Volume 65, Issue 1, 2021

Journal of Scientific Research

Institute of Science,

Banaras Hindu University, Varanasi, India.

Immobilization of Phytase from Rice Bean (*Vigna umbellata* Thunb.) on Glutaraldehyde Activated Chitosan Microspheres

K. Belho and P. K. Ambasht*

Department of Biochemistry, School of Life Sciences, North-Eastern Hill University, Shillong-793 022, Meghalaya, India.

Ivy5belho@gmail.com, pravin.ambasht@gmail.com*

***Abstract*: The immobilization of phytase from rice bean was optimized on the glutaraldehyde-activated chitosan microsphere, and characterized. The optimum percent immobilization was 77.48%, when microspheres were prepared with 1.5% chitosan, activated with glutaraldehyde (0.5%, for 4 h) and with the enzyme protein (0.2 mg/mL, and coupling time 4 h). The immobilized phytase exhibited elongated fibres and pores. FTIR results suggest the formation of a Schiff base with a band at 1638 cm^{-1}. The pH and temperature optimum of immobilized phytase was 4.0 and 40 °C, respectively. The energy of activation was 34.5 kJ/mol. The K_m and V_{max} values of immobilized phytase were 0.62 mM and 3.42 µmol/min. The immobilized enzyme when incubated at 50 °C, retained 59% activity after 75 min. The immobilized phytase showed 80% activity retention, after 14 days, when stored at 4 °C. The immobilized phytase could be reused for 4 cycles with 58% activity retention. The immobilized phytase, when incubated for 60 min in the presence trypsin and pepsin, showed 92.9 and 95% activity retention, respectively. The properties of immobilized phytase did not alter with respect to pH, and temperature optima. Immobilized phytase exhibited proteolytic resistance when incubated with pepsin for 1 h, and this can find application in animal feed.**

***Index terms*: Chitosan, Immobilization, Pepsin, Phytase, Proteolytic resistance, Rice bean, Storage stability.**

I. INTRODUCTION

Phytic acid is a derivative of inositol with six phosphate groups, each being attached to different carbon atoms. Due to the presence of negative charges on phosphate groups, attracts different metal ions for binding. The metal ions, therefore are not in a free-state for absorption, and result in their deficiencies. The enzyme phytase hydrolyses phosphate groups, and thus relieves the chance of metal ion chelation. The study of the phytase is therefore considered to be very important. It has been purified from some plant sources and characterized (Gibson & Ullah, 1988; Konietzny et al., 1995; Mahajan & Dua, 1997; Phillippy, 1998; Greiner et al., 1998, 2001; Greiner & Alminger 1999; Greiner, 2002; Andriotis & Ross, 2003; Belho et al., 2016; Belho & Ambasht, 2017). Immobilization of phytase has been pursued mostly from microbial sources like *Aspergillus ficuum* (Liu et al., 1999; Moraes et al., 2008), *A. niger* (Menezes-Blackburn et al., 2011; Trouillefou et al., 2015; Coutinho et al., 2020), *A. heteromorphus* (Lata et al., 2014), *Escherichia coli* (Menezes-Blackburn et al., 2011; Cho et al., 2011; El-Shora et al., 2019), *Candida krusei* (Quan et al. 2003), and mushrooms (Onem & Nadaroglu, 2014; Onem et al., 2016). The literature on the enzyme immobilization, and the use of different matrices, has been extensively discussed (Mosbach, 1971; Mateo et al., 2007; Spahn & Minteer, 2008; Liu et al., 2018; Sastre et al., 2020). Immobilized phytase has more comprehensive applications, like use as amperometric biosensor for the detection of phytate (Moraes et al., 2008) as fertilizer, and as a source of exogenous Pi (Trouillofou et al., 2015), as feed (Cho et al., 2011; Coutinho et al., 2020), as bioreactors (Greiner & Konietzny, 1996), and reduction of phytic acid content from soymilk (Chen et al., 2018).

There are only fewer reports on immobilization of a plant phytase viz. from wheat, and avocado (Celem & Onal, 2009a, b; Chen et al., 2018). The wheat phytase used is commercially available with low specific activity, i.e., 0.1 U/mg protein (Chen et al., 2018). Immobilization studies have been carried out in our laboratory, and reported earlier with other enzymes like alpha amylase (Kharkrang & Ambasht, 2013, 2018), and acid phosphatase (Belho et al., 2014; Kalita et al., 2019; Kalita et al.,

* Corresponding Author

2020). Chitosan has been used in our earlier study on acid phosphatase from wheat germ (Belho et al., 2014). Chitosan is derived from chitin, and is a linear polysaccharide composed of D-glucosamine, and N-acetyl D-glucosamine linked through β-1→4 linkage. Its properties like hydrophilicity, and biodegradability make it better support for the enzyme immobilization (Payne & Sun, 1994). Glutaraldehyde is known to act as a cross-linking agent (Migneault et al., 2004). The $-NH_2$ group from D-glucosamine reacts with the aldehydic group of glutaraldehyde. The use of chitosan has been reviewed in several publications (Knaul et al., 1999; Krajewska, 2004; Gupta & Jabrail, 2007; Wahba, 2017). Bacterial phytase has been immobilized on chitosan (El-Shora et al., 2019). Since limited information on plant phytase immobilization is available, the present paper is an effort in that direction. In the present paper, we describe the results of optimization of immobilization, kinetic characterization and stability studies of phytase from rice bean (*Vigna umbellata*) on the glutaraldehyde-activated chitosan microsphere.

II. MATERIALS AND METHODS

Chemicals were procured from Sigma Aldrich, USA, Jebsen & Jessen Gmbh & Co., Germany, and sd. Fine Chemicals, India. The other chemicals were of AR Grade from India. Phytase purified from germinated rice bean seeds was used (Belho et al., 2016). De-ionized water was collected from the Milli-Q system (Millipore USA).

A. Optimization of immobilization of rice bean phytase on chitosan microspheres and activity assay

The process of the preparation of chitosan microspheres, their activation in the presence of glutaraldehyde, and the immobilization of phytase, was optimized by varying different parameters, one at a time. The parameters varied were: concentrations of chitosan (1.5-2.5%), and glutaraldehyde (0.2-3.0%), activation time (2-24 h), enzyme protein concentration (0.05-0.4 mg), and coupling time (2.0-24.0 h). The immobilization is expressed in terms of percent immobilization.

A 1.5% solution of chitosan was prepared by dissolving chitosan flakes in 1 M acetic acid. The solution was then steadily dropped using a peristaltic pump (20 mL/h) into 15% NaOH - 95% ethanol solution with constant stirring. The microspheres formed were kept in the solution for 16 h. Microspheres were then washed in 50 mM sodium acetate buffer, pH 5.0, and stored in the same buffer until further use. The above process was carried out at 4 °C. Chitosan microspheres were activated with 0.5% glutaraldehyde for 4.0 h. Activated microspheres were then washed with 50 mM sodium acetate buffer, pH 5.0, to remove any excess glutaraldehyde on the surface. The glutaraldehyde-activated chitosan microspheres were suspended in 1.0 mL free diluted enzyme (0.2 mg) for 4.0 h. Microspheres were then washed with 50 mM sodium acetate buffer pH 5.0, till washings were free from unbound phytase. The % immobilization was determined, as described earlier (Belho et al., 2014).

The free phytase activity was assayed by discontinuous spectrophotometric method, using 100 mM sodium acetate buffer, pH 4.0, as assay buffer as described earlier (Belho et al., 2016). For the assay of immobilized phytase activity, the reaction mixture contained 1.5 mM sodium phytate (0.35 mL), and the assay buffer (0.05 mL) was maintained at 40 °C. The assay was initiated with the addition of two phytase immobilized microspheres, and was incubated for 30 min. The reaction was stopped by removal of the microspheres. The rest of the process was as described earlier (Belho et al., 2016).

B. Photography, Scanning Electron Micrograph (SEM), and Fourier Transform Infrared Spectrum (FTIR)

The microspheres were thoroughly washed with 50 mM sodium acetate buffer, pH 5.0 (storage buffer), and were then placed on a watch glass. Microspheres were photographed using *Nikon Cool Pix L810 camera*. For SEM, protocols were followed as reported earlier using *Scanning Electron Microscope SEM-JSM 6360, JEOL (Japan)*. (Belho et al., 2014). For FTIR, the fresh chitosan microspheres were rinsed with 20 mM Tris-HCl buffer, pH 7.0. The method reported earlier was followed using FTIR instrument *Sample-Perkin-Elmer (Spectrum400) FT-IR /FT-FIR Spectrophotometer (USA)* for recording the FTIR spectra (Kalita & Ambasht, 2019).

C. Steady-state kinetics of immobilized phytase

The variation of enzyme activity with pH has been studied using 0.1 M buffers in the pH range 2.0-8.0. The buffers used were: glycine-HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), Tris-maleate (pH 6), and Tris-HCl buffer (pH 7.0-8.0). From the data, plots of the rate of reaction versus pH were made. The effect of temperature on the rate of the immobilized phytase catalysed reaction has been studied in the range of 20 to 80 °C using 0.1 M sodium acetate buffer, pH 4.0. The reaction mixture was maintained at a particular temperature. The concentration of sodium phytate was 1.5 mM, as in the routine activity assay. From the data, a plot of rate of reaction versus temperature, was made for the determination of optimum temperature. Arrhenius plot from the above data, was made to determine the energy of activation. Thermal inactivation studies were carried out at 50 °C for phytase immobilized on chitosan microspheres. The latter was maintained at 50 °C. At different time intervals, microspheres were taken out and were chilled in ice. The chitosan microspheres were tested for residual activity at 40 °C. The effect of substrate concentration on the rate of reaction of the immobilized phytase catalysed reaction was determined. The rate of reaction was monitored at different concentrations of sodium phytate (0.091-

1.75 mM) in the test solution. From this data, the Lineweaver Burk plot was made, and values of K_m and V_{max} were determined.

D. Storage, reusability, and proteolytic resistance of the immobilized phytase

Immobilized phytase microspheres were routinely stored in 50 mM sodium acetate buffer, pH 5.0, at 4 °C. In one set of experiment, enzyme activity was routinely tested on different days using different microspheres. A plot of percent residual activity versus time was made. In another set of experiment, the activity of a particular set of microspheres was assayed on different days for the number of cycles of reuse. From the data, a plot of percent residual activity versus cycles of reuse was made.

For proteolytic resistance studies, immobilized phytase microspheres were incubated with pepsin (0.1 mg/mL) in 50 mM glycine-HCl buffer (pH 2.5) and with trypsin (0.1 mg/mL) in 50 mM Tris-HCl buffer (pH 8.0) at 37 °C. The immobilized phytase microspheres were taken out at regular intervals of time and were chilled in an ice bath. The phytase activity was assayed routinely. A plot of percent residual activity versus time was made.

III. RESULTS AND DISCUSSION

A. Optimization of immobilization of phytase

The results of the optimization of immobilization of phytase on the glutaraldehyde activated chitosan microspheres are summarized in Table I. It is evident that 1.5% chitosan solution was the most suited for the preparation of microspheres and yielded maximum %immobilization. At higher concentrations, however, there is a reduction in the % immobilization. Attempts were made to prepare microspheres from a 1.0% chitosan solution. Microspheres formed were fragile, and therefore were not used for immobilization in the present study. Further, proper microspheres could not be formed, when an attempt was made to use a 3.0%, chitosan solution, due to very high viscosity. The maximum phytase % immobilization was observed when microspheres were activated with 0.5% glutaraldehyde. A fall in the phytase % immobilization was observed beyond 0.5% glutaraldehyde, because the latter not only acts as a cross-linking reagent, but is also a denaturant. The activation was incomplete in the presence of glutaraldehyde, when exposed for 2 h, and was complete in 4 h. Exposure of glutaraldehyde beyond 4 h brought a reduction in the %immobilization.

The maximum %immobilization was observed with the enzyme protein concentration 0.2 mg/mL, and coupling time 4 h. The glutaraldehyde activated chitosan microsphere has limited capacity; therefore, even if the protein concentration is increased beyond saturation, it did not affect the %immobilization. In the present study, the maximum % immobilization achieved was 77.48%. The yield in the present study is higher in comparison to some other phytases (soybean and avocado) immobilized on Cepabead EC-EP (Celem & Onal, 2009a, b). The fungal enzyme entrapped inside carrageenan blocks, and calcium alginate showed a little higher yield (Lata et al., 2014).

Table 1: Optimization of the immobilization of phytase on the glutaraldehyde activated chitosan: Effect of variation in different parameters on % immobilization of phytase.

Chitosan (%)	Glutaral dehyde (%)	Activation time (h)	Protein (mg/L)	Coupling time (h)	% Immobilization ± SD
1.5	**2.0**	**6.0**	**0.2**	**12.0**	**63.96 ± 2.3**
2.0	2.0	6.0	0.2	12.0	52.25 ± 3.4
2.5	2.0	6.0	0.2	12.0	41.44 ± 2.9
1.5	0.2	6.0	0.2	12.0	61.26 ± 3.2
1.5	**0.5**	**6.0**	**0.2**	**12.0**	**69.36 ± 3.6**
1.5	1.0	6.0	0.2	12.0	65.77 ± 3.3
1.5	2.0	6.0	0.2	12.0	63.96 ± 3.9
1.5	3.0	6.0	0.2	12.0	56.76 ± 3.1
1.5	0.5	2.0	0.2	12.0	64.86 ± 2.6
1.5	**0.5**	**4.0**	**0.2**	**12.0**	**72.97 ± 2.3**
1.5	0.5	6.0	0.2	12.0	69.36 ± 2.2
1.5	0.5	12.0	0.2	12.0	61.26 ± 3.1
1.5	0.5	24.0	0.2	12.0	56.76 ± 2.4
1.5	0.5	4.0	0.05	12.0	62.16 ± 1.8
1.5	0.5	4.0	0.1	12.0	68.47 ± 2.1
1.5	**0.5**	**4.0**	**0.2**	**12.0**	**72.97 ± 1.7**
1.5	0.5	4.0	0.4	12.0	49.55 ± 1.9
1.5	0.5	4.0	0.2	2.0	65.77 ± 2.3
1.5	**0.5**	**4.0**	**0.2**	**4.0**	**77.48 ± 2.8**
1.5	0.5	4.0	0.2	12.0	72.97 ± 2.5
1.5	0.5	4.0	0.2	24.0	63.06 ± 2.5

B. Photographs and SEM

The photographs of the non-activated, and glutaraldehyde activated chitosan microspheres are shown in **Plate Ia** and **Ib**.

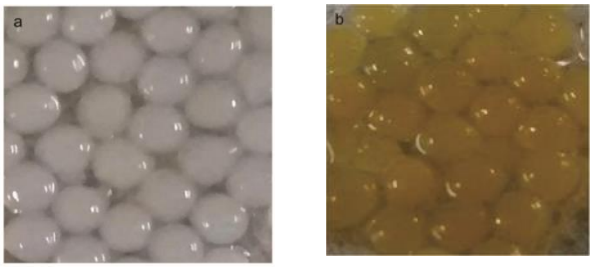

Plate I: Photographs of chitosan microspheres.
a. Non-activated chitosan microspheres,
b. glutaraldehyde activated chitosan microspheres.

It is evident that the non-activated microspheres were white, while the activated microspheres attained yellowish brown colour. Similar results were seen in the earlier report (Belho et al., 2014). The change in colour is primarily due to the reaction between the aldehyde and the primary amine, and the formation of the Schiff base.

The SEM of chitosan microspheres is shown in **Plate II**. In the **Plate IIa**, magnified (x110), the whole microsphere is shown. The surface is not smooth. In the Plate **IIb**, the surface of the non-activated microsphere has been shown at a magnification of x9000. It gives a condensed and granular appearance. The surface of the glutaraldehyde activated microsphere is shown in **Plate IIc**, where fibres with pores are noticed. The surface of the activated microsphere with immobilized phytase is shown in **Plate IId**. Upon phytase immobilization, the cross-linking is more visible and prominent.

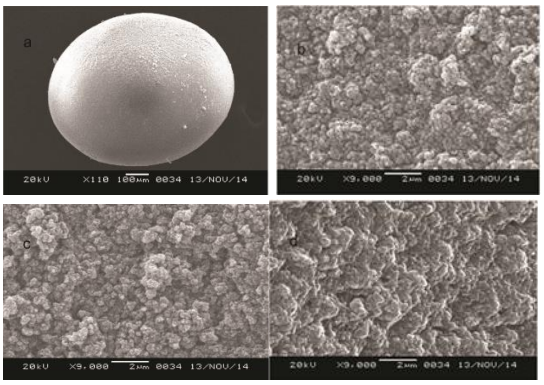

Plate II: SEM of chitosan microspheres. (a) Whole view of chitosan microsphere, (b) Non-activated chitosan microsphere, (c) Glutaraldehyde-activated chitosan microsphere, and (d) Phytase immobilized on the glutaraldehyde-activated chitosan microsphere.

C. FTIR of the microspheres of chitosan, glutaraldehyde activated chitosan and immobilized phytase

The results of FTIR of chitosan, glutaraldehyde activated chitosan, and the immobilized phytase on the activated chitosan microsphere is shown in **Fig. 1**. It is evident that in all these spectra, the band at 1638 cm^{-1} is typical. The FTIR of the chitosan band at 1638 cm^{-1}, represents $–NH.CO.CH_3$ of N-acetyl-glucosamine, with a high % transmittance (31-32%). In the glutaraldehyde activated chitosan, band at 1638 cm^{-1} was observed with significantly reduced % transmittance (6-8%). The band between 1615-1650 cm^{-1} is designated for the stretching vibration of C=N in Schiff's base (Cinarli et al., 2011). The latter is formed between glutaraldehyde and chitosan. There is also the presence of free $–NH.CO.CH_3$. In some other reports, close to 1660 and 1664 cm^{-1}, a strong reaction of glutaraldehyde, and chitosan was observed (Knaul et al., 1999; Gupta & Jabrail, 2007).

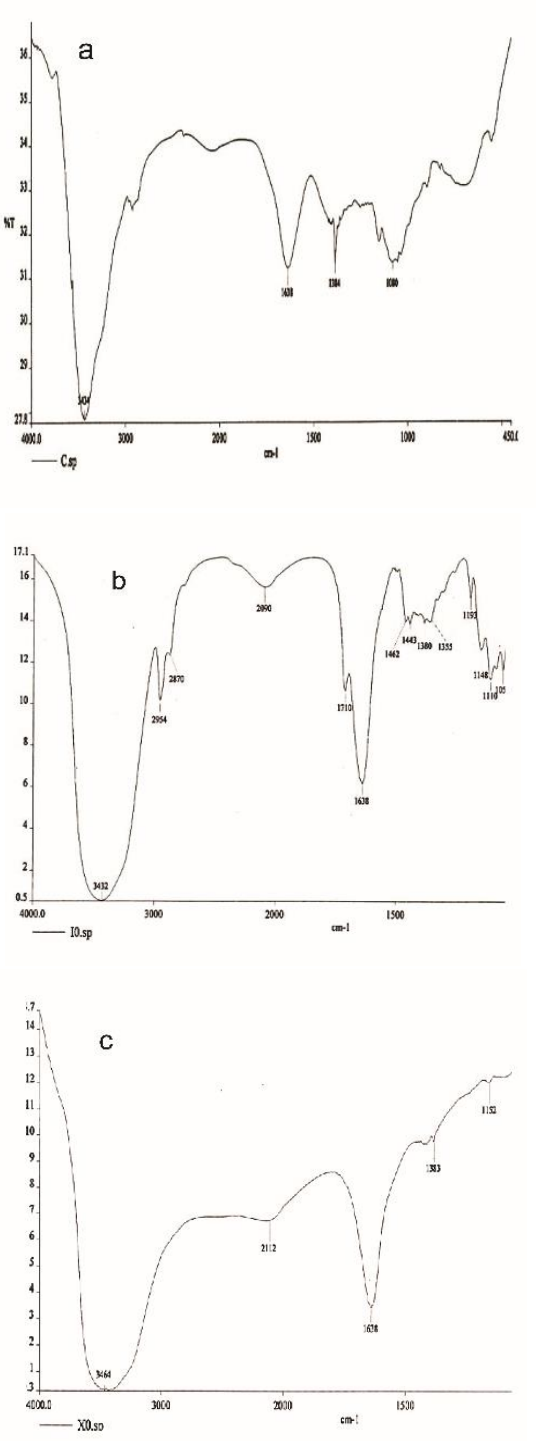

Fig. 1: FTIR spectrum: (a) Non-activated chitosan, (b) Glutaraldehyde-activated chitosan, and (c) Phytase immobilized on glutaraldehyde- activated chitosan microspheres.

The missing band at 1910 cm^{-1} provides cross-linking, not via acetalization. The band 1910 cm^{-1} represents R-O-R str. (Gupta & Jabrail, 2007). The band at 1710 cm^{-1} shows the presence of the

str. aldehyde group. In the immobilized phytase on the glutaraldehyde-linked chitosan, band at 1638 cm^{-1} was retained; however, the band (1710 cm^{-1}) disappeared. In the case of the immobilized enzyme, the band at 1638 cm^{-1}, exhibits a further reduction in % transmittance (3-4%), suggesting cross-linking between the enzyme and the glutaraldehyde activated chitosan. The other prominent bands are at 3432, 3434, and 3464 cm^{-1}, and 1384, 1380, and 1383 cm^{-1} represent the –OH str. hydrogen-bonded and -OH bending, respectively.

D. Effect of pH and temperature on free and immobilized phytase

The results of the effect of pH on the immobilized, and the free phytase catalysed reaction are shown in **Fig. 2a**.

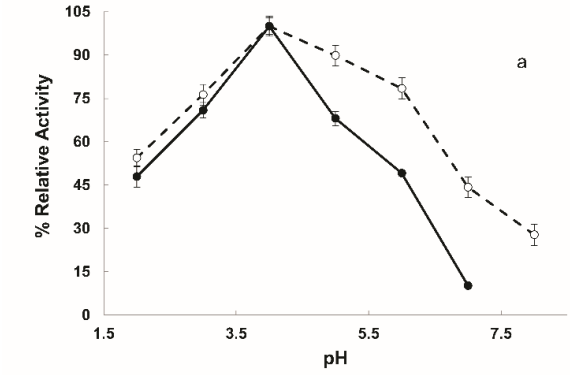

Fig. 2a: Effect of pH on relative activities of the free (●) and the immobilized (○) phytase.

The pH optimum of the immobilized enzyme was at pH 4.0, like that of free enzyme, without any shift. Our results are consistent with some other reports, where no shift in pH optimum has been reported (Liu et al., 1999; In et al., 2007; Celem & Onal, 2009b; Trouillefou et al., 2015). Further, it is evident that the immobilized enzyme showed higher activity at any pH value. At pH 6.0, i.e., two pH units above optimum pH, the immobilized enzyme retained more than 78% activity, while the free enzyme showed less than 50% activity. The immobilized enzyme was still functional at pH 8.0, with more than 25% activity showing better stability in the alkaline range.

The plot of % relative activities of the free and the immobilized phytase at different temperatures are shown in **Fig. 2b**. The temperature optimum in both free, and immobilized enzymes was 40 °C. It is interesting to note that the free enzyme showed better relative activities at lower temperatures (20 and 30 °C), but the immobilized enzyme showed better relative activity at higher temperatures (50-80 °C). Though the optimum temperature is 40 °C, both the free and the immobilized enzymes are active at 80 °C with 36 and 44% activities, respectively. In some other cases also, there is no change in optimum temperatures of both the free, and the immobilized phytase (Celem & Onal, 2009a,b; Cho et al., 2011; Onem & Nadaroglu, 2014). At higher temperatures, the microspheres tend to become softer, as the incubation time is also 30 min.

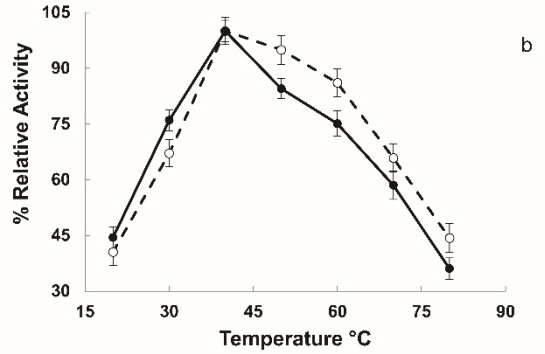

Fig. 2b: Effect of temperature on the free (●) and the immobilized (○) phytase.

In some cases, the immobilized enzyme had a higher temperature optimum, in comparison to the free enzyme (Liu et al., 1999; In et al., 2007; Awad et al., 2015).

E. Immobilized phytase: Activation energy and thermal stability

The activation energy of immobilized phytase was determined using the Arrhenius plot, and is shown in the **Fig. 3a**. The activation energy value was found to be 34.5 kJ/mol, which is marginally higher in comparison to that for the free phytase (32.2 kJ/mol) (Belho et al., 2016). The above increase may be due to the changes in the enzyme micro-environment by the supporting matrix. In some other reports, the activation energy of the immobilized enzyme was also higher in comparison to the free enzyme (Celem & Onal, 2009a, b; Menezes-Blackburn et al., 2011). The rice bean phytase showed similar activation energies to the phytase from *A. niger* (Menezes-Blackburn et al., 2011). It is interesting to note that acid phosphatase from wheat germ, immobilized in agar-agar, and gelatin, and exhibited very close values of activation energies with respect to the free enzyme (Kalita et al., 2020).

The results of the thermal inactivation of the immobilized phytase studied at 50 °C is shown in **Fig. 3b**. The temperature selected was 10 °C above the optimum temperature (40 °C). The immobilized enzyme retained 59% activity, even after 75 min of incubation. The free enzyme under similar conditions, retained only 30% activity (Belho and Ambasht, unpublished work). The immobilized enzyme has some protection, due to the shielding effect, and therefore has better thermal stability.

F. Effect of sodium phytate concentration variation on the rate of immobilized phytase-catalysed reaction

The result of the effects of variation of sodium phytate concentration on the immobilized phytase catalysed reaction, has been presented in the form of Lineweaver-Burk plot (**Fig. 4**).

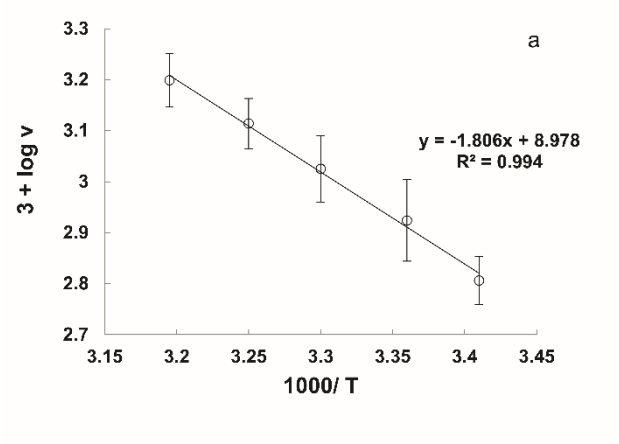

Fig. 3a: Arrhenius plot of the immobilized phytase.

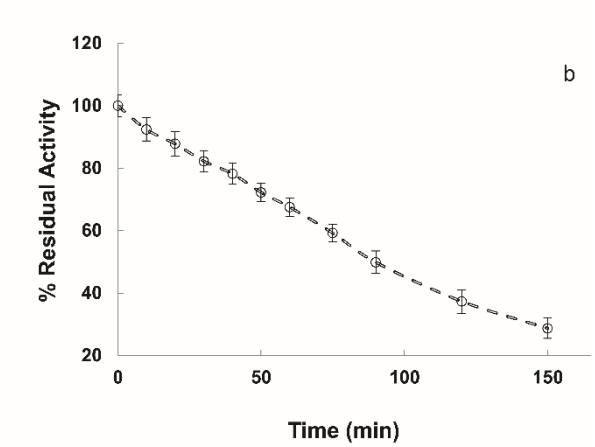

Fig. 3b: Thermal inactivation kinetics of the immobilized phytase at 50 °C.

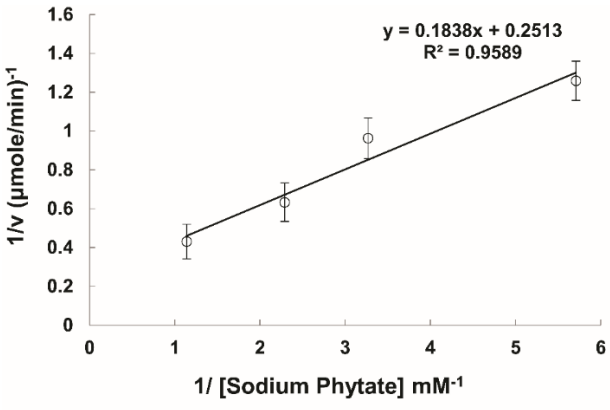

Fig. 4: Lineweaver Burk plot for the immobilized (○) phytase at different concentrations of sodium phytate.

The values of K_m and V_{max} were 0.62 mM and 3.42 µmol/min, respectively. The K_m value of the immobilized enzyme is almost 3.1 times higher in comparison to that of the free enzyme (Belho et al., 2016). In most of the reports, immobilized phytase showed a higher value of K_m in comparison to the free enzyme (Greiner & Konietzny, 1996; Liu et al., 1999; Celem & Onal, 2009a, b). In the case of the free enzyme, there is no restriction in its movement, and therefore both enzyme and substrate move, and their collision is easy. On the other hand, in the case of the immobilized enzyme, the movement of the enzyme is restricted, and therefore collision with the substrate is less facilitated. Similar reasons for the increase in K_m have been reported earlier (Celem & Onal, 2009 a, b).

G. Storage stability and reusability

The results of storage stability (4 °C) of the free and the immobilized phytase are shown in **Fig. 5a**. It is evident that the free enzyme is very labile, retains only 75% activity on the 7th day, and loses more than 50% activity on the 14th day. The corresponding immobilized enzyme, however, retained more than 90% activity on the 7th day and close to 80% activity on the 14th day. The immobilized enzyme, thus showed a better shelf-life in comparison to the free enzyme. The immobilization brought a better storage stability (Celem & Onal, 2009a, b; Lata et al., 2014; Awad et al., 2015). The present result showed better stability with respect to phytase entrapped, and cross-linked inside the carragenan (Lata et al., 2014).

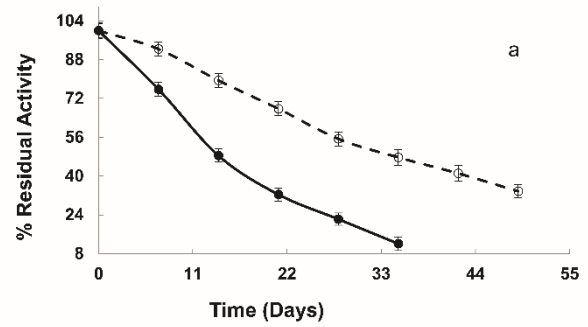

Fig. 5a: Storage stability of the free (●), and the immobilized (○) phytase stored in 50 mM sodium acetate buffer pH 5.0 at 4 °C.

The results of the efficiency of phytase activity retention, with respect to the reusability of a particular set of microspheres, are shown in the **Fig. 5b**. Immobilized phytase on the glutaraldehyde activated chitosan microspheres, could be reused for 4 cycles, with retention of close to 58% residual activity at 40 °C. On the 7th cycle of the reuse, 36% activity was retained. Further reuse (8th

cycle) brought significant loss in activity (20% residual activity). It was observed that upon reuse, the microspheres became harder, with cracks, and resulted in leaching of the enzyme.

H. Stability study of immobilized phytase in the presence of proteolytic enzymes

The result of the effect of incubation of trypsin and pepsin on the free phytase for 60 min has been described earlier, with 91 and 72% activity retention, respectively (Belho & Ambasht, 2017). There are reports on the study of proteolytic resistance against free phytase (Igbasan et al., 2000; Zhang et al. 2010). The results of the effect of trypsin and pepsin incubation for 1 h, on the immobilized phytase activity is shown in **Fig. 6**.

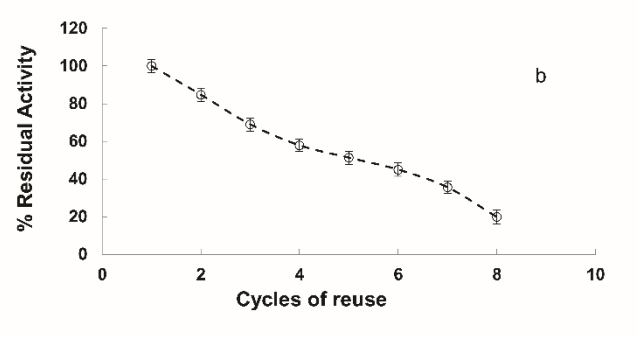

Fig. 5b: Number of cycles of reuse of immobilized phytase.

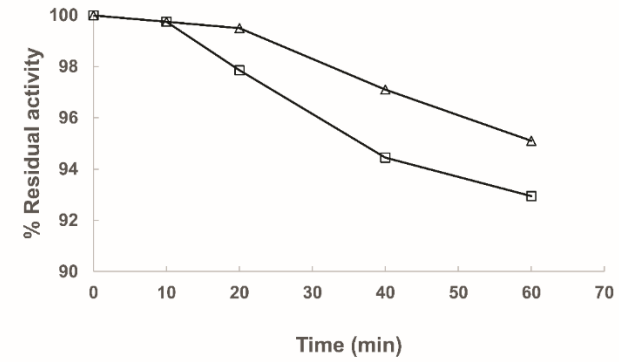

Fig. 6: Effect of the trypsin (□) and pepsin (Δ) incubation on the immobilized phytase.

It is evident that the immobilized phytase upon incubation of trypsin brought marginal improvement towards proteolytic stability, with 92.9% retention of activity. In case of the pepsin incubation, however, immobilization brought much improvement in the stability with 95% activity retention in comparison to the free enzyme (72%). The present result is the first report on the use of the immobilized phytase, in the study on proteolytic resistance.

CONCLUSION

To the best of our knowledge, this is the first report on the immobilization of a plant phytase on chitosan microspheres. Immobilization, did not bring a change in the properties of phytase like optimum pH, and temperature. Rice bean phytase immobilized on chitosan microsphere showed better storage stability at 4 °C, and resistance in the presence of trypsin and pepsin in comparison to the free phytase. Further, this may find useful applications in the future with respect to agriculture.

ACKNOWLEDGMENTS, COMPLIANCE WITH ETHICAL STANDARDS AND CONFLICT OF INTEREST

The authors acknowledge the research facilities furnished in the Department through UGC DRS III. Ms. K. Belho is grateful for Maulana Azad Senior Research Fellowship. The authors acknowledge Head, Department of Chemistry, NEHU, for allowing FTIR facility. Conflict of interest is ruled out. This article does not contain any of the experiments with human subjects or animals.

REFERENCES

Andriotis, V.M.E., & Ross, J.D. (2003). Isolation and characterisation of phytase from dormant *Corylus avellana* seeds. *Phytochemistry* 64, 689-699.

Awad, G.E.A., Esawy, M.A., El-Gammal, E.W., Ahmed, H.M., Elnashar, M.M., & Atwa, N.A. (2015). Comparative studies of free and immobilized phytase, produced by *Penicillium purpurogenu* GE1, using grafted alginate/ carrageenan beads. *Egyptian Pharmaceutical Journal* 14, 87-93.

Belho, K., & Ambasht, P.K. (2017). Characterization of phytase isolated from rice bean cotyledons (*Vigna umbellata* Thunb.) with respect to influence of different factors. In MN Bhattacharjee, DL Buam, C Masharing, BM Laloo (eds) Exploring Chemistry -Interface with Human welfare. Eastern Book House Publishers, Guwahati, pp. 157-166.

Belho, K., Nongpiur, S.R., & Ambasht, P.K. (2014). Immobilization of acid phosphatase (Type I) from wheat germ on glutaraldehyde activated chitosan beads: Optimization and characterization. *Journal of Proteins and Proteomics* 5, 177-183.

Belho, K., Nongpiur, S.R., & Ambasht, P.K. (2016). Purification and partial characterization of phytase from rice bean (*Vigna umbellata* Thunb.) germinated seeds. *Journal of Plant Biochemistry & Biotechnology* 25, 27-30.

Celem, E.B., & Onal, S. (2009a). Immobilization of avocado phytase on epoxy-activated Sepabead EC-EP and its

application in soymilk phytate hydrolysis. *Artificial Cells, Blood Substitutes, and Biotechnology* 37, 195-202.

Celem, E.B., & Onal, S. (2009b). Immobilization of phytase on epoxy-activated sepabead EC-EP for the hydrolysis of soymilk phytate. *Journal of Molecular Catalysis B- Enzymatic* 61, 150-156.

Chen, K.I., Chiang, C.Y., Ko, C.Y., Huang, H.Y., & Cheng, K.C. (2018). Reduction in phytic acid in soymilk by immobilized phytase system. *Journal of Food Science* 83, 2963-2969.

Cho, E.A., Kim, E.J., & Pan, J.G. (2011). Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores. *Enzyme and Microbial Technology* 49, 66-71.

Cinarli, A., Gurbuz, D., Tavman, A., & Birteksoz, S. (2011). Synthesis, spectral characterizations and antimicrobial activity of some Schiff bases of 4-chloro-2-aminophenol. *Bulletin of the Chemical Society of Ethiopia* 25, 407-417.

Coutinho, T.C., Tardioli, P.W., & Farinas, C.S. (2020). Phytase immobilization on hydroxyapatite nanoparticles improve its properties for use in animal feed. *Applied Biochemistry and Biotechnology* 190, 270-292.

El-Shora, H.M., Khalaf, S.A., & El-Solia M.M. (2019). Modification and immobilization of bacterial phytase. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 10, 529-541.

Gibson, D.M., & Ullah, A.H.J. (1988). Purification and characterization of phytase from cotyledons of germinating soybean seeds. *Archives of Biochemistry and Biophysics* 260, 503-513.

Greiner, R. (2002). Purification and characterization of three phytases from germinated lupine seeds (*Lupinus albus* Var. Amiga). *Journal of Agricultural and Food Chemistry* 50, 6858-6864.

Greiner, R., & Alminger, M.L. (1999). Purification and characterization of a phytate-degrading enzyme from germinated oat (*Avena sativa*). *Journal of the Science of Food and Agriculture* 79, 1453-1460.

Greiner, R., & Konietzny, U. (1996). Construction of a bioreactor to produce special breakdown products of phytate. *Journal of Biotechnology* 48, 153-159.

Greiner, R., Konietzny, U., & Jany, K.D. (1998). Purification and properties of a phytase from rye. *Journal of Food Biochemistry* 22, 143-161.

Greiner, R., Muzquiz, M., Burbano, C., Cuadrado, C., Pedrosa, M.M., & Goyoago, C. (2001). Purification and characterization of a phytate-degrading enzyme from germinated faba beans (*Vicia faba* Var. Alameda) *Journal of Agricultural and Food Chemistry* 49, 2234-2240.

Gupta, K.C., & Jabrail, F.H. (2006). Glutaraldehyde and glyoxal cross-linked chitosan microspheres for controlled delivery of centchroman. *Carbohydrate Research* 341, 744-756.

Igbasan, F.A., Manner, K., Miksch, G., Borriss, R., Farouk, A., & Simon, O. (2000). Comparative studies on the *in vitro* properties of phytase from various microbial origins. *Archives of Animal Nutrition* 53, 353-373.

In, M.J., Kim, K.H., & Oh, N.S. (2007). Phytate degradation by immobilized *Saccharomyces cerevisiae* phytase in soybean-curd whey. *Biotechnology and Bioprocess Engineering* 12, 348-353.

Kalita, T., & Ambasht, P.K. (2019). Immobilization and characterization of acid phosphatase from wheat germ (Type I) in agarose gel. *Journal of Proteins and Proteomics* 10, 291-297.

Kalita, T., Sangma, S.D., Bez, G., & Ambasht, P.K. (2020). Immobilization of acid phosphatase in agar-agar and gelatin: Comparative characterization. *Journal of Scientific Research* 64, 191-200.

Kharkrang, K., & Ambasht, P.K. (2013). Immobilization of α-amylase from *Pennisetum typhoides* inside gelatin beads and its characterization. *Journal of Proteins and Proteomics* 4, 183-189.

Kharkrang, K., & Ambasht, P.K. (2019). Characterization of α-amylase from *Pennisetum typhoides* immobilized inside calcium alginate. *Journal of Scientific Research* 63, 53-67.

Knaul, J. Z., Hudson, S. M., & Creber, K.A.M. (1999). Crosslinking of chitosan fibers with dialdehydes: Proposal of a new reaction mechanism. *Journal of Polymer Science B-Polymer Physics* 37, 1079-1094.

Konietzny, U., Greiner, R., & Jany, K.D. (1995). Purification and characterization of a phytase from spelt. *Journal of Food Biochemistry* 18, 165-183.

Krajewska, B. (2004). Application of chitin-and chitosan-based materials for enzyme immobilizations: a review. *Enzyme and Microbial Technology* 35, 126-139.

Lata, S., Rastogi, S., Kapoor, A., & Imran, M. (2014). Immobilization of phytase produced by fungal strain *Aspergillus heteromorphus* MTCC 10685. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 5, 1615-1631.

Liu, B.L., Jong, C.H., & Tzeng, Y.M. (1999). Effect of immobilization on pH and thermal stability of *Aspergillus ficuum* phytase. *Enzyme and Microbial Technology* 25, 517-521.

Liu, D.M., Chen, J., & Shi, Y.P. (2018). Advances on methods and easy separated support materials for enzymes immobilization. *Trends in Analytical Chemistry* 102, 332-342.

Mahajan, A., & Dua, S. (1997). Nonchemical approach for reducing antinutritional factors in rapeseed (*Brassica campestris* var. Toria) and characterization of enzyme phytase. *Journal of Agricultural and Food Chemistry* 45, 2504-2508.

Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., & Fernandez-Lefuente, R. (2007). Improvement of enzyme

activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology* 40, 1451-1463.

Menezes-Blackburn, D., Jorquera, M., Gianfreda, L., Rao, M., Greiner. R., Garrido, E., & Mora, M. (2011). Activity stabilization of *Aspergillus niger* and *Escherichia coli* phytases immobilized on allophanic synthetic compounds and montmorillonite nanoclays. *Bioresource Technology* 102, 9360-9367.

Migneault, I., Dartiguenave, C., Bertrand, M.J., & Waldron, K.C. (2004). Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques* 37, 790-802.

Moraes, M.L., Oliveira Jr., O.N., Filho, U.P.R., & Ferreira, M. (2008). Phytase immobilization on modified electrodes for amperometric biosensors. *Sensors and Actuator B: Chemical* 131, 210-215.

Mosbach, K. (1971). Enzymes bound to artificial matrixes. *Scientific American* 224, 26-33.

Onem, H., Cicek, S., & Nadaroglu, H. (2016). Immobilization of a thermostable phytase from Pinar melkior (*Lactarius piperatus*) onto magnetite chitosan nanoparticles. *CyTA-Journal of Food* 14, 74-83.

Onem, H., & Nadaroglu, H. (2014). Preparation and properties of purified phytase from oakbug milkcap (*Lactarius quietus*) immobilised on coated chitosan with iron nano particles and investigation of its usability in food industry. *Journal of Food and Nutrition Research* 2, 938-945.

Payne, G.F., & Sun, W.Q. (1994). Tyrosinase reaction and subsequent chitosan adsorption for selective removal of a contaminant from a fermentation recycle stream. *Applied and Environmental Microbiology* 60, 397-401.

Phillippy, B.Q. (1998). Purification and catalytic properties of a phytase from Scallion (*Allium fistullosum* L.) leaves. *Journal of Agricultural and Food Chemistry* 46, 3491-3496.

Quan, C.S, Fan, S.D., & Ohta, Y. (2003). Immobilization of *Candida krusei* cells producing phytase in alginate gel beads: an application of the preparation of *myo*-inositol phosphates. *Applied Microbiology and Biotechnology* 62, 41-47.

Sastre, D.E., Eduardo, A.R., & Netto, C.G.C.M. (2020). Strategies to rationalize enzyme immobilization procedures. *Methods in Enzymology* https://doi.org/10.1016/bs.mie.2019.09.003 1-30.

Spahn, C., & Minteer, S.D. (2008). Enzyme immobilization in Biotechnology. *Recent Patents on Engineering* 2, 195-200.

Trouillefou, C.M., Le Cadre, E., Cacciaguerra, T., Cunin, F., Plassard, C., & Balamie, E. (2015). Protected activity of a phytase immobilized in mesoporous silica with benefits to plant phosphorus nutrition. *Journal of Sol-Gel Science and Technology* 74, 55-65.

Wahba, M.I. (2017). Chitosan-glutaraldehyde activated calcium pectate beads as a covalent immobilization support. *Biocatalysis and Agricultural Biotechnology* 12, 266-274.

Zhang, G.Q., Dong, X.F., Wang, Z.H., Zhang, Q., Wang, H.X., & Tong, J.M. (2010). Purification, characterization and cloning of a novel phytase with low pH optimum and strong proteolysis resistance from *Aspergillus ficuum* NTG-23. *Bioresource Technology* 101, 4125-4131.

***