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Optimization of Extracellular Phytase Production from *Bacillus amyloliquefaciens* PFB-02 *G*rown on Selected Agricultural Wastes

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ABSTRACT

The undigested phytic acid excreted in animal waste causes phosphorus pollution from agriculture. Addition of microbial phytases to animal feeds is a promising approach to overcoming challenges posed by phytic acid. In this study, influence of physicochemical parameters on growth and production of phytase from Bacillus amyloliquefaciens PFB-02 was investigated over 96 h cultivation period. Effects of some readily available and low cost agricultural wastes on phytase production from B. amyloliquefaciens PFB-02 were also investigated. Maximum growth with phytase yield of 5.3 U/ml was achieved in the basal medium at 48 h cultivation period, pH 5.0, 40 °C and 180 rpm. The optimized conditions were used to study phytase production from B. amyloliquefaciens PFB-02 grown on wheat bran, black-eyed bean skin and rice bran based media under submerged fermentation conditions. Remarkably, wheat bran supported highest enzyme yield of 17.0 U/ml with 317.5% increase over yield in basal media. Rice bran and black-eyed bean skin also supported phytase production from B. amyloliquefaciens PFB-02 with yield of 4.6 U/ml and 3.5 U/ml, respectively. The results suggest that agricultural wastes are effective low cost substrates for phytase production from B. amyloliquefaciens PFB-02 for potential application in animal feed formulation.

Keywords: Agricultural wastes; *Bacillus amyloliquefaciens* PFB-02; optimization; phytase; production

INTRODUCTION

Phytase (*myo*-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolase catalyzes the hydrolysis of phytic acid (myo-inositol hexakisphosphate) to inorganic phosphate and myoinositol phosphate derivatives (Wyss *et al*., 1999). Phytic acid, the storage form of phosphorus in many cereals and legumes fed to animals has negative impact on animal nutrition and environment (Raboy, 2007).

Ruminants digest phytic acid by phytases from fungi and bacteria present in their gut microflora. However, phytic acid is poorly digested by monogastric animals which lack sufficient phytase in their digestive tracts. The undigested phytic acid is excreted in animal waste and has been reported as a major source of phosphorus pollution from agriculture (Raboy, 2007). Furthermore, the reduced bioavailability of essential mineral cations to animals results from the tight binding of phosphate in phytic acid to Ca^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (Bohn *et al*., 2008; Ravindran *et al*., 1995; Torre *et al*., 1991). Phytic acid has been reported to have negative impact on amino acid digestibility thus making it a limiting factor on protein availability (Selle *et al.*, 2006). In fact, protein-phytate interactions are fundamental to the detrimental impact of phytate on protein/amino acid availability in pig and poultry nutrition (Selle *et al*., 2012).

A promising approach to overcoming these challenges posed by phytic acid is based on addition of microbial phytases to animal feeds as supplements to improve digestibility and dietary phytate-phosphorus utilization (Liao *et al.*, 2005; Stahl *et al.*, 2004;). Consequently, phytases have become important industrial enzymes and presently, there is continuous search for phytases from different microbial sources which have potentials for industrial use. The major challenge in this approach is the cost of microbial phytase production which could be a barrier to their utilization. Hence, there is an urgent need to screen for phytase production from variety of microbial sources and investigate conditions that support high yield of the enzyme at very low cost. Reduction in the production cost of microbial phytases could greatly reduce the cost of the enzyme for use as supplements in animal feeds. Efficient, scalable and economical process for improved yield of phytase production is critically required (Bhavsar and Khire, 2014).

Consequently, this study investigated the production of phytase from *Bacillus amyloliquefasciens* PFB-02 and optimization of the enzyme yield using inexpensive and readily available agricultural wastes at predefined conditions.

MATERIALS AND METHODS

Materials

Media components were products of Sigma-Aldrich (St Louis, MO, USA). Rice bran, black-eyed bean skin and wheat bran were purchased from a local market in Akure, Southwest Nigeria, sundried and powdered into fine particles. Further processing on the powdered substrates was carried out using standard sieve. All other chemicals used were of analytical grade.

Microorganism, Culture Preparation and Phytase Production

The microorganism used was a bacterium isolated from soil sample of a poultry litter site. The strain was identified as

Bacillus amyloliquefasciens by the Biotechnology Unit of Federal Institute of Industrial Research, Lagos, Nigeria based on morphological and biochemical methods described in Bergey's Manual of Systematic Bacteriology (Vos et al., 2009). This bacterial strain was designated PFB-02 and maintained on nutrient agar slants at 4 °C. Culture preparation was done as previously reported by Fasimoye *et al*., 2014). The medium for growth of seed culture contained 5.0 g/L glucose, 3.0 g/L peptone, 2.0 g/L NaCl and 0.5 g/L of $CaCl_2$. Peptone was allowed to dissolve before the addition of salts and pH was adjusted to 5.0 and the medium was sterilized in an autoclave at 121 ^{0}C for 15 minutes and allowed to cool. The medium was inoculated with *B. amyloliquefasciens* PFB-02 using wire loop and incubated for 24 h at 40 ^{0}C in a shaking incubator (Stuart, UK) at 180 rpm. The 24-hour old seed culture was used to inoculate the production medium constituting 5% v/v and then incubated for 96 h at 40 ^{0}C with shaking at 180 rpm. At the end of the cultivation period, the broth was centrifuged at 10,000 g for 15 minutes at 4 °C. The cell free supernatant was recovered as crude enzyme preparation.

Assay of Phytase Activity

Activity of phytase was assayed following the method of Heinonen and Lahti (1981) with slight modifications. The reaction mixture was made up of 2.0 ml of 200 mM sodium acetate buffer pH 5.0 containing 1.0 mg/ml sodium phytate and 1.0 ml of enzyme solution. The mixture was incubated for 30 min at 40 ^{0}C. Termination of the reaction was done by the addition of 1.0 ml of 15% trichloroacetic acid and color development was done by the addition of 1.0 ml of colouring reagent (3.66 g of $FeSO_4.7H_2O$, 0.5 g of $(NH_4)_6Mo_7O_{24}.4H_2O$ and 1.6ml of concentrated H_2SO_4 in 50.0 ml of distilled water). The assay mixture was kept on ice for 10 min. Absorbance was then taken at 700 nm. Sodium phosphate was used as standard for the phytase activity. One unit of phytase activity was defined as the amount of enzyme that liberates 1 µmol of inorganic orthophosphate per minute per ml under assay conditions.

Growth Kinetics and Phytase Production

Growth kinetics of *B. amyloliquefaciens* PFB-02 over the cultivation period and production of phytase were studied by cultivating the bacteria for 6, 12, 24, 36, 48, 60, 72, 84 and 96 h at pH 5.0 and 40 °C with shaking at 180 rpm. The growth of the microorganism was determined by measuring the absorbance of culture at 600 nm. The cultures were centrifuged at the end of each cultivation period and the supernatants were used for determination of phytase activity which was the measure of phytase production.

Influence of pH on Growth and Phytase Production

Cultures of *B. amyloliquefaciens* PFB-02 were grown in basal media at temperature of 40 °C with varied pH of 2.0 - 9.0 at 180 rpm for 48 h using 50 mM of the following buffer solutions: glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0 - 5.0), sodium citrate (pH 6.0), Tris- HCl (pH 7.0 to 8.0) and glycine-NaOH (pH 9.0) for media preparation. Optimal pH for growth and phytase production from *B. amyloliquefaciens* PFB-02 was determined at the end of the cultivation period by measuring the absorbance of culture at 600 nm and determining phytase activity in the cell free supernatant obtained after centrifugation at 10,000 rpm and 4°C for 15 min.

Influence of Temperature on Growth and Phytase Production

Optimal temperature for growth and phytase production from *B. amyloliquefaciens* PFB-02 was determined by investigating growth kinetics and phytase production in basal media at pH 5.0 with varied temperatures. Cultures were grown at 30, 40, 50 and 60 °C with shaking at 180 rpm and pH 5.0 for 48 h. Optimal temperature for growth and phytase production by *B. amyloliquefaciens* PFB-02 was determined at the end of the cultivation period by measuring the absorbance of culture at 600 nm and determining phytase activity as earlier described.

Investigation of Phytase Production from B. *amyloliquefaciens* PFB-02 Grown on Selected Agricultural Wastes under Optimized Conditions

The influence of some selected agricultural wastes as carbon sources on the production of phytase from *B. amyloliquefaciens* PFB-02 was investigated by substituting glucose in the production media with 5.0 g/L of black-eyed bean skin, rice bran and wheat bran, respectively. The pH of the production medium in each study was adjusted to 5.0 and sterilized in an autoclave for 15 min and allowed to cool before it was inoculated with 24 hour old seed culture of *B. amyloliquefaciens* PFB-02. This was incubated at 40 °C for 72 h in a shaking incubator at 180 rpm. Growth of *B. amyloliquefaciens* PFB-02 and phytase production were monitored over the 72 h cultivation period at 6, 12, 24, 36, 48, 60 and 72 h durations. At the end of each cultivation duration, the absorbance was taken at 600 nm to determine the microbial growth and the culture was centrifuged 10,000 g for 15 min at 4 °C before assaying for phytase activity according to the standard method earlier described.

RESULTS AND DISCUSSION

Growth Kinetics and Phytase Production from B. *amyloliquefaciens* PFB-02

Growth kinetics of *B. amyloliquefaciens* PFB-02 and phytase production were studied to determine the cultivation period that favours maximal yield of enzyme and evaluate the effect of growth of organism on phytase production. Growth of organism was exponential from 12 to 48 h, followed by a deceleration phase (Figure 1). Phytase production was exponential between 24 and 36 h however optimum enzyme production was recorded at 48 h which corresponds to the time favouring maximum growth of *B. amyloliquefaciens* PFB-02. Phytase production from *B. amyloliquefaciens* PFB-02 is clearly found as growth dependent. The observed deceleration phase of bacterial growth after 48 h is due to the exhaustion of nutrients in the cultivation medium (Leroy and De Vuyst, 2011). Reduced phytase production by *B. amyloliquefaciens* PFB-02 after 48 h might be associated with synthesis of secondary metabolites in the medium which

repressed the synthesis of phytase from the bacteria under study. There is intimate relationship between primary metabolic pathway and secondary metabolism in some bacterial strains as demonstrated in the study by Craney *et al.* (2012).

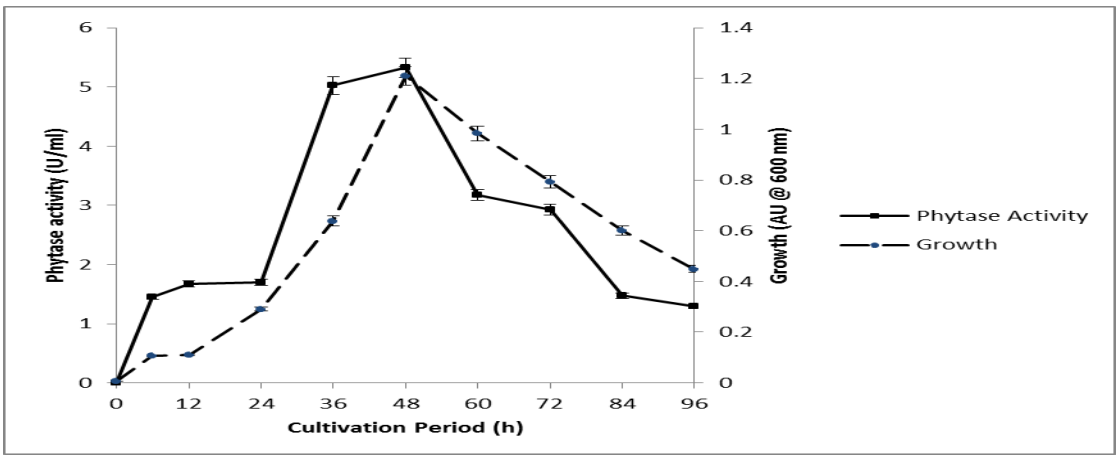


Figure 1: Growth kinetics and phytase production from *Bacillus amyloliquefaciens* PFB-02 over 96 hour cultivation period. Phytase activity (U/ml) was used to measure phytase production. Symbols and bars represent mean values and standard deviations of triplicate determinations.

Effect of pH on Growth and Phytase Production from B. *amyloliquefaciens* PFB-02

B. amyloliquefaciens PFB-02 grew maximally over a narrow pH range of 4.0 to 6.0 with highest growth recorded at pH 5.0 (Figure 2). Surprisingly, phytase production was observed over a broad pH range of 3.0 to 9.0 with optimal yield of 5.16 U/ml at pH 5.0 at 48 h of cultivation (Figure 2). Gulati *et al.* (2007) reported similar optimum pH of 5.5 for phytase production from *Bacillus laevolacticus* however, the enzyme yield of 1.80 U/ml from *B. laevolacticus* was relatively low when compared to the yield of phytase obtained from *B. amyloliquefaciens* PFB-02 in this study. On the contrary, Demirkan *et al.* (2014) and Fu *et al*. (2011) reported optimum phytase production from *Bacillus sp.* at alkaline pH values of 7.5 and 8.0. It can be speculated that phytase production from *Bacillus* spp at specific pH is source and strain dependent (Sun *et al.,* 2013; Papke and ward, 2004).

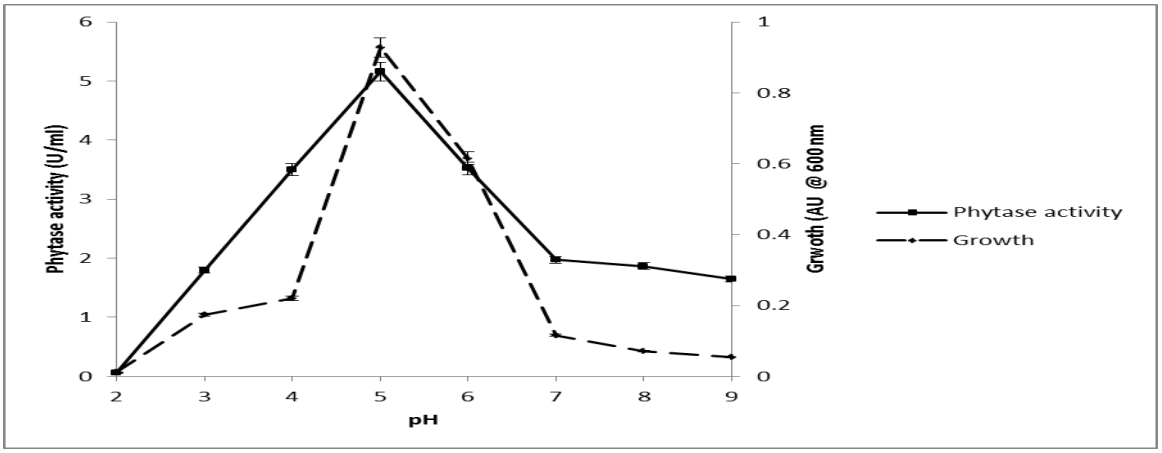


Figure 2. Effect of pH on growth and phytase production from *Bacillus amyloliquefaciens* PFB-02 over 48 h cultivation period. Phytase activity (U/ml) was used to measure phytase production from *Bacillus amyloliquefaciens* PFB-02. Symbols and bars represent mean values and standard deviations of triplicate determinations.

Effect of Temperature on Bacterial Growth and Phytase Production from B. *amyloliquefaciens* PFB-02

Phytase production from *B. amyloliquefaciens* PFB-02 was studied over temperature range of 30 to 60 °C and growth of the bacteria was also monitored. Growth of *B. amyloliquefaciens* PFB-02 was maximum at 40 °C with drastic growth decline at temperatures above 40 °C. Similarly, *B. amyloliquefaciens* PFB-02 produced phytase maximally in the temperature range of 30 to 40 °C with optimal yield of 5.28 U/ml at 40 °C at the end of 48 h cultivation period (Figure 3). However, phytase production began to decrease at temperatures above 40 °C with relative enzyme production of 83% and 62% at 50 and 60 °C, respectively. This confirms the observation made earlier on growth kinetics of *B. amyloliquefaciens* PFB-02 and phytase production that these two properties are interdependent. The

optimum temperature for phytase production from *Bacillus* spp. is in the range of 35 - 50 °C (Demirkan *et al.*, 2014; Gulati *et al.*, 2007; Kerovuo *et al.*, 1998). *Bacillus sp.* produced phytase optimally at 35 °C (Dermikan *et al.*, 2014) while in an earlier study, *B. subtilis* had highest yield of phytase at 37 °C (Kerovuo *et al.*, 1998). Higher temperature had been reported to support phytase production in *B. laevolacticus* which exhibited optimum enzyme production at 50 °C (Gulati *et al.*, 2007).

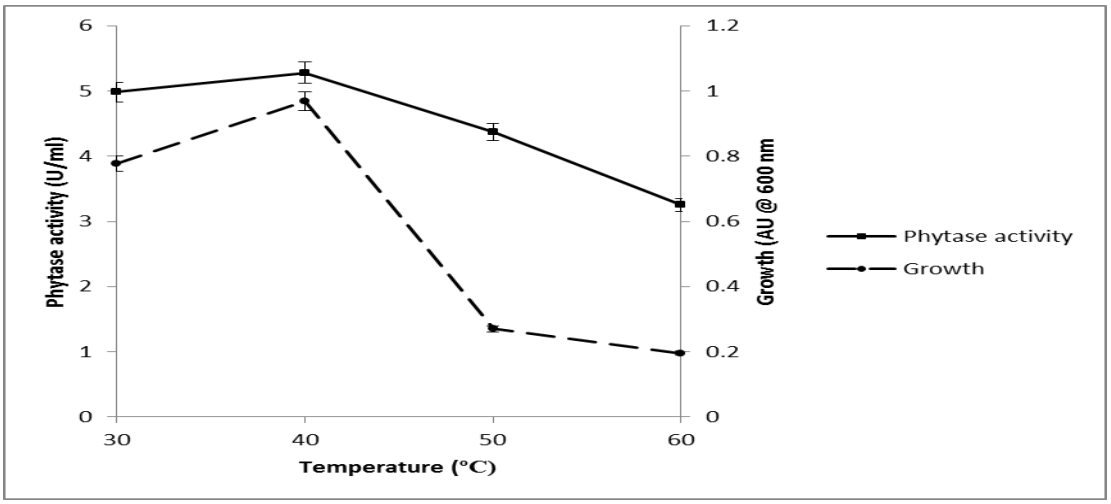
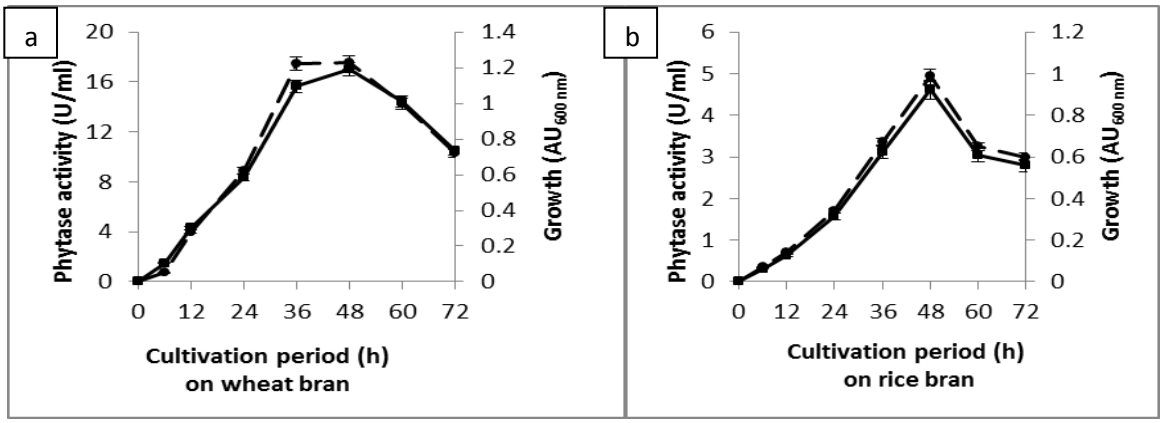


Figure 3. Effect of temperature on growth and phytase production from *Bacillus amyloliquefaciens* PFB-02 over 48 h cultivation period. Phytase activity (U/ml) was used to measure phytase production from *Bacillus amyloliquefaciens* PFB-02. Symbols and bars represent mean values and standard deviations of triplicate determinations.

Influence of Selected Agricultural Wastes on Phytase Production from B. *amyloliquefaciens* PFB-02

Three agricultural wastes, black-eyed bean skin, wheat bran and rice bran were selected as alternative carbon sources for phytase production from *B. amyloliquefaciens* PFB-02. The selection was based on their ready availability and cost effectiveness. All the carbon sources supported growth and phytase production from *B. amyloliquefaciens* PFB-02 when compared with control which had glucose as carbon source (Figure 4). Wheat bran was the best carbon source for phytase production from *B. amyloliquefaciens* PFB-02 supporting maximum yield of phytase (17 U/ml) followed by rice bran which supported enzyme yield of 4.6 U/ml. The lowest yield of phytase (3.5 U/ml) was obtained in the presence of black-eyed bean skin (Figure 4). This result is highly remarkable considering the 317.5 % increase of phytase yield over what was obtained in the basal medium. The yield in the present study is relatively higher than enzyme yield from previous studies which reported wheat flour as the best carbon source for phytase production from certain *Bacillus* species (Demirkan *et al.*, 2014; Idriss *et al.*, 2012; Kim *et al.*, 1998). It is however surprising that in the study by Gulati *et al.* (2007), wheat bran did not support phytase production from *B. laevolacticus*. Results from the present study have clearly demonstrated that the improved phytase production from *B. amyloliquefaciens* PFB-02 over earlier reports is a cooperative effect of optimum physicochemical and nutritional factors. Rice bran and black-eyed bean skin supported phytase production from *B. amyloliquefaciens* PFB-02 with relative yield of 83% and 62%, respectively. Popanich *et al.* (2003) also reported phytase production from a soil bacterium in a medium supplemented with rice bran and soybean meal extract. It is very important to note that this is the first report on black-eyed bean skin as carbon source for phytase production from bacteria which interestingly supported phytase production from *B. amyloliquefaciens* PFB-02.



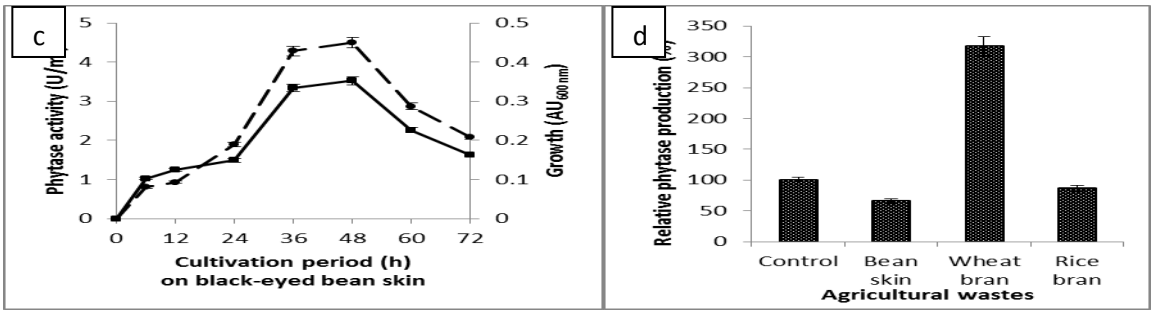


Figure 4. Growth kinetics (**– – –)** and phytase production (---) from *Bacillus amyloliquefaciens* PFB-02 on selected agricultural wastes (a) wheat bran based medium at 40 °C and pH 5.0, (b) rice bran based medium at 40 °C and pH 5.0. and (c) bean skin based medium at 40 °C and pH 5.0. (d) Relative phytase production from *Bacillus amyloliquefaciens* PFB-02 grown on selected agricultural wastes under defined and optimized physicochemical conditions. Phytase activity (U/ml) was used to measure phytase production from *Bacillus amyloliquefaciens* PFB-02. Symbols and bars represent mean values and standard deviations of triplicate determinations.

CONCLUSION

B. amyloliquefaciens PFB-02 is a good producer of phytase under optimized physicochemical and nutritional conditions defined in this study. Results from this study suggest that agricultural wastes which are readily available and affordable can be used as alternative carbon sources for phytase production from *B. amyloliquefaciens* PFB-02. Studies are in progress to investigate the effects of varying concentration of the selected agricultural wastes and possible inducers on yield of phytase with detailed characterization of the enzyme. Ultimately, our results should enhance reduction of production cost and improve phytase yield for industrial and biotechnological purposes especially in animal feed formulation.

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