim of this study is to evaluate the production of phytase enzyme from *R. mucilaginosa* RG-PK20 using a phytic acid source (substrate) from agricultural waste, with in vitro testing in poultry feed.

MATERIALS AND METHODS

Rhodotorula mucilaginosa RG-PK20 Preculture

R. mucilaginosa RG-PK20 active isolates from a 50% glycerol stock were cultivated in a 300 mL Erlenmeyer flask with 100 mL of yeast extract-peptone-dextrose (YPD) liquid media. The cultures were then incubated for three days at 25 °C at 120 rpm in a shaking water bath. A reddish turbid medium indicates a healthy yeast culture (El-Ziney *et al*., 2018). Additionally, *R. mucilaginosa* RG-PK20 yeast cells were observed both macroscopically and microscopically.

Phytase Activity Qualitative Test

PSMA (Phytase Selective Medium Agar) selective medium was used to perform a qualitative assessment of phytase enzyme activity. The medium was prepared with the following composition: 15 g of glucose, 5 g of NH4NO3, 0.5 g of KCl, 0.5 g of MgSO4, 0.01 g of FeSO4, 0.01 g of MnSO4, 2 g of CaCl2, 20 g of microbiological agar, and 5 g of Na-phytate in 1000 ml of distilled water (Casey & Walsh, 2004). Additionally, the acidity of the medium was adjusted to pH 5. After being cultivated on PDA media, single colonies were streaked onto PSMA media in one quadrant and then incubated at 25, 30, and 37 °C for 72 hours. A clear zone surrounding the phytase enzyme indicates its activity.

Substrate Preparation Using Agricultural Waste

Agricultural wastes (rice bran, soybean meal, and corn cob) were oven-dried until the dry matter (DM) reached 90%, then mashed to a size of 0.3-0.7 mm (Pires *et al*., 2019). Each sample was weighed to 10 g and placed into heat-resistant plastic containers. Next, the samples were then sterilized using an autoclave at 1 atm pressure and a temperature of 121°C for 15 minutes.

Phytase Production by Solid State Fermentation (SSF)

The fermentation medium was mixed with a quantity of the substrate mixture at a ratio of agricultural waste, substrate medium, and yeast culture (1:1:1 w/v/v) in a 100 mL Erlenmeyer flask. A total of 10 g of each agricultural waste substrate (rice bran, corn cob, and soybean meal) was added to 10 mL of PSMB media solution (composed of 15 g glucose, 5 g NH4NO3, 0.5 g KCl, 0.5 g MgSO4, 0.01 g FeSO4, 0.01 g MnSO4, and 2 g CaCl2 dissolved in 1000 mL distilled water). In addition, 10 mL of *R. mucilaginosa* RG-PK20 culture was added to each medium. The mixture was homogenized and the acidity was adjusted to pH 3, 4, 5, and 6 using 1% HCl. Fermentation was carried out in a shaker incubator at 25, 28, and 30 °C for 4 days.

Extraction of Phytase Crude Extract

Phytase crude extract is obtained from solid-phase fermentation (SSF). Extraction was performed by putting 1 g of SSF into a falcon tube and adding 4 mL of sterile distilled water. The sample was homogenized by shaking for 15 minutes, then 2 mL was taken and put into an Eppendorf tube and centrifuged at 8000 rpm, at 4 °C, for 10 minutes. The supernatant obtained is a sample of phytase crude extract (Seprianto *et al*., 2023b).

Preparation of Phosphate Standards

The standard curve uses KH2PO4 with concentrations of 0, 10, 20, 30, 45 and 50 ppm. Stock solution (1000 ppm) was made by weighing KH2PO4 as much as 0.1432 g and put into an Erlenmeyer flask containing 100 mL of distilled water. The standard was analyzed using a UV-Vis spectrophotometer with λ= 700 nm. The standard curve was made by plotting the phosphate concentration data on the X-axis and the absorbance data on the Y-axis, following the linear equation Y= aX + b.

Phytase Activity

Phytase activity was measured by placing 50 µL of crude phytase extract into a test tube and adding 50 µL of 0.5% (b/v) Na-phytate in an acetic acid buffer (0.1 M, pH 5). The samples were then incubated at 30 °C for 30 minutes. Subsequently, the reaction was stopped by adding 100 µL of 15% TCA. Next, 160 µL of phosphate-molybdate solution was added to the sample and incubated at 30 °C for 1 h. A positive result was indicated when the solution turned blue. The solution was analyzed using a spectrophotometer at a wavelength (λ) of 700 nm.

Optimization of Phytase Production by the Addition of Carbon and Nitrogen

Production optimization was made by selecting the optimal pH and temperature conditions for each substrate in phytase production. Phytase re-production was also performed by adding different amounts of glucose and urea in quantities of up to 0%, 0.5%, and 1.0% of the total substrate, respectively. In addition, extraction and phytase efficacy tests were again conducted to determine the optimum level of the addition of carbon and nitrogen sources.

In Vitro Test of Phytase on Poultry Feed (Riviere *et al.*, 2021)

Crude phytase extract was obtained from the phytase enzyme assay, which was the best treatment. A total of 50 µL of the extract was added to a microtube, while 50 µL of distilled water was added to another microtube as a negative control. Poultry feed was weighed at 1 g and then ground. After grinding, the feed was dissolved in 20 mL of sterile distilled water. The 5% milled poultry feed was added to each phytase sample up to a volume of 50 µL. The samples were then incubated at 30 °C for 30 minutes, and the reagent mixture (50ul) was added to each sample. The samples were allowed to stand for 20 minutes until a blue color appeared. The phytic acid content of the feed, both prior to and following *in vitro* treatment, was quantified using a spectrophotometer at a wavelength of 880 nm (Kanti, 2017).

Phytase Essay - SDS-PAGE

SDS-PAGE was used to determine the molecular weight of the protein samples following the method from Sumengen *et al.* (2013). SDS gel electrophoresis was performed on a 10% homogeneous gel using a vertical electrophoresis system. Crude phytase samples were mixed with a sample loading buffer in Eppendorf tubes, which were placed in boiling water for 5 min. The denatured samples were separated by SDS-PAGE on a 12% separating gel coated with a 5% stacking gel. After electrophoresis, the gel was stained to visualize the phytase activity. Protein bands were detected by immersing the gel in 0.1% Coomassie Brilliant Blue in a solution of methanol (50%) and acetic acid (10%) for 2 h. After staining, the gel was destained overnight in a solution of methanol (5%) and acetic acid (7%). The gel was visualized with a CCD camera.

Data Analysis

Data were statistically analyzed using a two-way analysis of variance (two-way ANOVA). If the data analysis results show that the null hypothesis is rejected, further tests, such as the Duncan test, can be carried out.

RESULTS

Pre-culture of *Rhodotorula mucilaginosa* RG-PK20

Figure 1 displays the findings of both the macroscopic and microscopic examinations of the yeast cell *R. mucilaginosa* RG-PK20. A single pink colony was produced as a result of cell rejuvenation. According to the macroscopic observations, *R. mucilaginosa* RG-PK20 cells have a round colony shape, a pale pink surface with a pink circle in the center, a slightly loose and viscous texture, and intact rounded edges (Figure 1A). The cells were oval in shape and ranged in size from 5 to 20 micrometers, as shown by the microscopic observations made using methylene blue cell staining (Figure 1B).

The Activity of the Phytase on PSMA-Specific Media

When a clear zone forms surrounding the yeast streak, it results in phytase selective medium agar (PMSA); this indicates the presence of the phytase. The clear zone measurements demonstrated the capacity of various phytase activities at 25, 28, and 30 °C. The clear zone diameter of 37.6 mm at 25 °C on day 4 had the maximum activity (Figure 2A), although it was not statistically significant (Table 1).

Phytase Production by Solid State Fermentation (SSF)

Table 2 shows that the concentration of phytase generated by *R. mucilaginosa* RG-PK20 with rice bran

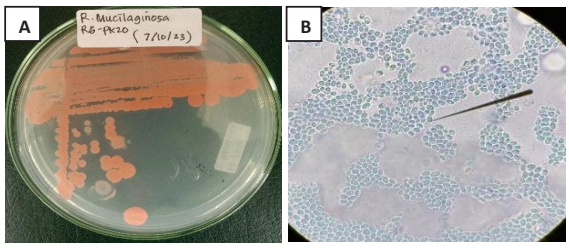

Figure 1. Macroscopic and microscopic observations of *Rhodotorula mucilaginosa* RG-PK20 (A) colony shape and color (B) cell shape.

Table 1. Clear zone diameter as an indicator of phytase activity by *Rhodotorula mucilaginosa* RG-PK20 after incubation at 25, 28, and 30 °C

Temperature °C	Diameter of clear zone (mm)
25	37.6
28	33.6
30	37.1

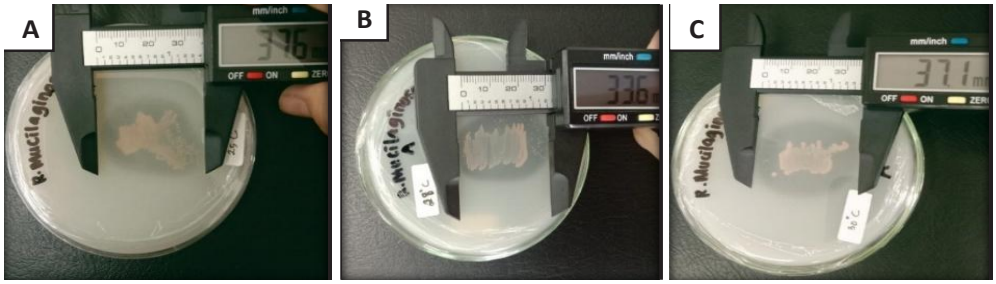

Figure 2. Qualitative test results of phytase enzyme activity. (A) 25 °C on day 4; (B) 28 °C on day 4; (C) 30 °C on day 4.

Table 2. Phytase concentration (mg/mL) of *Rhodotorula mucilaginosa* RG-PK20 cultivated with different substrates under various temperature (25, 28, and 30 °C) and pH (3–7) conditions

pH	Temperature (°C)			Average	Significantly (Pv)		
	25	28	30		pH	Temp	pH x Temp
		Rice bran					
3	8.14±0.01^{d}	0.01±0.01^{e}	0.01±0.01^{e}		***	**	***
4	19.20±0.57^{a}	16.59±0.18^{c}	19.57±0.57^{a}				
5	16.91±0.23^{c}	18.05±0.29^{b}	19.38±0.23^{a}				
6	18.95±0.24^{ab}	18.15±0.14^{b}	16.49±0.25^{c}				
K+	14.81±0.10^{d}	14.28±0.27^{d}	17.41±0.17^{bc}				
		Soybean meal					
3	0.04±0.05	8.11±0.25	5.37±0.05	4.51±3.33^{b}	***	NS	NS
4	6.25±0.26	11.72±0.08	8.98±0.01	8.98±2.47^{ab}			
5	6.85±0.19	6.05±0.52	10.43±0.43	7.78±2.27^{ab}			
6	8.95±0.31	9.70±0.12	9.64±0.39	9.43±0.40^{a}			
K+	7.91±0.52	11.14±0.63	12.34±0.19	10.46±2.28^{a}			
		Corn cob					
3	24.28±0.01	24.38±0.03	25.44±0.02		NS	NS	NS
4	25.05±0.15	24.52±0.02	24.79±0.06				
5	24.66±0.06	24.67±0.01	24.95±0.02				
6	24.83±0.02	25.29±0.00	25.29±0.04				
K+	25.41±0.11	24.85±0.01	24.98±0.12				

Note: Different superscripts in columns and rows within a substrate indicate significant differences in results at p<0.05 for 2 stars (**) and p<0.01 for 3 stars (***). NS= not significant.

substrate was found to be significantly impacted by both pH and temperature (p<0.05). The synthesis of the phytase was strongly influenced by the interplay between pH and temperature (p<0.01). In contrast, the pH factor was the sole component that had an impact on enzyme production using soybean meal substrate (p<0.05). Throughout the pH and temperature treatments, even the corncob substrate generated consistent concentrations (p>0.05). The highest phytase concentrations using rice bran, soybean meal, and corn cob substrates were 19.58, 11.72, and 25.44 phytase mg/mL, respectively.

Phytase Activity

In the rice bran substrate, the phytase activity extracted from the culture was influenced by temperature, pH, and a combination of the two (p<0.01). The corn cob substrate generated phytase with very steady activity and was unaffected by the three parameters (p>0.05), whereas the substrate's phytase activity of the soybean meal substrate was only influenced by temperature (p<0.05). Table 3 shows the overall activity of the phytase. Tables 2 and 3 demonstrate the highest concentration and activity in each substrate, which led to the selection of the rice bran (pH: 4, temperature: 30), soybean meal (pH: 5, temperature: 25), and maize (pH: 6, temperature: 30) substrate for further testing with the addition of glucose and urea.

Optimization of Phytase Production by the Addition of Carbon and Nitrogen

Figure 3 displays the outcomes of the evaluation of how adding glucose and urea affected the synthesis of phytase. Phytase synthesis was impacted by the addition of glucose to the corn cob substrate but was not affected by the addition of soybean meal to rice bran. Phytase production using rice bran substrate was reduced by adding urea but using corn cobs and soybean meal substrates was unaffected. However, there was no discernible drop in phytase production in any of the three substrates.

In Vitro Test of Phytase on Poultry Feed

Table 4 presents the results of the phytase activity derived from corn cob substrate in an *in vitro* assay

Table 3. The activity of phytase from *Rhodotorula mucilaginosa* RG-PK20 with different substrates (unit/mL)

pH	Temperature (°C)			Average	pH*Temp
	25	28	30		
		Rice bran			
3	1.43±0.0017^{b}	0.00±0.0004^{a}	0.00±0.000 ^{a}	0.47±0.7372	Pv=0.009*
4	3.37±0.6279^{e}	2.91±0.2141^{cde}	3.43±0.6657^{e}	3.24±0.4916	
5	2.97±0.1909^{cde}	3.17±0.3377^{cde}	3.39±0.2722^{e}	3.18±0.2868	
6	3.32±0.1222^{de}	3.18±0.1652^{cde}	2.89±0.2876^{cde}	3.13±0.2524	
K	2.59±0.1449^{cd}	2.51±0.3189^{c}	3.05±0.2079^{cde}	2.72±0.3192	
Average	2.74±0.7834	2.35±1.2789	2.56±1.3901		
		Soybean meal			
3	2.33±0.0589	2.00±0.2979	2.73±0.0637	2.35±0.3534	Pv=0.252
4	2.67±0.3039	2.75±0.0905	2.58±0.0159	2.67±0.1613	
5	3.89±0.2336	3.35±0.6084	3.20±0.5064	3.48±0.4908	
6	3.57±0.3646	3.13±0.1416	2.53±0.4561	3.07±0.5401	
K	3.70±0.6112	3.46±0.7432	3.03±0.2306	3.39±0.5366	
Average	3.23±0.7035	2.94±0.6514	2.81±0.3654		
		Corn cob			
3	4.26±0.0098	4.27±0.0309	4.46±0.0236	4.33±0.1039	Pv=0.284
4	4.39±0.1813	4.29±0.0318	4.35±0.0614	4.35±0.0974	
5	4.33±0.0701	4.32±0.0063	4.38±0.0271	4.34±0.0441	
6	4.36±0.0253	4.43±0.0530	4.44±0.0530	4.41±0.0480	
K	4.46±0.1262	4.35±0.0206	4.38±0.1423	4.40±0.0980	
Average	4.36±0.1045	4.34±0.0614	4.40±0.0708		

Note: Different superscripts in columns and rows within a substrate indicate significant differences in results at p<0.05.

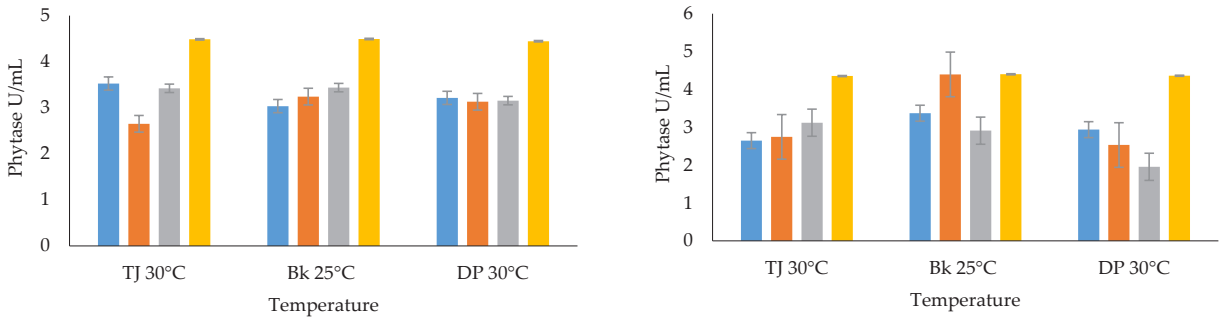

Figure 3. Phytase activity with the addition of glucose (A) and nitrogen (B) sources at the optimal temperature. TJ=corn cob, Bk=soybean meal, DP=rice bran. The control (yellow) is a medium that does not utilize agricultural waste, glucose, or urea; instead, sodium phytate is added as the substrate. 0% (■), 0,50% (■), 1% (■), Control (■).

conducted on poultry feed. The selection of this phytase was based on the highest enzymatic activity observed. Phytic acid levels in three commercial chicken feeds hydrolyzed with phytase ranged from 1 to 1.4 mg/g, which were successfully reduced by 74% to 81%. The percentage that can be hydrolyzed tends to increase with the amount of phytic acid present.

Phytase Essay - SDS-PAGE

According to the SDS-PAGE data, the protein phytase molecule is represented by a band at 55 kDa (Figure 4), although the band was discernible only in the soybean meal substrate treatment.

DISCUSSION

Pre-cultivation was conducted to guarantee the precision of yeast identification and to confirm the lack of contamination. For this reason, both microscopic and macroscopic analyses were conducted. Despite the use of various strains, the macroscopic and microscopic observations of *R. mucilaginosa* RG-PK20 in this study were consistent with findings from earlier research. Depending on the particular carotenoids generated and their concentrations, *R. mucilaginosa* colonies can be smooth, shiny, and pigmented, ranging in color from pink to orange (Hue *et al*., 2023; Zohari *et al*., 2021). The colonies seen in this study had a thick, somewhat mucilaginous texture (Gattlen *et al*., 2011) and were grouped in a spiral pattern (Da Cunha *et al*., 2009), which is consistent with the traits of *R. mucilaginosa*.

R. mucilaginosa RG-PK20 was cultivated on phytase selective medium agar (PSMA), which is initially turbid because of the presence of sodium phytate, in order to verify the existence of a clear zone as a sign of phytase activity. The presence of a clear zone around colonies on PSMA indicates successful phytate degradation (Li *et*

Table 4. Result of *in vitro* test of phytase on poultry feed

Kind of feed	Concentration of phytic acid (mg.g-1)		Degradation of phytic acid in feed (%)
	Before hydrolysis	After hydrolysis	
Juwawut seed (BJ)	10.810.286	0.2745049	74.61
Niger seed (BNS)	12.472.367	0.2306453	81.51
Sawi seed (BS)	14.678.872	0.2706833	81.56

al., 2013). Normally, the clear zone diameter on the phytase microorganism medium ranges from 14 to 20 mm (Mohammadi-Kouchesfahani et al., 2019; Priyodip & Balaji, 2024). In our study, *R. mucilaginosa* achieved this range under optimal fermentation conditions at a temperature of 30 °C (Naghavi *et al*., 2014). The variation in the clear zone diameter in this research compared to previous reports can be attributed to several factors, including differences in microorganism strains, inhibitor factors, and the composition of the medium, especially phytic acid concentrations (Sasirekha *et al*., 2012).

A clear zone is evidence of phytase synthesis, showing the hydrolysis of phytate-bound phosphorus into free phosphate, as indicated by the development of a clear zone around the colonies (Tariq *et al*., 2017). In detail, phytase hydrolysis of myo-inositol hexakis (dihydrogen phosphate) into inorganic phosphate and myo-inositol occurs through the cleave of phosphate ester bonds at the hydroxyl position on carbon 1 through to 6 of the inositol ring (Sapna *et al*., 2013; Singh *et al*., 2011). These findings suggest that *R. mucilaginosa* RG-PK20 can be cultivated on inexpensive and plentiful natural media to produce phytase.

In this study, the production of phytase from *R. mucilaginosa* RG-PK20 varied greatly depending on the substrate and fermentation conditions. Among the substrates, corn cobs are superior to soybean meal and rice bran in the production of phytase (Table 2), a fact related to the concentration of phytic acid with values of 6%-7%, 6.9%, and 1%-2% for corn cobs, rice bran, and soybean meal, respectively (Jain & Singh, 2017; Koni *et al*., 2024; Singh *et al*., 2011). The presence of phytic acid in the fermentation substrate, especially when free phosphate is scarce, stimulates the formation of phytase by yeast (Vilanculos *et al*., 2024). This implies that phytic acid triggers the synthesis of phytase in some microbes in addition to acting as a substrate. When exposed to phytic acid, for instance, other yeasts (for example, *Aspergillus niger*) also can produce phytase (Heydari-Majd *et al*., 2024). The production of phytase by yeast is also affected by pH and temperature.

Overall, the study showed that both pH and temperature affected the concentration of phytase from *R. mucilaginosa* RG-PK20 in rice bran and soybean meal but not in corncobs as substrates. Optimum pH levels for rice bran, soybean meal, and corncob fermentation are 4, 5, and 3, respectively. These results are consistent with those of a previous study, which showed that *R. mucilaginosa* can be cultured in the pH range of 3.0-7.0, with optimal conditions at 5.0 (Yu *et al*., 2015).

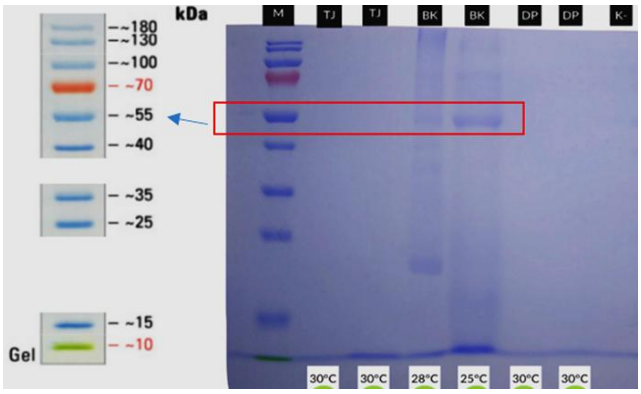

Figure 4. Results of SDS-PAGE of crude phytase molecules at the optimal temperature. (M): Marker, Broad range protein ladder; (Tj): corn cob; (Bk): Soybean meal; (Dp): rice bran; (K-): Negative control obtained from PSMB medium without treatment.

Fermentation pH can be adjusted, as there is evidence of a complex interaction between pH and temperature (Scarcella *et al*., 2017).

In our study, the ideal temperature range for phytase production varied from 28 °C for soybean meal as a substrate to 30 °C for rice bran and corn cobs, with corresponding values of 11.72, 19.57, and 25.44 phytase mg/mL. These findings are consistent with an earlier study that described how the mutant *Rhodotorula minuta* reached its maximum phytase synthesis at 30 °C (26.735 mg/mL) and 35° C (21.620 mg/mL) (Gowthami & Gunashree, 2023). Nevertheless, *R. mucilaginosa*, a cold-adapted strain, exhibited the highest phytase activity at 50 °C, with significant activity maintained at lower temperatures (37 °C) (Yu *et al*., 2015). In addition to measuring the quantity of an enzyme, its activity offers vital information about its catalytic effectiveness.

Phytase activity is defined by its ability to degrade phytate, which is crucial for enhancing the bioavailability of minerals and phosphates in animal feeds (Qvirist *et al*., 2015). This activity is influenced by various factors, including substrate type and assay conditions (Gocheva *et al*., 2023). Phytase production employing rice bran, soybean meal, and corn cobs as substrates yielded the greatest phytase activity at 3.37, 3.89, and 4.46 units/mL, respectively. Rice bran production phytase activity is unstable, depending on temperature and pH fermentation conditions ($p<0.01$), while soybean meal and corn cobs' phytase activity was unaffected by these variables ($p>0.05$). This is in line with Yu *et al.* (2015) finding that the phytase activity of *R. mucilaginosa* is stable between pH 3.0 and 7.0 and at low temperatures.

The best substrate for phytase production using *R. mucilaginosa* RG-PK20 is shown in detail by the data in Tables 2 and 3, together with the results of the statistical analysis. Corn cob is the most efficient choice. However, the best conditions for each substrate were subsequently subjected to further testing with the addition of urea and glucose. Based on Figure 3, this additional glucose and urea did not influence phytase activity for all substrates. Our results contradict those

of previous research. According to Coban and Demirci (2014), phytase activity rose 11% when 60 g of glucose was added during fermentation. On the other hand, additional urea reduced phytase activity after 10 hours (Wei *et al*., 2006). Overall, the data indicate that the addition of glucose and urea at low concentrations (0%, 0.5%, and 1%) did not have a significant effect on phytase activity. These findings suggest that simple carbon sources such as glucose and nitrogen do not positively enhance phytase activity under specific temperature conditions for each substrate tested. The presence of both organic and inorganic nitrogen in the substrate provides the yeast with a greater nitrogen source for protein synthesis, which can influence cell biomass and enzyme production (Chen *et al*., 2018). It is essential to acknowledge the significance of this issue, particularly in light of the further results obtained from *in vitro* tests on poultry feed.

Poultry feed containing 1.0, 1.2, and 1.4 mg/g of phytic acid was hydrolyzed in vitro using a phytase from *R. mucilaginosa* RG-PK20. After 20 minutes, the phytic acid level was reduced by up to 81% (Table 4). This level was less than those indicated in earlier research, which found that after hydrolyzing feed for 24 hours, phytases from *Aspergillus oryzae* decreased phytic acid by 90%–97% (Pragya *et al*., 2023). The results of this study, however, refer to the degradation time of 15 to 30 minutes, during which the feed is present in the chicken crop (Classen *et al*., 2016). Given that the poultry crop has a pH of around 4.5, the enzyme activity found can also withstand acidic environments (Naves *et al*., 2012). Supplementation of poultry feed with the *R. mucilaginosa* solid-state fermentation product, which is rich in carotenoids, improved the yolk color and increased the carotenoid content, thereby improving the intestinal health of hens (Sun *et al*., 2020). Therefore, the testing of phytase from *R. mucilaginosa* RG-PK20 *in vivo* shows much promise.

SDS-PAGE was also performed for further details. The confirmed phytase from *R. mucilaginosa* RG-K20 had a molecular weight of 55 kDa (Figure 4), consistent with the normal range of 37-55 kDa for microbial phytases (Jain *et al*., 2016). However, a strain of *R. mucilaginosa* from Antarctic marine sediment synthesize phytase with a distinct molecular weight of 63 kDa (Yu *et al*., 2015). According to previous studies, phytase with a lower molecular weight can hydrolyze phytic acid more quickly *(Quan et al*., 2004). The phytase protein band was only observed in the soybean substrate, while no band was detected in the corn cob or rice bran substrates. The absence of the phytase protein band in these samples could be attributed to suboptimal protein extraction processes for the two materials. The physical and chemical matrices of corn cobs and rice bran differ significantly, which affects the efficiency of protein isolation. Both corn cobs and rice bran contain high levels of phenolic compounds (Sadh *et al*., 2018). Furthermore, these materials contain inhibitory compounds such as polyphenols, tannins, or other complex compounds that may bind to proteins, thereby hindering the extraction process. As a result, the target protein concentration in corn cob and rice bran extracts was below the detection limit of SDS-PAGE. Based on the study findings, this validates the claim that phytase could be employed in the chicken feed sector.

CONCLUSION

According to the findings, *R. mucilaginosa* RG-PK20 can be effectively cultivated utilizing agricultural waste to produce phytase, as demonstrated by its molecular weight determined by SDS-PAGE examination. The most efficient substrate for phytase generation among the different types of agricultural waste examined were corn cobs, which produced phytase concentrations of up to 25.29 mg/mL phytase, with an activity of 4.46 units/mL. Furthermore, *in vitro* hydrolysis of phytic acid in poultry feed by phytase from *R. mucilaginosa* RG-PK20 reduced phytic acid levels by 81%. These results imply that the phytase produced by this yeast has great potential for use in the feed sector.

CONFLICTS OF INTEREST

We affirm that none of the authors has any conflicts of interest related to the study or the publication process.

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