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Green reduction of phytic acid in *Artocarpus heterophyllus* (Jackfruit) seed using phytase from *Aspergillus aculeatus* JF3

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Microbial phytase enhances food quality by hydrolysing phytate into simpler derivatives and releasing elements that serve as macroelements. Fungi were isolated from the pulp and seeds of jackfruit on potato dextrose agar. The fungi were screened for phytase production, and the best phytase producer was selected and identified molecularly using the ITS region. Phytase production by the selected fungus was optimised. The effects of temperature, pH, and cations on the produced phytase were determined. Jackfruit was treated with the selected fungus, and proximate analyses of both treated and untreated jackfruits were carried out. The top phytase producer, with a hydrolytic index of 2.67, was identified as *Aspergillus aculeatus* JF3, with accession number OQ992634. Maximum phytase production was recorded in a medium containing Na^+, sucrose, peptone, and Triton X-100. Phytase activity of *Aspergillus aculeatus* JF3 was relatively stable between 35 and 85°C and from pH 3 to 9. Iron II positively influenced the phytase activity of *Aspergillus aculeatus* JF3. The phytic acid content of jackfruit treated with *Aspergillus aculeatus* JF3 decreased by 18% compared to the untreated samples. The phytase-producing *Aspergillus aculeatus* JF3 isolated from *Artocarpus heterophyllus* exhibited stability across different temperatures and pH levels, enhancing nutrient bioavailability in *Artocarpus heterophyllus*.

Keywords: *Aspergillus* species, Enzyme characteristics, Hydrolysis, Phytate, Proximate analyses

The phosphorus stored in seeds, nuts, legumes, and unprocessed whole grains (concentrated in the outer bran layers) is called phytic acid, or phytates[1]. These foods vary significantly in their phytic acid content, even within the same food, depending on the type of seed used, the climate, the environment, and the soil quality[2]. The inhibition of iron, magnesium, zinc, and calcium absorption by phytic acid has drawn much interest because it may lead to mineral deficiencies. Consequently, phytic acid is often described as an anti-nutritional factor that coexists with normal rumen contents and other animal diets. Additionally, releasing a large amount of phosphate from phytic acid into soil or water could cause eutrophication[3].

Phytases are hydrolases that liberate phosphate from phytic acid, thereby making phosphate, divalent cations (calcium, magnesium, zinc, and iron), and myo-inositol available for digestion and absorption[3,4]. Naturally, fungi produce phytases because of their enzymatic ability to withstand varying temperatures, pH, and other harsh environmental conditions. *Aspergillus* species have been reported several times in the production of phytases[2,4-9].

Jackfruit (*Artocarpus heterophyllus*) is a rich source of nutrients and minerals[10] that can serve as feed for animals instead of other highly sought-after foods for human consumption[11]. It is also abundant in phosphorus, which is stored as phytic acid in its pulp and seed. Despite the richness of nutrients in jackfruit[10], phytic acid in it complexes with metals, proteins, carbohydrates, lipids, and minerals[3,4], thereby hindering nutrient bioavailability and making it difficult to access the nutrients embedded in this fruit. Therefore, the full potential of the fruit can be better harnessed after hydrolysing the phospho-monoester bonds of phytic acid to release inorganic phosphate, less phosphorylated myo-inositol, and the associated proteins and divalent metal ions[12]. Existing methods for reducing phytate are neither eco-friendly nor cost-effective. Thus, further research is required on phytase-producing organisms whose

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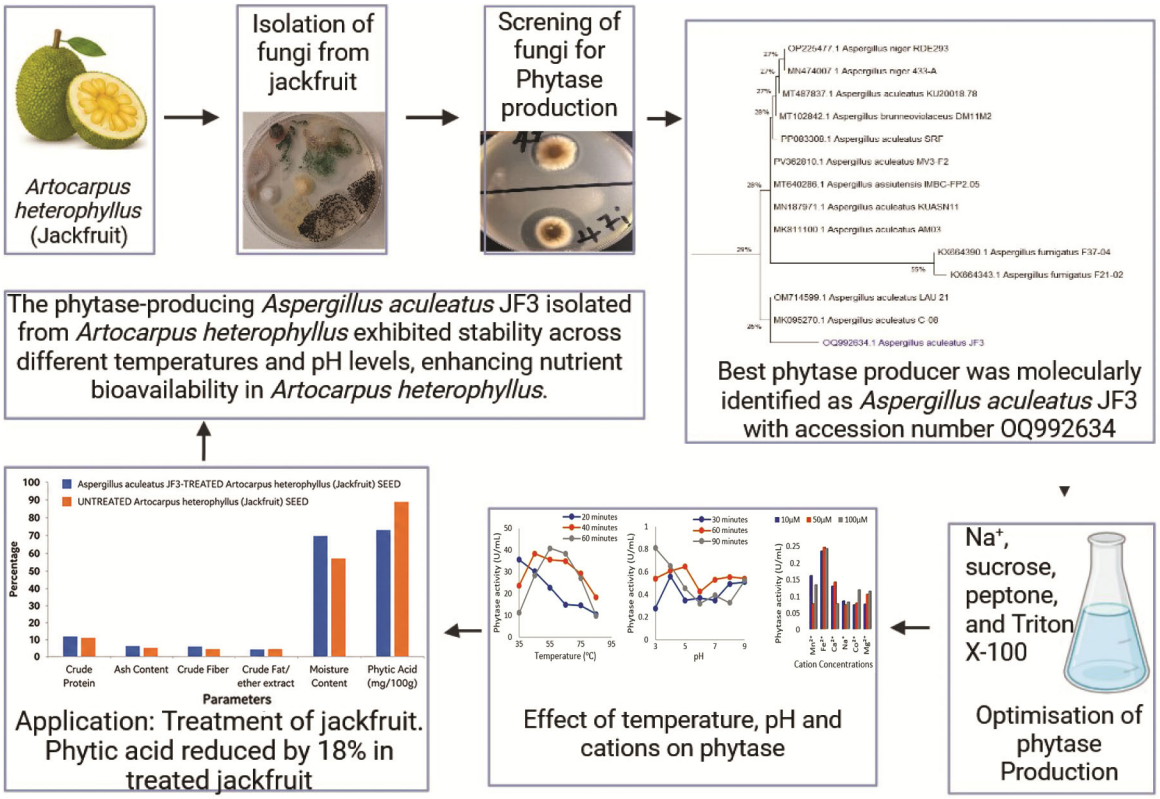

Graphical abstract

enzymes, which are eco-friendly, can be utilised in relevant industrial processes to cleave these bonds without affecting other components[12]. The work focused on isolating phytase-producing fungi from jackfruit samples, optimising their phytase production conditions, and reducing the proximate phytic acid content of jackfruit treated with selected phytase-producing fungi in a solid-state fermentation process.

Materials and Methods

Isolation of Filamentous Fungi

Filamentous fungi were isolated from the pulp and seed of spontaneously degrading jackfruits collected from the Botanical Garden of the University of Ibadan and as a kind gift from a colleague residing at Akobo, Ibadan, using the pour plate method on potato dextrose agar. Degrading samples were also carefully placed on solidified potato dextrose agar plates, and Petri dishes were observed for fungal growth over seven days.

Screening for Phytase

The screening medium is known as Phytate Screening Medium (PSM), which contained the following in g/L: glucose 20, Na-phytate 4, $CaCl_2$ 2, NH_4NO_3 5, KCl 1.0, $MgSO_4.H_2O$ 1.0, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, Agar 15. The screening medium was steam-sterilised at 121°C for 15 min and allowed to cool. Next, the medium was inoculated with the isolated fungi and incubated at 28±2°C for 72 h. A positive screening occurs when a clear zone forms around the fungus that could produce phytase on the screening medium. Phytase-selective agar plates were sequentially washed with various solutions to determine whether the zones were due to phytase action or organic acid generation during microbial fermentation. First, the screening plates were flooded with a 2% cobalt chloride solution and left to sit at room temperature (28±2°C) for 5 min. They were then removed and flooded with a freshly made combination of 6.25% ammonium molybdate and 0.42% ammonium vanadate. The solution was discarded after incubating the plates for 5 min at room temperature, and plates with persistent zones of clearance were considered to contain phytase-producing fungi, with zones measured in millimetres[6]. The Solubilisation Index (SI) was calculated as a ratio of the sum of the fungal diameter and hydrolysis zone to the fungal diameter.

$$SI = \frac{(\text{Colony diameter} + \text{Halo diameter})}{\text{Colony diameter}}$$

Phytase production

The phytase production medium was made up of the Phytate Screening Medium (glucose 20, Na-phytate 4, $CaCl_2$ 2, NH_4NO_3 5, KCl 1.0, $MgSO_4.H_2O$ 1.0, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, in g/L) but devoid of the agar component and used in a submerged fermentation process. Fungi that exhibited high zones of phytase production during screening were selected for submerged fermentation. For each production parameter, 25 mL of the amended medium was sterilised at 121℃ for 15 min, allowed to cool, inoculated with two 7 mm agar plugs of the selected fungus, and incubated at 28±2℃ for 96 h. The production medium was filtered, and the separated mycelium was dried to a constant weight at 80°C to determine the influence of culture composition on mycelial proliferation. Phytase contained in the cell-free production broth was quantified as described in the modified phytase assay[13]. Enzyme (125 µL) was added to 125 µL of 1.5 mM sodium phytate in sodium acetate buffer (0.1 M, pH 5.0). The mixture was incubated in a water bath at 55℃ for 30 min, and the reaction was stopped by the addition of 0.25 mL of 15% trichloroacetic acid. The released inorganic phosphate was measured by incubating the entire mixture with 2 mL of water, 2.5% ammonium molybdate, 6 N H_2SO_4, and 10% ascorbic acid in a 2:1:1:1 ratio at 55℃ for 30 min and then allowed to cool to room temperature. A spectrophotometer was used to measure absorbance at 700 nm. One unit of phytase activity was defined as the quantity of enzyme needed to release one unit of phosphate per minute under assay conditions.

Phylogenetic analysis

Phytase-producing fungi were initially identified by their colour on the plate, micromorphology, conidial characteristics, and sporulating structure[14,15]. The best phytase-producing fungus was molecularly identified by its ribosomal Internal Transcribed Spacer (ITS) region[16,17]. The DNA was extracted, amplified, and sequenced[16,17]. The sequence was compared with the National Centre for Biotechnology Information database for identification and submitted for an accession number[16,17]. The maximum likelihood method was utilised in MEGA 11 to analyse the molecular phylogenetic diagram[18,19].

Effect of carbon and nitrogen source, metal ions, and surfactants on phytase production

Different carbon sources (20 g/L each of glucose, maltose, sucrose, fructose, or mannose) were substituted in the fermentation medium to determine their influence on phytase production. The impact of various nitrogen sources on phytase production was assessed using 5 g/L NH_4NO_3, NH_4SO_4, KNO_3, Peptone, and NH_4Cl in the fermentation medium. The effects of 10-100 mM concentrations of chloride salts of metal ions (Na^+, Mn^{2+}, K^+, and Ca^{2+}) in the fermentation medium on phytase production were evaluated. The fermentation medium was supplemented with 10-100 mM concentrations of Tween 80, Urea, and Triton X-100 for surfactants to examine their effects on phytase production.

Effect of temperature on phytase activity over different incubation periods

The effect of various temperatures and incubation periods on phytase stability and activities was determined by incubating a mixture of the enzyme and sodium phytate in sodium acetate buffer, as previously described in the phytase assay, at 35, 45, 55, 65, 75, and 85℃ for 20, 40, and 60 min. Phytase activity was assessed according to the method outlined in the phytase assay.

Effect of pH on phytase activity

The effect of pH on phytase activity was determined using sodium phytate prepared in acetate buffers (pH 3-5), citrate-phosphate buffer (pH 6-8), and Tris-HCl buffer (pH 9). Equal volumes of the buffered sodium phytate were incubated with the fungal phytase for 30 min, followed by phytase assay.

Effect of cations on phytase activity

The influence of different cations (Mn^{2+}, Fe^{2+}, Ca^{2+}, Na^+, Co^{2+} and Mg^{2+}) at varying concentrations (10, 50 and 100 µM) on phytase activity was assessed by incubating equal volumes of phytase enzymes with these cations. Phytase activity was measured as described in the phytase assay.

Phytate degradation in milled *Artocarpus heterophyllus* seed and proximate analysis

Phytate degradation was examined in milled jackfruit seeds using solid substrate fermentation. Five grams (5 g) of milled jackfruit seed samples were sterilised in a conical flask plugged with cotton wool, containing 10 mL of a mineral solution used during phytase screening at pH 5.0. The set-up was allowed to cool, inoculated with two 7 mm agar plugs

of the selected fungus, and incubated for 5 days. The proximate analysis of the uninoculated and the fungus-treated seed was then conducted to determine the effect of the fungus on the phytate content of the seed. The crude protein, crude fat/ether extract, dry matter and moisture content, ash content, and crude fibre were analysed chemically according to the methods described by the Association of Official Analytical Chemist[20]. All analyses were carried out in duplicate.

Results

Isolation, screening, and identification of phytase-producing filamentous fungi

Filamentous fungi isolated from decaying jackfruit pulp and seed samples ranged from 2.5×10^5 to 9.0×10^6 and 4.6×10^4 to 1.2×10^6, respectively, as shown in (Table 1). Seven out of the initially selected (23) isolates, which showed zones of hydrolysis after the counterstaining assay, are reported in (Table 2). The three isolates with the highest phytase activities were selected, and their solubility index, quantitative phytase assay, and probable identities were reported (Table 2). Isolate JF3, which exhibited the highest hydrolytic index of 2.67, was identified molecularly using the ITS region as *Aspergillus aculeatus* JF3 with accession number OQ992634. It was thus selected for enzyme studies. Figure 1 showed that *Aspergillus* JF3 is closely related to *Aspergillus aculeatus* LAU 21 and *Aspergillus aculeatus* C-08 but distantly related to *Aspergillus flavus* FMB 0222.1, *Aspergillus fumigatus* F37-04 and *Aspergillus niger* 433-A.

Table 1 — Isolation of filamentous fungi from decaying jackfruit and seed samples

Jackfruit Sample	Pulp Sample (CFU/g)	Seed Sample (CFU/g)
A	2.5×10^5	4.6×10^4
B	2.3×10^5	1.3×10^5
C	9.0×10^6	1.2×10^6

Effect of carbon and nitrogen source, metal ions, and surfactants on phytase production

In Figure 2A, different carbon sources influenced the growth of *A. aculeatus* JF3 and its phytase production. The addition of glucose to the culture medium resulted in the highest mycelial weight (0.114 g), followed closely by fructose (0.109 g) and mannose (0.109 g), while the lowest mycelial weight (0.077 g) was recorded when maltose was the carbon source. Phytase production was most enhanced in a medium supplemented with sucrose (30.020 U/mL), whereas the least phytase production was recorded in a maltose-supplemented medium (13.286 U/mL).

Figure 2B illustrates the effect of varying nitrogen sources on the growth of *A. aculeatus* JF3 and its phytase production. The highest mycelial weight (0.128 g) was recorded with NH_4NO_3, as a nitrogen source, which was closely followed by $(NH_4)_2SO_4$ (0.119 g), while the lowest mycelial weight (0.079 g) was observed when peptone was used as the nitrogen source. The highest phytase production (26.852 U/mL) was observed in a medium containing peptone, followed by potassium nitrate (24.192 U/mL). The lowest phytase production (8.352 U/mL) was recorded in the medium containing NH_4NO_3.

The addition of different cations to the culture medium of *Aspergillus aculeatus* JF3 affected mycelial weight and phytase production of the organism (Fig. 2C). The highest mycelial weight (0.112 g) was recorded when K^+ was added to the culture medium, closely followed by the addition of Na^+ (0.106 g), while the lowest (0.081 g) was observed with the addition of Ca^{2+}. The introduction of monovalent metal ions (K^+ and Na^+) resulted in higher mycelial weight than the addition of divalent metal ions (Mn^{2+} and Ca^{2+}). Phytase production, however, was most enhanced in a medium supplemented with Na^+ (44.51 U/mL), whereas the least production (9.00 U/mL) was recorded in a Ca^{2+}-supplemented medium.

Table 2 — Qualitative screening for phytase activity

Microbial code	Fungal diameter on Day 3	Zone of fungal colony + hydrolysis (mm)	Solubilisation Index (SI)	Probable Identity
JF3	15	40	2.67	*Aspergillus* sp.
JF47i	17	33	1.94	*Aspergillus niger*
JF 47	7	10	1.43	*Aspergillus* sp.
JF2	7	8	1.14	*Aspergillus* sp.
JP2	7	8	1.14	ND
JP6	7	8	1.14	ND
JP7	7	8	1.14	ND

Key: 'ND' is "Not Determined"

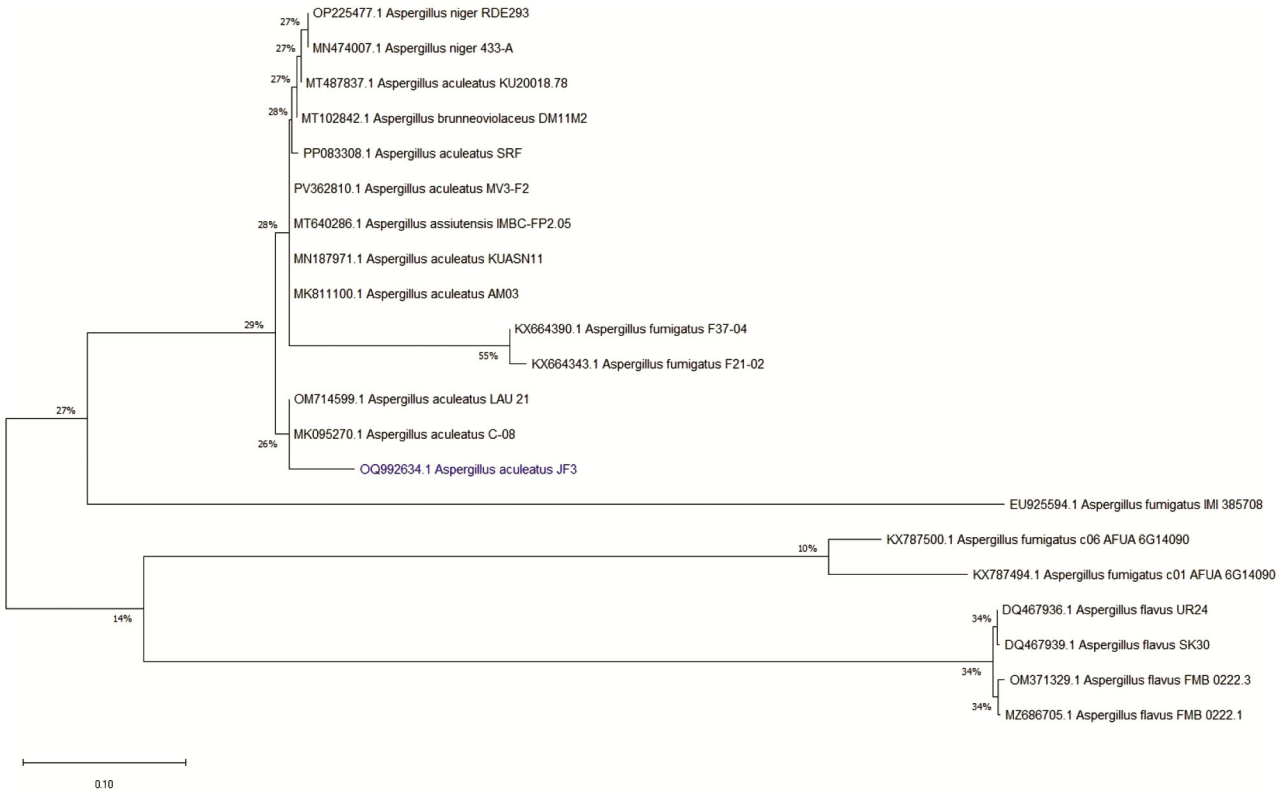

Fig. 1 — Phylogenetic analysis of *Aspergillus aculeatus* JF3

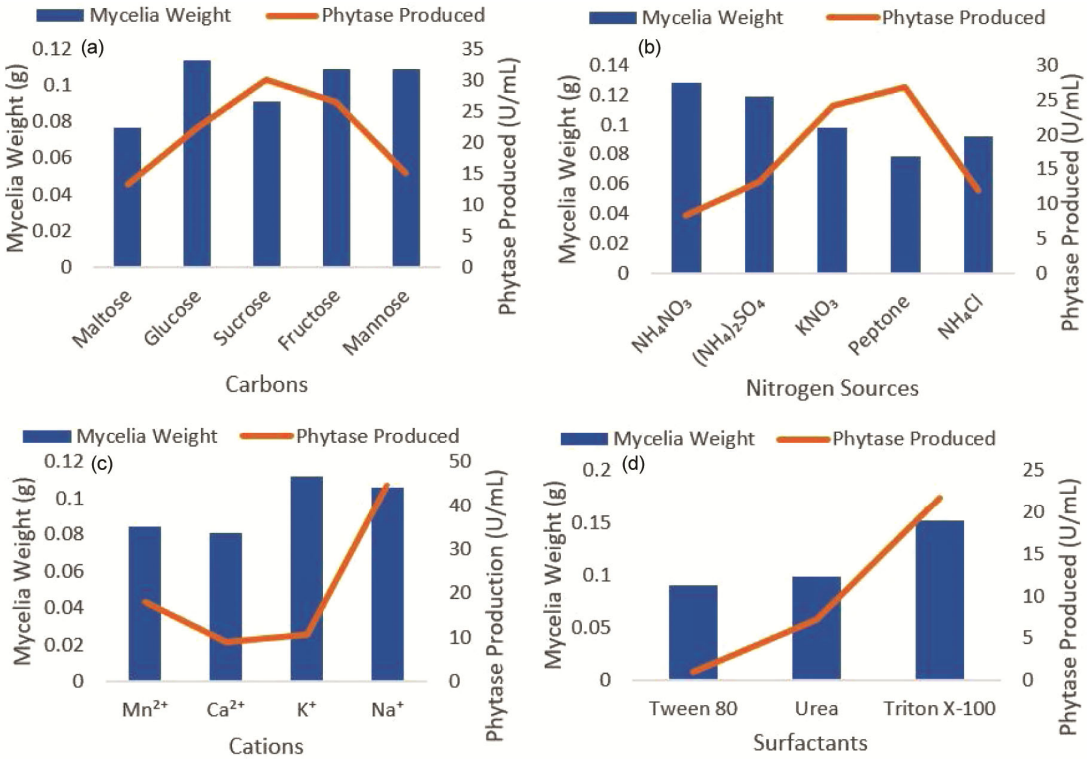

Fig. 2 — Effect of different (A) carbon, (B) nitrogen, (C) cations and (D) surfactants on the phytase produced and biomass weight by *Aspergillus aculeatus* JF3

Among the surfactants used to supplement the fermentation medium of *A. aculeatus* JF3, Triton X-100 supported the highest mycelial weight (0.152 g), followed closely by Urea (0.099 g) and Tween 80 (0.090 g) (Fig. 2D). Phytase production followed the same order as that of the mycelial weight with its highest yield (21.653 U/mL) in Triton X-100 and the lowest (7.163 U/mL) in Tween 80.

Effect of temperature, pH, and cation on phytase activity

The effect of varying temperatures (35, 45, 55, 65, 75, and 85°C) and incubation periods (20, 40, and 60 min) on phytase stability and activity is shown in (Fig. 3A). When phytase was incubated for 20 min, the highest phytase activity (35.531 U/mL) was recorded at 35°C, which then decreased with increasing temperature. The highest (38.388 U/mL) and lowest (18.224 U/mL) phytase activities recorded during 40 min of incubation were at 45°C and 85°C, respectively. Incubation for 60 min revealed the highest phytase activity (40.837 U/mL) at 55°C, while the lowest activity (9.612 U/mL) was recorded at 85°C.

Figure 3B shows the effect of pH and time on the activity of phytase from *Aspergillus aculeatus* JF3. The highest phytase activities were observed at pH 4 (0.56 U/mL), pH 5 (0.646 U/mL), and pH 3 (0.81 U/mL) after incubation times of 30, 60, and 90 min, respectively. The activity of phytase from *Aspergillus aculeatus* JF3 at various pH levels remained relatively stable during a 60 min incubation, except for the activity measured at pH 6 (0.425 U/mL), which was lower than those at the other pH levels after 60 min of incubation.

The influence of varying concentrations of cations on the phytase stability and activity is depicted in (Fig. 3C). The Fe^{2+} at a concentration of 50 µM supported the highest phytase activity (0.242 U/mL), and the enzyme activity with Fe^{2+} was most stable at all three concentrations 10, 50, and 100 µM. The Na^{+} and Co^{2+} showed the lowest phytase activity (0.072 U/mL) at 50 µM and 10 µM, respectively.

Phytate degradation in milled *Artocarpus heterophyllus* seed and proximate analysis

The proximate analysis of phytase-treated and untreated jackfruit seeds is shown in (Fig. 4). The crude protein of phytase-treated jackfruit seeds (11.37%) was higher than that of untreated jackfruit seeds (10.47%). The ash content (6.30%), crude fibre (5.20%), crude fat (2.54%), and moisture content (68.52%) of jackfruit seeds treated with phytase from *Aspergillus aculeatus* JF3 exceeded their respective values (2.20%, 3.97%, 3.33%, and 55.99%) in untreated jackfruit seeds. The phytic acid (72.52%) of treated jackfruit seeds is less than the phytic acid level (87.91%) found in phytase-treated jackfruit seeds.

Discussion

Phosphorus in phytic acid is not readily utilised because it is not easily accessible. This has prompted further research into enzymes capable of degrading phytic acid, which could be applied to break down phytate in phytic acid-containing substrates to release digestible feed. The presence of fungi in jackfruit pulp and seed samples indicates that these organisms could be potential phytase producers, as jackfruit contains a substantial quantity of phytic acids that phytase can break down to release phosphates and minerals[21,22]. The observation of a clear zone around the screened organism on the plate might result from acid production, but a clear zone after counterstaining with

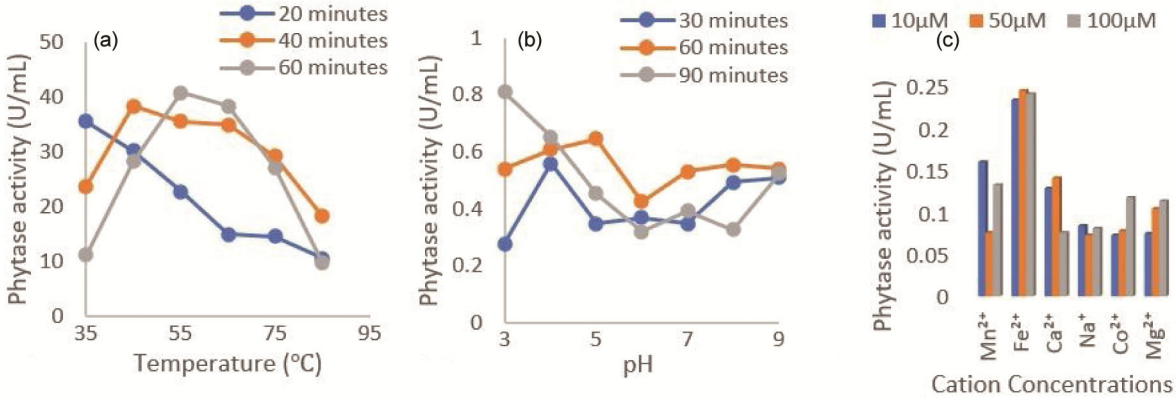

Fig. 3 — Effect of (A) temperature and time (B) pH and time, and (C) cation concentrations on the activity of phytase from *Aspergillus aculeatus* JF3

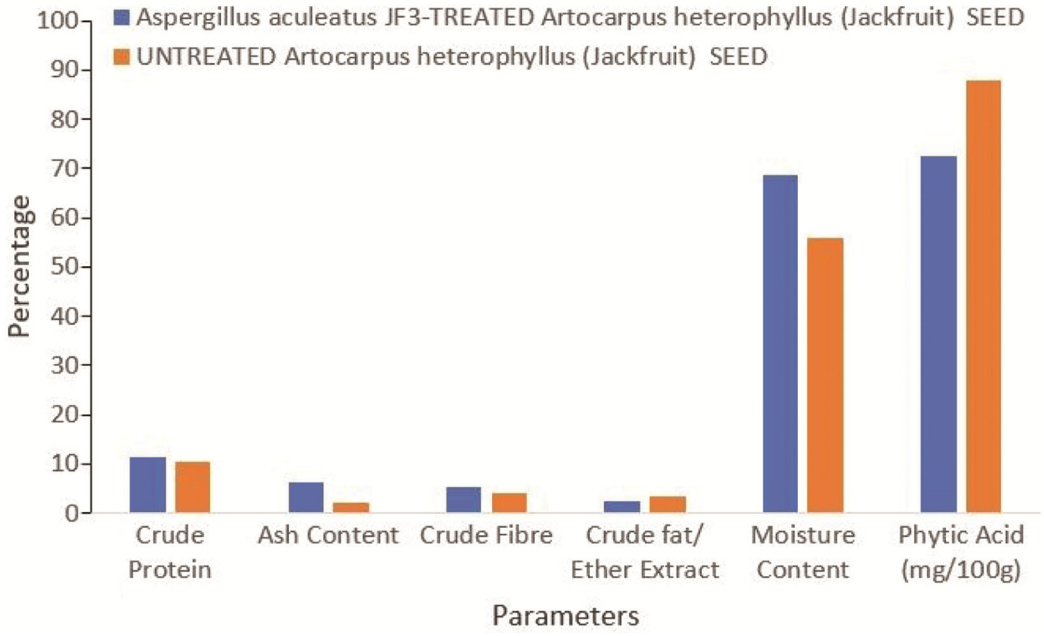

Fig. 4 — Proximate analysis of *Aspergillus aculeatus* JF3-treated and untreated jackfruits seeds

cobalt chloride and ammonium molybdate/ammonium vanadate solutions confirmed that it was due to phytase production. This work showed that the molecularly identified *Aspergillus aculeatus* JF3 is a phytase-producing fungus. Many *Aspergillus* species have also been reported as phytase producers[4,5,7-9], and this research contributes to the sparse body of knowledge on phytase-producing *Aspergillus aculeatus* strains.

Carbon as an energy source influences the growth of organisms and the production of enzymes. Glucose positively impacted the growth of the phytase-producing *Aspergillus aculeatus* JF3, similar to the findings that reported glucose as the most effective among various carbon sources for supporting the growth of phytase-producing *Thermomyces lanuginosus* and *Talaromyces luteus*[23]. The maximum yield of phytase obtained in this study with glucose as the carbon source aligns with the work of researchers[24,25] who utilised wild-type *Aspergillus oryzae* and *Aspergillus tubingensis* to produce phytase. Some reports showed that the highest yield of phytase from *Rhizomucor pusillus* and mutant-type *Aspergillus oryzae* was obtained when starch was employed as the carbon source[23,24]. Variations in the yield of phytases reported by different researchers using the same carbon source could be attributed to the organism's genetic makeup or environmental conditions.

The influence of nitrogen sources on the growth of organisms and the production of enzymes has been established[25-27]. The nitrogen source (peptone) that supported the least growth of *Aspergillus aculeatus* JF3 had the most significant positive influence on phytase production in this study. This aligns with findings that reported the highest production of phytase with peptone as a nitrogen source, while this same peptone resulted in the least growth for various strains of bacteria (B9, L3, and RT)[26]. However, the results obtained in this study contrast with a work, which recorded the highest phytase production with the same nitrogen source that supported the highest growth[23]. Inorganic nitrogen source (ammonium sulphate) was identified as the most effective for producing phytase by *Aspergillus oryzae*[5,24]. Additionally, another work reported peptone as the optimal nitrogen source among those tested for producing phytase by *Aspergillus tubingensis*[25]. The production of phytase by *Aspergillus aculeatus* JF3 was influenced by the presence of metal ions. In this research, Na^{+} supported the highest phytase enzyme production, followed by Mn^{2+}, whereas the lowest production occurred with a medium containing Ca^{2+}.

According to reports, surfactants enhance membrane permeability and alter lipid metabolism to improve enzyme secretion, resulting in a positive impact on enzyme synthesis[28]. Among the surfactants examined, higher phytase production was observed in

the medium containing Triton X-100 than in the medium containing Tween 80, which contradicts the findings of a study reporting greater phytase production by *Aspergillus niger* during fermentation with Tween 80 than with Triton X-100[28].

The observed activities of the phytase from *Aspergillus aculeatus* JF3 at different temperatures and times indicate that the enzyme activity is relatively stable. With varying incubation times, the maximum activity of phytase from *Aspergillus aculeatus* JF3 ranged between 35 and 65℃, which aligns with reports from several studies[4,7,28]. A similar report recorded the maximum activity of phytase from different organisms at 45 and 55℃[9], while another obtained the highest activity at 60℃[28].

Phytases used in the feed industry are expected to remain active at the pH levels found in the digestive systems of animals (pH 2 – 4)[7]. The maximum phytase activity of *Aspergillus aculeatus* JF3 was observed at pH 3, indicating that this phytase can effectively degrade phytic acid-containing substrates into feed that animals can absorb. The ability of the phytase from *Aspergillus aculeatus* JF3 to function across a broad pH range suggests that the enzyme can also be applied in varying pH conditions.

Metal ions positively influenced phytase activity in this study, likely by enhancing interactions between the enzyme's active site and its substrates. The most effective enhancer for the phytase activities of *Aspergillus aculeatus* JF3 was Fe^{2+}. A similar enhancement in the presence of Fe^{2+} ion was noted in the enzymatic characterisation[29]. In contrast to the findings of this study, where Fe^{2+} significantly bolstered phytase activity, and Co^{2+} exhibited the least positive effect on phytase performance, Fe^{2+} inhibited phytase activity while Co^{2+} greatly enhanced the activity of phytase produced by *Aspergillus niger* NT7[28].

The reduction observed in phytic acid content in jackfruit treated with *Aspergillus aculeatus* JF3 demonstrates that this phytase-producing fungus can effectively break down phytic acid in phytate-containing substrates. This decrease in phytic acid content following treatment indicates a reduction in the antinutritional content of jackfruit, aligning with some findings where a decrease in phytate content in jackfruit after pretreatment was observed[30,31]. An increase in crude protein, ash content, and crude fibre in jackfruit treated with *Aspergillus aculeatus* JF3 signifies that this fungus was able to enhance the nutrient content of jackfruit.

Conclusion

Phytase-producing *Aspergillus aculeatus* JF3 was isolated from jackfruit. The optimum production of phytase by *Aspergillus aculeatus* JF3 was observed when sucrose served as a carbon source, peptone as a nitrogen source, Na^+ as a cation, and Triton X-100 as a surfactant. Temperature, pH, and cations influenced the phytase activity of *Aspergillus aculeatus* JF3. The ability of *Aspergillus aculeatus* JF3 to reduce phytic acid and increase crude protein, ash content, and crude fibre in jackfruit establishes it as a potential organism for breaking down phytic acid-containing substrates, thus releasing its nutrients.

Conflict of interest

All authors declare no conflict of interest.

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