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# Poly(vinyl alcohol)-based Electrospun Nanofibers: Characterization and Phytase Immobilization

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**Abstract:** The aim of this study is to immobilization phytase obtained from cowpea seeds into nanofiber-based on poly(vinyl alcohol) (PVA) and to investigate kinetic properties, optimal pH, and temperature of free and immobilized phytase. The structural analysis and morphological properties of the nanofibers are carried out via SEM and XRD. The results indicated that enzyme stability, pH, and thermal stability are increased after immobilizing phytase into the nanofiber. The optimum pH and temperature of the free and immobilized phytase are found as pH 5.0 and 45-65 $^{o}$C, respectively. These results indicated that the immobilized phytase could be a good candidate for agriculture, animal feed, food, and medical applications.

**Keywords:** phytase; cowpea seeds; biocatalyst; nanofiber.



## 1. Introduction

Catalysts are materials that accelerate any reaction without being consumed. Recently, most reactions are effectively performed in the presence of a catalyst, and the efficiency of a complex reaction can be enhanced by using a suitable catalyst. They can be classified in mainly four groups such as biocatalyst, inorganic, organic, and organometallic catalysts [1,2]. Among these catalysts, biocatalysts or enzymes that originated from a natural source can be catalyzed biochemical reactions. They have exhibited superior behaviors in terms of working conditions such as pH and temperature [3,4]. Also, they have shown good substrate selectivity performance, high efficiency, and productivity with very low energy consumption compared to the other catalysts [5].

Phytase (myo-inositol hexaphosphate phosphohydrolase) is one of the most noticeable enzymes in human health, environmental protection, and nutrition [6,7]. Phytase as a biocatalyst is an enzyme that catalyzes the hydrolysis of myo-inositol phosphates, Myo-inositol, and inorganic phosphate by producing *ortho*-phosphate groups from the inositol core of phytate [8]. According to the literature, phytase has also been widely used in the human diet, food industry, agriculture, and pharmaceutical sectors [9,10]. Moreover, this biocatalyst's most preferred industrial application is a feed supplement for some animals such as fish, pigs, and poultry [11]. On the other hand, it is a thermally unstable enzyme that loses its enzymatic activity at high temperatures [12]. The use of phytase in the industry is unfavorably affected due to the thermally unstable property. Therefore, there is still a need to obtain phytase thermally stable.

Microbial origin enzymes have been generally used in industrial applications of phytase. Therefore, there is a need to develop more economical and effective new sources of phytase. In this study, a new plant origin enzyme source was used for the purification of phytase.

Electrospinning is a versatile technique used to fabricate micro to nanoscale fibers by using inorganic materials, polymers, ceramic, and composites [12-15]. Electrospun fibers have superior properties such as large surface area, biocompatibility, non-toxicity, and porosity [16-18]. The fabricated electrospun fibers also have a wide range of applications such as biomedical, biological dressing, drug release, tissue engineering, membrane, catalysis, and enzyme immobilization [19,20]. Immobilization of the enzyme on nanomaterials gains strong stability to the enzyme and protects the enzyme against leaching and 3-D structure loss [21]. Also, the enzyme is placed into the porous structure of the fibers, and the pH or temperature resistance of the enzyme has been improved after the immobilization of the enzyme into the nano/micro-fiber.

In this study, plant-based biocatalyst (cowpea seeds) was used to isolation of phytase, and the isolated enzyme was immobilized into poly(vinyl alcohol) (PVA) nanofibers to obtain recoverable, recyclable, and stable phytase preparations and to improve thermal stability, and catalytic activity of the plant origin phytase. The reason for the new Optimum pH temperature, kinetic parameters such as $K_m$ and $V_{max}$ of the enzymes were also determined, and the obtained results for the free and immobilized enzyme were compared.

## 2. Materials and Methods

### *2.1. Materials.*

Cowpea seeds (*Vigna unguiculata*) were supplied from the local market. Poly(vinyl alcohol) (PVA, molecular weight 60 kDa) was purchased from Merck (Germany). The other chemicals were purchased from Sigma Aldrich (Germany), and they were used without any purification.

### *2.2. Purification of phytase from cowpea seeds.*

10% (w/v) Cowpea seeds solution was prepared in 0.1 M sodium acetate (NaOAc) buffer, and the pH value of the buffer solution was adjusted as 5.0. The solution was retained in a refrigerator at +4 $^{o}$C overnight. The next day, they were homogenized with a Waring blender. After homogenization, the homogenate was filtered using a fine muslin, and it was centrifuged at +4 $^{o}$C for 30 min at 10 000 rpm. After centrifugation, the pellet was cast out, and the supernatant was acted to 60% ammonium sulfate (*w:v*) saturation and the solution was dialyzed by using a dialysis sac, and the dialyzed enzyme was used for phytase activity analysis and was stored at -20 $^{o}$C for further experiment [22].

### *2.3. Preparation of phytase-PVA solution.*

Poly(vinyl alcohol) solution (8%, *w/v*) was prepared in 10 mL 0.1 M NaOAc buffer (pH 5.0). Then, phytase was directly added into PVA solution at different enzyme concentrations (0.5-2.0%; *w/v*), and it was mixed for 1 h using magnetic stirring.

*2.4. Immobilization of phytase via electrospinning.*

Phytase-PVA solution was placed into a 5 mL syringe (0.07 mm diameter needle). The flow rate was adjusted in the range from 0.5 to 2.0 mL/h, and it was controlled via an infusion pump (New Era Pump System Inc.). The distance between the collector plate and the pump was 13 cm, and the applied voltage was adjusted to 23 kV from DC high voltage power source (PW1010, Elektrosis). Also, PVA fiber was fabricated under the following conditions: voltage 23 kV, distance 13 cm, and flow rate 1 mL/h as the control group for characterization (Figure 1).

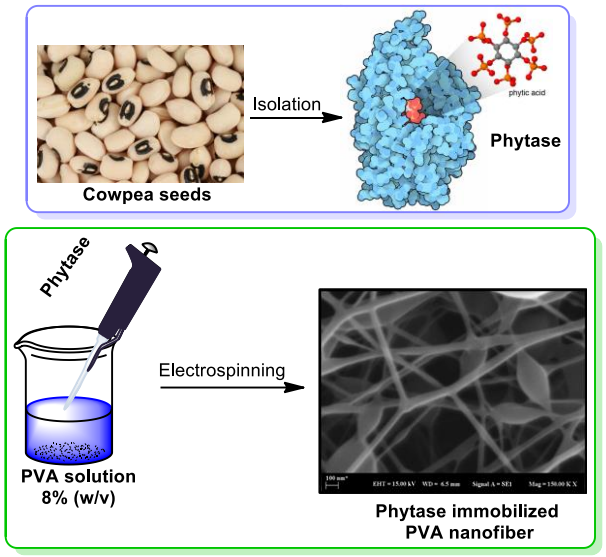


**Figure 1.** Fabrication of the enzyme-containing nanofibers.

*2.5. Measurements.*

The X-ray diffraction (XRD) measurement of PVA and phytase-PVA nanofibers was carried out using an X-ray diffractometer (Panalytical X'Pert PRO XRD, Cambridge, UK). The size distribution of the fibers was determined via Image J using 100 different fiber diameters to determine the average diameter of the fibers. Morphological properties of immobilized phytase into PVA nanofiber were also recorded by an FE-SEM (Zeiss EVO LS 10, Oberkochen, Germany). FT-IR spectra of the nanofibers and sodium phytate were measured using an FT-IR spectrophotometer (Thermo Scientific (Nicolet iS10), Paisley, UK) with an ATR sample unit.

*2.6. Phytase standard assay.*

Free phytase activity was determined as described via the Harland method [23] with slight modification as follows: 2 mM substrate (sodium phytate) solution (100 µL) was prepared in 0.1 M NaOAc buffer (pH 5.0), and 350 µL NaOAc buffer solution was placed into the substrate solution. This mixture was incubated in a water bath for 10 min at 50 $^{o}$C. Then, 150 µL enzyme was added to the mixture containing the substrate. This mixture was incubated for 30 min at the mentioned temperature above. After incubation of phytase with the substrate, the reaction was stopped by denaturing the phytase with 400 µL trichloroacetic acid (15%, *w/v*). The mixture was centrifuged at 5000 rpm for 10 min. To determine phytase activity, 0.2 mL supernatant was stirred with 1.8 mL distilled water and 2 mL color reagent (3:1:1; $H_2SO_4$ (1 M), ascorbic acid (10%; *w/v*), and ammonium molybdate (2.5%; *w/v*) was added to the

mixture. The mixture was incubated for 15 min at 50°C, and then it was cooled to ambient temperature, and the absorbance value was measured at 820 nm via a UV-Vis spectrophotometer. The same procedure was applied to the immobilized enzyme (0.3 mg) into PVA nanofiber containing phytase.

*2.7. pH and temperature effect on phytase activity.*

The effect of pH was investigated by incubating the enzymes with the substrate (Na-phytate) in 0.1 M different buffer solutions: glycine-HCl (pH 2.0-3.0), NaOAc (pH 4.0-6.0), and Tris-HCl (pH 7.0-9.0). Besides, the temperature effect was determined in the range from 25 to 95 °C, and the incubation temperature was changed at the mentioned temperatures.

*2.8. Thermal stability and kinetic parameters of free and immobilized phytase*.

The thermal stability of the enzyme was also studied, and they were incubated for 10, 20, and 30 min in the range from 30 to 90 °C. The enzymatic activity was determined under the phytase standard assay to determine the enzymatic activity of phytase. Phytase activity was studied at different sodium phytate concentrations (50-120 mM) under the phytase activity assay. $K_m$ and $V_{max}$ values were found from the Lineweaver-Burk plot [24,25].

## 3. Results and Discussion

*3.1. Morphological properties and characterization of electrospun fibers.*

SEM images of PVA and PVA nanofiber containing phytase were given in Figure 2. As can be seen in Figure 2, PVA and phytase-PVA electrospun nanofibers have a smooth surface with a nanofiber structure. The fiber homogeneity was increased with the addition of the enzyme into the PVA solution. Moreover, phytase-PVA nanofiber was contained beads with an average diameter in the range of 94 to 197 nm. This nanofiber structure with beads can offer protection and stability to the enzyme. Rathnayake and et al. stated that nanofibers containing a large number of beads in the structure were provided to extra stability to the enzyme, and this structure has protected the enzyme from the external condition [12].

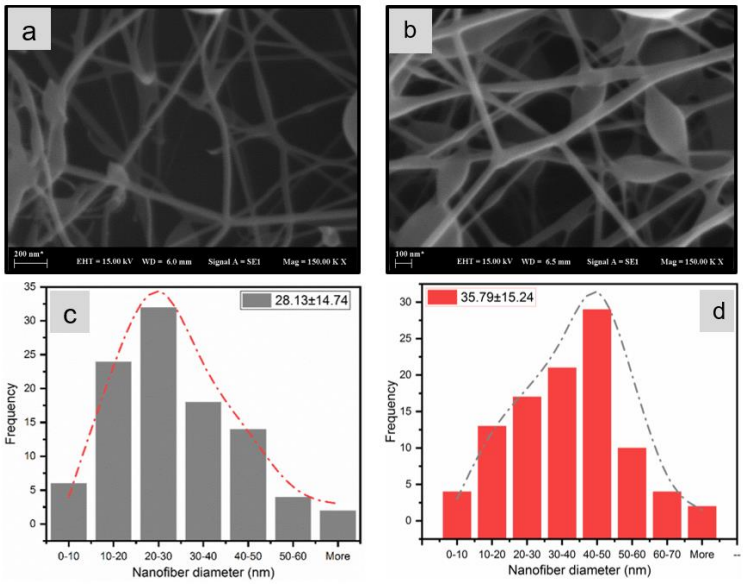


**Figure 2.** SEM image and size distribution of the fabricated PVA (a, c) and phytase-PVA (b, d) nanofibers at ambient conditions.

The average diameter of the nanofibers is given in Figure 2. The fiber diameter of PVA and phytase-PVA nanofibers was determined in the range from 0 to 60 and 0 to 70 nm, respectively, and the average diameter of the nanofibers was determined as 28.13±14.74 and 35.79±15.24 nm for PVA and phytase-PVA, respectively.

X-ray diffraction (XRD) analysis of PVA and phytase-PVA nanofibers were carried out to evaluate the crystalline structure (Figure 3). The neat PVA nanofiber exhibited peaks at 9.08, 13.89, 17.16, 23.58, 25.86, and 27.22°. The peak observed around 23.5° could be considered a crystalline peak for PVA, and it was recorded due to hydrogen bonds in PVA [26]. The XRD peaks of phytase–PVA nanofiber were observed at 7.73, 13.02, 17.16, 19.59, 25.50, and 27.22°. These peaks demonstrated that XRD peaks in the structure of phytase-PVA nanofiber were shifted to a lower 2θ angle. Moreover, some XRD peaks in the range from 35 to 60° disappeared in the XRD pattern of phytase-PVA fiber due to the good dispersion of phytase into the polymer solution [16].

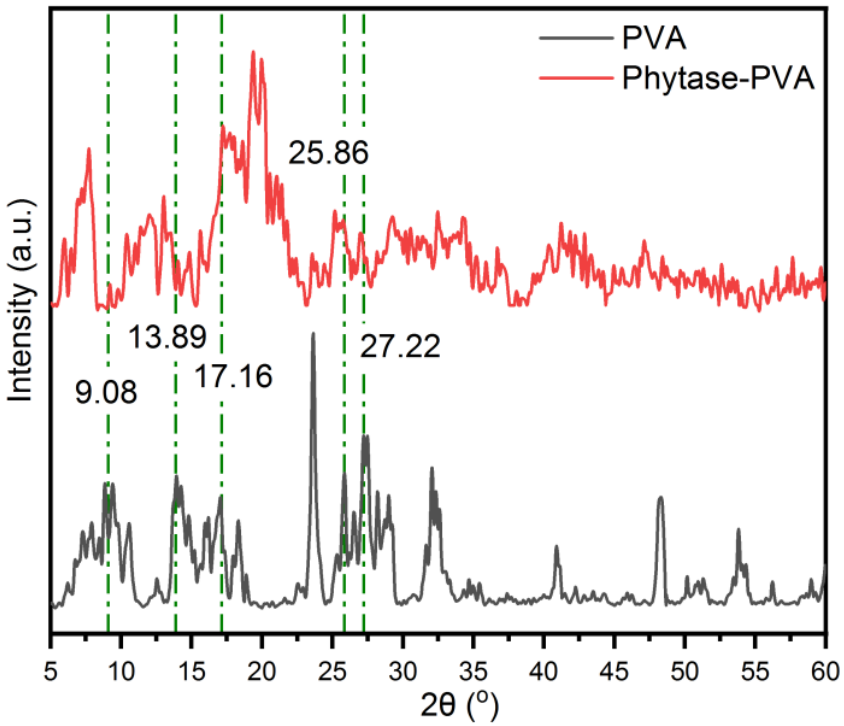


**Figure 3.** XRD pattern of the fabricated PVA and phytase-PVA nanofibers.

FT-IR spectra of the nanofibers and sodium phytate were given in Figure 4. The characteristic hydroxyl stretching (-OH) vibration of PVA was seen at 3289 $cm^{-1}$. Aliphatic –CH (Al-CH) stretching vibration and -C-O stretching vibration of the acetyl group of PVA were observed at 2909 and 1076 $cm^{-1}$, respectively [27]. A broad –OH stretching vibration of 1.5% phytase containing nanofibers was seen at 3264 $cm^{-1}$. Al-CH and –C-O stretching vibrations were also observed at 2919 and 1090 $cm^{-1}$ for nanofibers containing 1.5% phytase, respectively. $PO_4^{3-}$ and –C-O-P stretching vibrations were seen at 1186 and 902 $cm^{-1}$, respectively, in the substrate structure [28]. These stretching vibrations of PVA fibers containing 1.5% phytase were observed at 1320 and 916 $cm^{-1}$, respectively. FT-IR results indicated that –OH, -C-O, $PO_4^{3-}$ and –C-O-P stretching vibrations in the structure of fibers containing 1.5% phytase showed a small displacement due to hydrogen bonding interaction between phytase and the hydroxyl group of PVA [29].

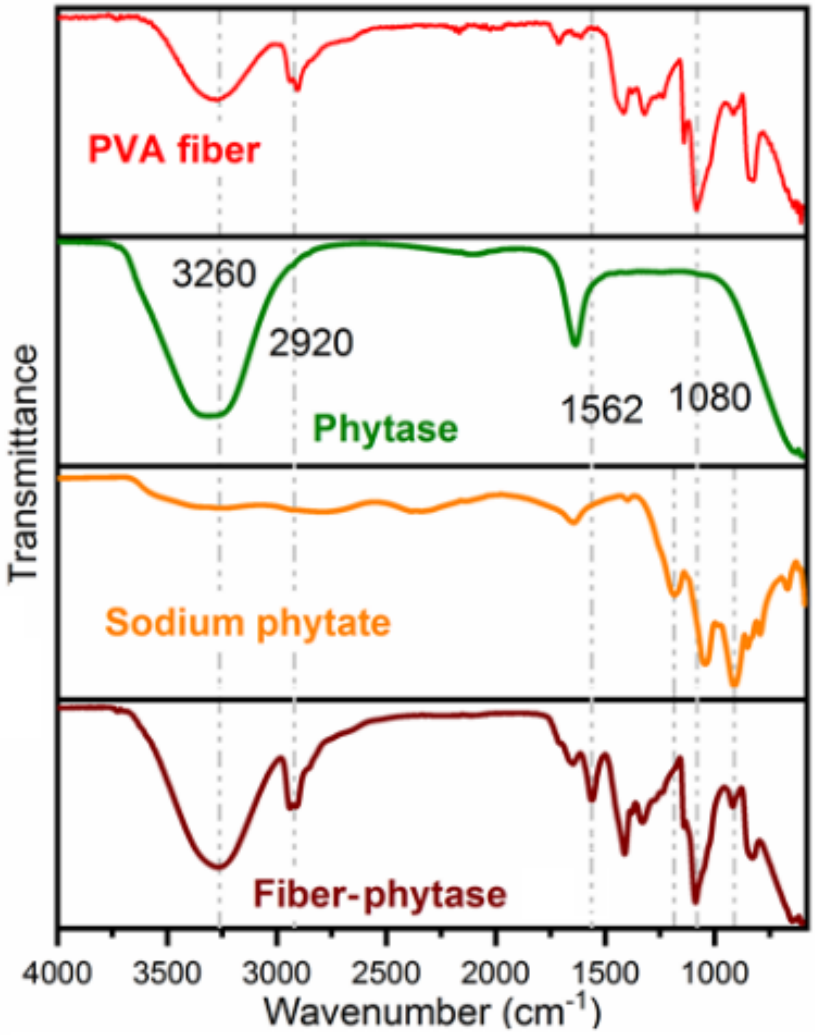


**Figure 4.** FT-IR spectra of PVA fiber, phytase, sodium phytate, and phytase containing nanofiber.

*3.2. pH and temperature effect on phytase activity.*

In the presence of amino acids, side chains of enzymes are affected by enzymatic activity at different pH values via protonation or deprotonation [30]. Figure 5a shows the pH effect on the free or immobilized phytase activity in the range of pH between 2 to 9. Relative enzymatic activity of free phytase was found around 80, 100, and 70% for pH 4, 5, and 6, respectively, and the enzyme was lost its activity around pH= 8 and 9. Relative activity of immobilized phytase was determined as 82, 84, 88, 100, 70, and 65% for pH 2, 3, 4, 5, 6, and 7, respectively, and it was preserved its activity with 58 and 20% relative activity at pH 8 and 9. According to these results, both free and immobilized enzymes have optimum enzymatic activity at pH 5. Moreover, pH effect results demonstrated that the pH stability of immobilized phytase showed higher relative enzymatic activity at a wide range of pH values. Phytase can be classified as acidic (pH 5.0), neutral (pH 7.0) and alkaline (pH 8.0) related to the enzymatic activity values at optimum pH. This value of the plant origin phytase was acidic (pH= 5.0) [31,32]. Moreover, acidic phytase is more preferred for industrial applications due to substrate specificity, digestibility, and applicability of this phytase type [33].

The temperature effect of the free or immobilized enzyme was studied using 2.5 mg PVA fiber containing 1.5% phytase (Figure 5b) under optimum conditions. Relative phytase activity of the free enzyme was increased between 25 and 45°C, and the maximum enzymatic activity was obtained at 45 °C. After 45 °C, its enzymatic activity was decreased. Relative

enzymatic activity of immobilized phytase was increased between 25°C and 65°C, and its maximum enzymatic activity was determined as 65°C, and then it was decreased very slowly. These results showed that the relative enzymatic activity of immobilized phytase was shifted to 65°C, and the optimum temperature of the immobilized phytase was increased compared to the free phytase. This increase could probably be the secondary interaction such as ionic or hydrophobic interactions and hydrogen bonds between phytase and support materials. Poly(vinyl alcohol) (PVA), which is a hydroxyl group (-OH) rich polymer, has been used to support material in this study. This polymer has been changed the optimum temperature of the immobilized enzyme because of its ability to withstand high temperatures [34]. Moreover, the results obtained from enzymatic activity studies for pH and temperature indicated that immobilized phytase has catalytic activity in a wider pH range and a higher optimum temperature. These properties are quite important for industrial applications of phytase.

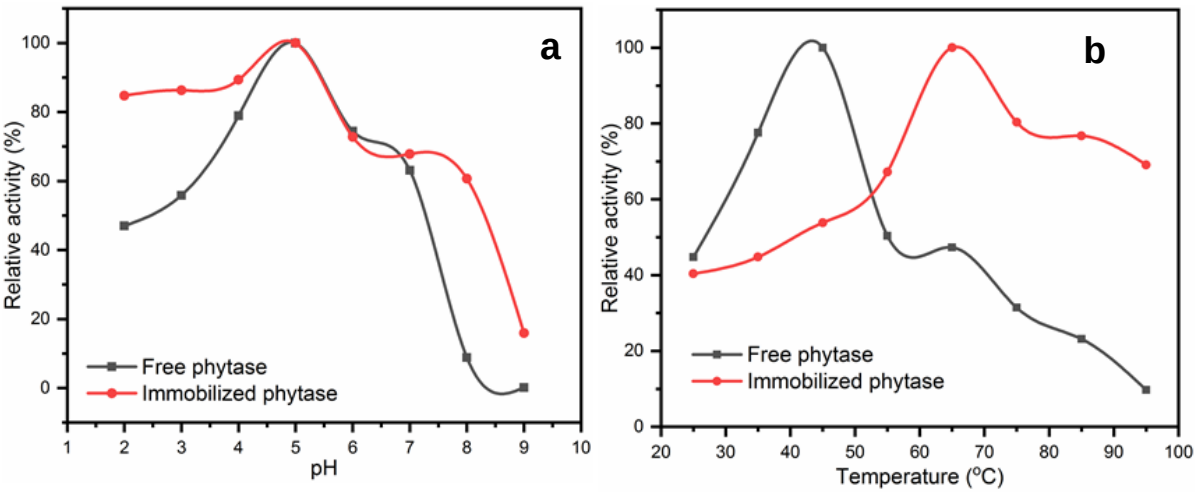


**Figure 5.** pH (a) and temperature (b) effect on free and immobilized phytase activity in the presence of 2.5 mg nanofiber containing 1.5% phytase.

*3.3. Thermal stability and kinetic parameters.*

Figure 6a shows the thermal stability of free and immobilized phytase at different temperatures (30-90°C). As seen in the thermal stability curve, the free phytase preserved 84.3% initial enzymatic activity during 50°C, and the relative activity of phytase dramatically decreased after this temperature. The relative activity of immobilized phytase was increased in the range from 30 (69.4%) to 50°C (80.3%), and it was slightly decreased at 60 and 70°C. Also, immobilized phytase showed higher thermal stability at 80 and 90°C, and it exhibited significant thermal stability against temperature change. Naghshbandi *et al.* stated that the enhanced thermal stability of the immobilized phytase was attributed to the structure of support materials, reduction in the molecular interaction between phytase and the supporting materials, and decrease in the mobility of protein [35].

The incubation time effect on relative enzymatic activity was given in Figure 6b at 50 and 80 °C. As shown in Figure 6b, the free enzyme has higher enzymatic activity at 50 °C than the immobilized phytase. Also, increasing the temperature, the enzymatic activity of immobilized phytase was increased, whereas the enzymatic activity of the free phytase was decreased, and it became more stable to the temperature after immobilization.

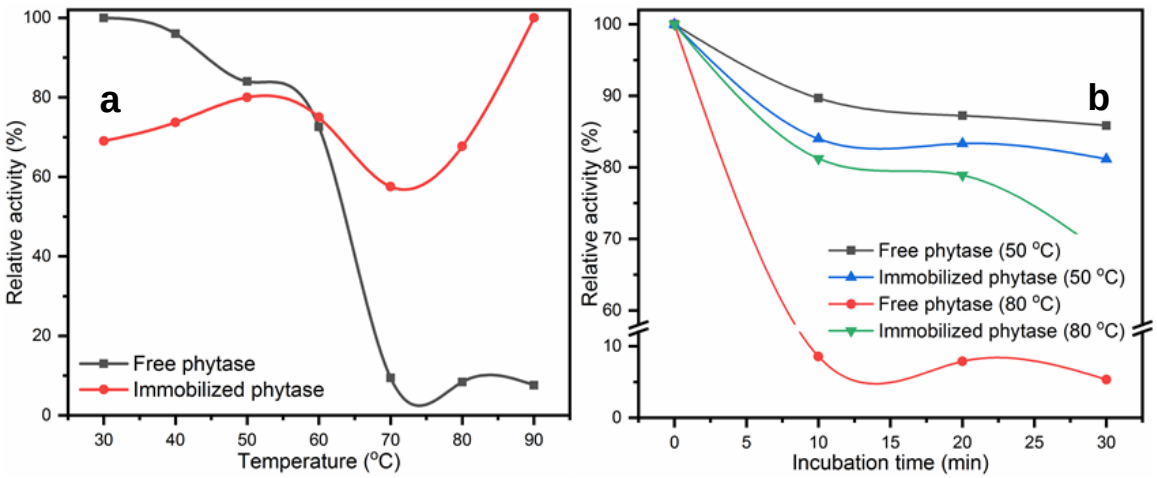


**Figure 6.** Thermal stability of free and immobilized phytase at 30 min incubation time (a), and incubation time effect on enzyme activity at 60 °C in the presence of 2.5 mg nanofiber at pH 5.0 (b).

Enzymatic activity was also investigated in the presence of different concentrations of sodium phytate to determine the $K_m$ and $V_{max}$ values. $K_m$ and $V_{max}$ values were calculated from the Lineweaver-Burk equation as 0.46 mM and 53.76 U/mg protein for the free phytase and 1.17 mM and 5.67 U/mg protein for immobilized phytase, respectively. These results showed that the Michaelis-Menten constant ($K_m$) value of immobilized phytase was increased after enzyme immobilization into PVA fiber. As known, enzyme-substrate affinity was decreased with an increased $K_m$ value. This could have probably pored the structure of the fibers. Isik *et al.* stated that the $K_m$ value of immobilized phytase was increased because of sodium phytate diffusion problems and steric hindrance [16]. In the presence of these factors, the enzyme active centers or porous structure of fiber may be blocked in the fiber fabrication process [36].

Fabrication of the polymer-based nanofibers for phytase immobilization as support material has not been studied except only in a few papers. Among these papers, Rathnayake *et al.* were fabricated rice bran-based electrospun nanofibers with a diameter of 20-50 nm, and the thermal stability of the immobilized enzyme was enhanced after immobilization [12]. In another paper, Harati *et al.* were obtained polyacrylamide and starch-based nanofibers for phytase immobilization. $K_m$ and $V_{max}$ values of the immobilized phytase were found as 56 µM, and 401 µmol/min mg, respectively, and the optimum pH and temperature were not changed after immobilization [37]. In the present manuscript, the kinetic parameters of the free and immobilized enzymes were calculated very closely. Also, the optimum temperature of the immobilized phytase was shifted to high temperature, and the immobilized phytase exhibited catalytic activity in a wider pH range.

The fabricated PVA nanofibers were also compared with the reported nano-based supporting materials in literature (Table 1) [11,22,37,38].

**Table 1.** Performance comparison of PVA nanofiber as supporting materials with the other materials.

| Supporting materials | Phytase source | Km | | References |
|---|---|---|---|---|
| | | Free enzyme | Immobilized enzyme | |
| Amino-multiwalled carbon nanotubes | Bacterial | 0.13 mM | 0.33 mM | 11 |
| PVA-CS nanofiber | Plant | 0.46 mM | 13.43 mM | 22 |
| Nanofibers-based on polyacrylamide | Microbial | 86 µM | 56 µM | 37 |
| Graphene oxide | Bacterial | 1.45 mM | 0.44 mM | 38 |
| PVA nanofiber | Plant | 0.46 mM | 1.17 mM | This study |

As can be seen from Table 1, the obtained results in this study are quite consistent with the results reported in the literature.

## 4. Conclusions

In this paper, phytase was partially purified from cowpea seeds, and it was immobilized into PVA nanofibers. Physicochemical characterizations of free and immobilized phytase such as kinetic ($K_m$ and $V_{max}$), optimum pH, and temperature were also done. Enzymatic activity results to determine optimum pH and temperature indicated that immobilized phytase has enzymatic activity in a wider pH range and the optimum temperature at a higher temperature. These properties are quite important for industrial application phytase. Thermal stability experiments were carried out by incubating the enzyme at different temperatures, and the enzymatic activity of immobilized phytase has higher thermal stability at high temperatures. The results showed that immobilized phytase on PVA nanofiber is highly suitable for agriculture, animal feed, food, and medical applications.

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## Conflicts of Interest

The authors declare no conflict of interest.

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