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Immobilization of Phytase produced by fungal strain *Aspergillus heteromorphus* MTCC 10685

Suman Lata*[1], Smita Rastogi[2], Ashima Kapoor[3] and Mohd. Imran[4]

[1] Department of Bioengineering, Integral University, Lucknow (UP), 226026, India.
[2]Department of Biotechnology, Delhi Technological University, Delhi, 110042, India.
[3]Department of Biotechnology, Meerut Institute of Engineering & Technology, Meerut (UP), 250005, India
[4]Department of Biotechnology, Integral University, Lucknow (UP), 226026, India.

ABSTRACT

The present study reports immobilization of *Aspergillus heteromorphus* phytase (MTCC 10685) by entrapment and cross-linking method. Among the different carriers used for entrapment, phytase entrapped in carrageenan blocks exhibited highest activity yield of 87%, followed with calcium alginate beads (82%) at optimized 3.0% alginate concentration. The phytase entrapped in polyacrylamide blocks gave minimum activity yield. The crude phytase immobilized on gelatin with cross-linking agent showed the highest activity and immobilization yield (90%). The optimum pH of the immobilized enzyme was shifted to a less acidic range compared with the free enzyme (from pH 4.0 to pH 4.5). The optimum temperature was determined to be 65^{o}C for the free enzyme, while the immobilized phytase showed optimum enzyme activity at 45^{o}C. The crude enzyme was found to be stable at 65^{o}C for 30 min. The carrageenan-entrapped and cross-linked phytase were stable at 45^{o}C for about 90 min. The thermal stability at the optimum temperature significantly improved by the immobilization process. Reusability of immobilized enzyme is a key factor for its cost effective industrial use. The carrageenan entrapped enzyme retained 80% of its initial activity after 4 cycles of reuse, while crosslinked phytase was able to retain 80% of its initial activity after 7 cycles. On storage at 4^{o}C, the carragenan entrapped phytase and cross-linked phytase exhibited appreciable activity after a week. The free phytase however could retain comparable enzyme activity upto 4 days and thereafter decreased sharply.

Keywords: *Aspergillus heteromorphus,* Crosslinked phytase, Entrapped phytase, Enzyme immobilization, Phytase production.

Corresponding author

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INTRODUCTION

Phytases (*myo*-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8) are phosphohydrolytic enzymes that initiate stepwise removal of phosphates and thereby release of free *myo*-inositol or the derivatives of *myo*-inositol from phytic acid (*myo*-inositol hexakisphosphate) or its salts [1]. The phytic acid is the major organic storage form of phosphorus which comprising 1-5% by weight present in oilseeds, cereal grains, legumes, pollens, nuts and others [2]. Phytase reduces the antinutritional properties of phytic acid and increases the bioavailability of essential metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} in monogastrics [3], which in turn reduces the total phosphorus pollution and eutrophication caused by the excretion of undigested phytic acid by monogastrics or simple stomached animals because of the lack of adequate levels of phytase in their digestive tracts [4]. Phytase occurs in a wide variety of organisms including plants, animals and micro-organisms; however, phytases have been isolated mostly from micro-organisms like bacteria, moulds and yeasts. The fungal isolates belonging to the genera *Aspergillus, Penicillium, Mucor* and *Rhizopus* are the most active microorganisms capable of producing phytase through submerged and solid-state fermentation [5,6].

Immobilized enzymes are currently the subject of considerable attention for their advantages over soluble enzymes and the steadily increasing number of applications for immobilized enzymes. Now days, immobilized enzymes are preferred over their free counterpart due to their prolonged availability or reusability that reduces or minimizes downstream and purification processes [7]. Enzymes can be immobilized by different techniques, such as adsorption, entrapment and cross linking [8]. Entrapment into gel matrices is a very common and cost effective method. The microbial cells and enzymes are entrapped in a gel matrix through which substrates and products diffuse easily. Agar, agarose, *k*-carrageenan, collagen, alginate, chitosan or cellulose has been used for immobilizing microbial enzymes as well as microbial cells by entrapment [9].

An ideal matrix must encompass characteristics like inertness, physical strength, stability and regenerability, ability to increase enzyme specificity/activity and reduce product inhibition, nonspecific adsorption and microbial contamination. Immobilization generates continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity [10].

With this view, the properties and applications of immobilized phytases from microorganisms have been studied by many researchers [11-16]. However, no report has yet been documented on the immobilization of fungal phytase which have phytase activity suitable for industrial exploitation. In the present investigation, an attempt has been made to select a suitable immobilization matrix, which could yield maximum enzyme activity from immobilized phytase of *Aspergillus heteromorphus*.

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MATERIALS AND METHODS

Chemicals

Calcium salt of phytic acid (Calcium phytate) of highest available purity was purchased from HiMedia Chemical Laboratories Pvt. Ltd. Company, Mumbai. All other chemicals and reagents were used of the highest available purity and obtained from leading manufacturers including BDH, Sigma and Glaxo.

Microorganism

The phytase producing fungal strain *Aspergillus heteromorphus* (MTCC Accession no. 10685) used in the present study was isolated from soil dumps of poultry farm waste [17]. It was deposited at Microbial Type Culture Collection, IMTECH, Chandigarh (India). It was maintained at 4^{o}C on Sabouraud's agar (SDA) medium which consisted of peptone (1%), dextrose (4%) and agar (1.5%).

Phytase production

The fungal strain *Aspergillus heteromorphus* was grown in the optimized production medium (Phytase Production Medium) [17], after incubation, the culture was filtered through Whatman no. 1 filter paper. The culture supernatant was used as a source of phytase for immobilization and estimation of phytase activity.

Phytase assay

Phytase activity was determined by measuring the amount of inorganic phosphate liberated from calcium phytate. The calcium phytate (0.2%) prepared in 0.1 M acetate buffer (pH 5.0) was used as a substrate.

For free phytase, the reaction mixture was prepared by the addition of 600 µl substrate with 150 µl of the crude free enzyme solution. After incubation for 20 min at 35°C, the enzymatic reaction was stopped by adding 750 µl of 5% (w/v) TCA solution and the released free orthophosphate (Pi) in the reaction mixture was measured by a modification of the method of Fiske and Subbarow [18]. Colour reagent (750 µl), was added to the sample solution (750 µl) and the production of phosphomolybdate was measured spectrophotometrically at 700 nm [19].

For the immobilized enzyme, assay mixture consisted of 1 ml substrate solution and immobilized phytase were incubated at 45°C for 40 min. After incubation, 750 µl TCA solution was added to stop the reaction. Further phytase assay procedure was similar as that of soluble phytase assay.

One unit of phytase is defined as the amount of enzyme that liberates one µg inorganic phosphate/ml/min under the assay conditions.

Immobilization of phytase

Entrapment of phytase in different matrices by entrapment method

The crude phytase enzyme was immobilized by entrapment method using different polymer matrices such as sodium alginate, *k*-carrageenan and polyacrylamide.

Entrapment of phytase in calcium alginate beads

A 10 ml suspension containing 3ml of phytase and 3% sodium alginate was extruded drop-wise into 0.2 M $CaCl_2$ solution using hypodermic syringe to form calcium alginate beads. After 2 hr, beads were washed with double distilled water and used for further studies.

Effect of different concentrations of sodium alginate on the activity of immobilized phytase

Effect of different concentrations of sodium alginate was studied using different concentrations of sodium alginate viz. 1%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5% and 5%. The enzyme assay was performed and phytase activity was calculated.

Effect of amount of enzyme entrapped in alginate beads

The effect of different amount of enzyme entrapped in sodium alginate solution used for bead formation was also investigated.

Effect of amount of alginate entrapped phytase on enzyme activity

Effect of number of beads of calcium alginate was studied using 1, 2, 3, 4, 5, 6 and 7 beads of calcium alginate in a reaction mixture. The enzyme assay was performed and phytase activity was determined.

Entrapment of phytase in k-carrageenan and polyacrylamide blocks

The 2ml phytase enzyme was thoroughly mixed with 8ml suspension of carrageenan (4%) and kept at 4°C to allow gelation. The carrageenan gel was soaked in cold 0.3 M KCl solution for 1 hr. Similarly, phytase was entrapped in polyacrylamide. The 2 ml phytase enzyme was thoroughly mixed with 8ml suspension of polyarylamide (10%) and kept at 4°C for 1 hr for gelation. The gels were cut into blocks of two sizes: (0.5 x 0.5 cm^2) and (1.0 x 1.0 cm^2). The blocks were washed with double distilled water. The enzyme activity of blocks was determined immediately and remaining blocks were stored at 4^oC.

Effect of amount of enzyme entrapped in carrageenan and polyacrylamide blocks

The effect of different amount of enzyme entrapped in carrageenan and polyacrylamide solution used for blocks formation were studied.

Effect of no. of blocks (carrageenan and polyacrylamide entrapped phytase) on phytase activity

Effect of number of blocks of two sizes was studied using 2, 3, 4 and 5 blocks of carrageenan and polyacrylamide in an individual reaction mixture. The enzyme assay was performed and phytase activity was calculated.

Immobilization of phytase by cross-linking method

Gelatin particles, as a solid support, were prepared as described by Kennedy et al. (1984) [20]. The cross linking reaction was achieved by adding 1.5 g of gelatin particles into a 2.5% aqueous solution of glutaraldehyde. The mixture was stirred for 90 min at room temperature. After the reaction was terminated, the glutaraldehyde was removed and the gelatin particles were washed with water. These cross linked gelatin particles were stored in 15% (w/v) NaCl at 4°C until used. Subsequently, phytases (5 ml) were coupled onto glutaraldehyde crosslinked gelatin particles (1.5 g) at 4°C for a period of 12 hr. Unbound enzymes was collected and the concentration was determined spectrophotometrically.

Effect of different volumes of substrate on the activity of immobilized phytase

Effect of different volumes (0.6 ml, 1 ml and 2 ml) of substrate solution of 0.2% concentration on the enzyme activity of cross-linked phytase was studied. The reaction mixture consisted of 0.5 g phytase coupled glutaraldehyde crosslinked gelatin particles and different volumes of substrate solution. The enzyme assay was performed and phytase activity was calculated.

Biochemical characterization of free and immobilized phytase

Effect of pH on phytase activity

The pH activity profile of free (150 µl), entrapped and cross-linked phytase was studied by incubating samples with calcium phytate (600 µl) in buffers of different pH ranging from 3.0 to 6.5 at 35°C for 20 min. The buffers (0.1M) used were Glycine-HCl (pH 2.5–3.5), acetate buffer (pH 4.0–6.0), HEPES (N-2-Hydroxy ethyl piperazine-N-ethane sulphonic acid) (pH 6.5 and 7.0) and Tris-HCl (pH 7.0–9.0).

Influence of temperature on enzyme activity

The temperature profile of the free and immobilized phytases (carragenan-entrapped phytase and cross-linked phytase) were determined by incubating with substrate at various temperatures ranging from 30^{o}C to 70^{o}C and phytase activity was calculated.

Thermal stability

To determine the thermal stability, free phytase of *Aspergillus heteromorphus* was incubated in the standard buffer at optimum temperature 65^{o}C for 60 min while the immobilized enzyme was incubated at 45^{o}C for 150 min and phytase activity was determined.

Reusability and Storage stability

The stability of immobilized phytase on repeated use was examined by measuring the enzyme activity after each successive run. The immobilized phytase was incubated with substrate solution and at the end of the reaction the immobilized enzyme was collected by filtration. It was washed with distilled water and re-suspended in 1 ml of freshly prepared substrate to start a new run. The supernatant was used to determine the phytase activity.

Free and immobilized enzymes were stored at 4^{o}C in 0.1 M of acetate buffer (pH 4.5). The storage stability of the free and immobilized enzyme was investigated by measuring their activities after being stored for a week and the remaining activity measurements were performed at regular intervals of 24 h.

Result analysis:

All the experiments and assays were carried out in triplicate and the mean values are presented.

RESULTS AND DISCUSSION

Immobilization of phytase

In the present study, the crude free phytase (29.0 U/ml) from *Aspergillus heteromorphus* was used for immobilization by entrapment and cross-linking method. For entrapment different matrices used were sodium alginate, carrageenan and polyacrylamide. Entrapment is one of the most convenient methods for enzyme immobilization. The major advantage of this technique is the simplicity and the beads formed are transparent, mechanically stable and non-toxic. This method is also cost effective and shows good performance in industrial applications.

Many investigators reported immobilization of phytase by using different matrices such as on glutaraldehyde-activated silicate [21], gelatin particles by cross linking [12], calcium alginate beads [14,22] and activated Sepharose [23] to study the various biotechnological

applications. Adsorption method was used for immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores [24]. However, there are very few reports on phytase enzyme immobilization as well by entrapment method, hence present study reports phytase immobilization by entrapment method.

Entrapment of phytase in alginate beads

Effects of different concentrations of sodium alginate solution on the activity of immobilized phytase

The effect of concentration of sodium alginate solution used for bead formation was studied in the range of 1.0-5.0%. The entrapped phytase activity in calcium alginate beads increased as the sodium alginate concentration increases up to 3.0% and decreased on further increase in concentration and shown maximum activity of 21.6 U/ml. Hence, 3.0% sodium alginate solution was used for bead formation in further studies (Table 1).

Table 1: Effect of concentration of sodium alginate on phytase activity

Concentration of alginate (%)	Enzyme activity (U/ml)
1.0	7.7
1.5	12.1
2.0	18.5
2.5	20.3
3.0	21.6
3.5	19.2
4.0	10.7
4.5	6.3
5.0	5.3

Effect of amount of enzyme entrapped in alginate beads

The effect of different amount of enzyme entrapped in sodium alginate solution used for bead formation was studied. The reaction mixture consists of 7 ml alginate solution and 3ml of enzyme of cultures in calcium alginate supported the maximum phytase activity of 23.8 U/ml (Table 2).

Table 2: Effect of amount of enzyme entrapped in alginate beads

Reaction mixture	Enzyme activity (U/ml)
8ml alginate solution + 2ml enzyme	21.6
7ml alginate solution + 3ml enzyme	23.8
6ml alginate solution + 4ml enzyme	20.1

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Effect of amount of alginate entrapped phytase on enzyme activity (no. of beads)

Since the rate of enzyme catalyzed reaction is dependent on concentration of enzyme, the effect of amount of beads ranging from 50-350 mg (which corresponds to 01 to 07 beads in a reaction mixture) on enzyme activity was studied. The rate of reaction increased up to 250 mg beads and shown maximum phytase activity of 23.8 U/ml when five beads (250 mg) of calcium alginate was used in the reaction mixture as compared to enzyme activity obtained with 04 beads (200 mg) (Table 3).

Table 3: Effect of number of calcium alginate beads in reaction mixture

Wt. of Calcium alginate bead (mg)	Enzyme activity (U/ml)
50	2.6
100	8.0
150	16.4
200	20.0
250	23.8
300	23.7
350	23.8

Weight of one bead = 50mg

Entrapment of phytase in carrageenan and polyacrylamide blocks

The crude phytase exhibiting 29.09 U/ml was entrapped in carrageenan and polyacrylamide gel blocks and immobilized phytase in blocks was used as a source of enzyme for the assay of phytase activity.

Effect of no. of blocks of carrageenan or polyacrylamide entrapped phytase in the reaction mixture

The phytase entrapped carrageenan and polyacrylamide gel was cut into blocks of two sizes; (0.5 x 0.5 cm^2) and (1.0 x 1.0 cm^2). The effect of number of carrageenan and polyacrylamide blocks of different sizes on phytase activity was studied. The results indicated that the rate of reaction increased up to four carrageenan blocks of 0.5 x 0.5 cm^2 size in a reaction mixture exhibiting 25.2 U/ml enzyme activity (Table 4), while in case of polyacrylamide gel, the maximum activity of 19.8 U/ml was observed with three blocks of same size in a reaction mixture (Table 5). It was observed in both the cases that the enzyme entrapped gel blocks of size 0.5 x 0.5 cm^2 showed higher activity as compared to blocks of size 1.0 x 1.0 cm^2 on interaction with substrate solution. It is due to increased surface area available with smaller blocks than the larger blocks.

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Table 4: Entrapment of phytase in *k*-Carrageenan blocks

Matrix	No. of blocks	Enzyme activity (U/ml)
Free enzyme	-	29.0
Carrageenan blocks (0.5 x 0.5 cm^2)	2	18.0
	3	21.2
	4	25.2
	5	25.0
Carrageenan blocks (1.0 x 1.0 cm^2)	2	10.5
	3	12.8
	4	15.0
	5	14.3

Table 5: Entrapment of phytase in polyacrylamide gel blocks

Matrix	No. of blocks	Enzyme activity (U/ml)
Free enzyme	-	29.0
Polyacrylamide blocks (0.5 x 0.5 cm^2)	2	16.2
	3	19.7
	4	18.2
Polyacrylamide blocks (1.0 x 1.0 cm^2)	2	12.0
	3	13.3
	4	13.5

Effects of amount of enzyme entrapped in carrageenan blocks and polyacrylamide blocks

The effect of different amount of enzyme entrapped in gel solution was investigated with a view to study its effect on enzyme-substrate interaction. It is because as the gel concentration increases, porosity decreases which affects the interaction of substrate with entrapped enzyme and the stability of immobilized enzyme preparation. From the results, it can be seen that in both cases, the optimum activity was obtained in the gel composition of 8 ml carrageenan (3%) or polyacrylamide solution (10%) and 2 ml of enzyme (29.09 U/ml) used for entrapment of enzyme. (Table 6).

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Table 6: Effect of amount of enzyme entrapped in carrageenan blocks and polyacrylamide blocks

Reaction mixture	Enzyme activity (U/ml)
Free enzyme	29.09
8ml Carrageenan sol. + 2ml enzyme	25.2
7ml Carrageenan sol.+ 3ml enzyme	23.0
6ml Carrageenan sol. + 4ml enzyme	24.1
8ml Polyacrylamide sol. + 2ml enzyme	19.8
7ml Polyacrylamide sol.+ 3ml enzyme	18.8
6ml Polyacrylamide sol. + 4ml enzyme	13.0

Immobilization of phytase by cross-linking method

Gelatin particles were used as solid support and crosslinking reaction was done with the treatment of gultaraldehyde. Phytase was finally coupled onto gultaraldehyde cross linked gelatin particles. On incubating the cross linked phytase (0.5 g) with different volumes of substrate solution (0.2%), the maximum phytase activity of 26.1 U/ml was obtained in the reaction mixture consisted of 0.5 g phytase crosslinked gelatin particles and 1 ml of substrate solution compared to the 600 µl substrate solution used earlier (Table 7).

Table 7: Activity yield of phytase bound to cross-linked gelatin particles

Reaction mixture	Enzyme activity (U/ml)	Activity yield (%)
Free enzyme (Control) -150µl enzyme + 600µl substrate solution	29.0	100
0.5g phytase cross linked gelatin particles + 600µl substrate solution	20.6	71.2
0.5g phytase cross linked gelatin particles + 1ml substrate solution	26.1	90.1
0.5g phytase cross linked gelatin particles + 2ml substrate solution	24.7	85.3

Activity yield of immobilized phytase by using different matrices

An attempt was made to achieve binding of high levels of enzyme with a high retention of its original or initial activity. Thus, the effect of different immobilization matrices on phytase

activity was investigated in terms of activity yield. The activity yield of the enzyme was defined as:

$$\text{Activity yield (\%)} = \frac{\text{activity of immobilized enzyme}}{\text{activity of free enzyme}} \times 100$$

Among the different matrices used, the maximum activity yield of 90% was obtained by phytase bound to crosslinked gelatin particles. The activity yield of alginate entrapped phytase and carrageenan entrapped phytase was found to be 82% and 87% respectively (Table 8). The polyacrylamide entrapped phytase however, showed only 68% activity yield.

Table 8: Effect of different matrices used for immobilization of phytase

Matrix	Enzyme activity (U/ml)	Activity yield (%)
Free enzyme	29.0	100
Ca-alginate beads	23.8	82
k- Carrageenan blocks	25.2	87
Polyacrylamide blocks	19.7	68
Glutaraldehyde crosslinked gelatin particles	26.1	90

On the basis of activity yield, phytase immobilized in carrageenan blocks and cross-linked on gelatin particles were selected for further immobilization studies.

Biochemical properties of free and immobilized phytase

The following properties of the immobilized crude phytase were examined and compared them with those of the free counterpart produced by *Aspergillus heteromorphus*.

Effect of pH on phytase activity

The pH activity profile of free, entrapped and cross-linked phytase was studied by incubating enzyme samples with substrate (600 µl) in buffers of different pH ranging from 3.0 to 6.5 at 35°C. As can be seen from the Fig.1, the optimum pH of free enzyme was found to be 4.0 while that of immobilized phytase was pH 4.5. The carrageenan entrapped phytase at optimum pH also exhibited increase in activity yield and became comparable to the cross linked phytase. Above pH 5.0, remarkable decrease in the activity also was observed in all forms of enzyme.

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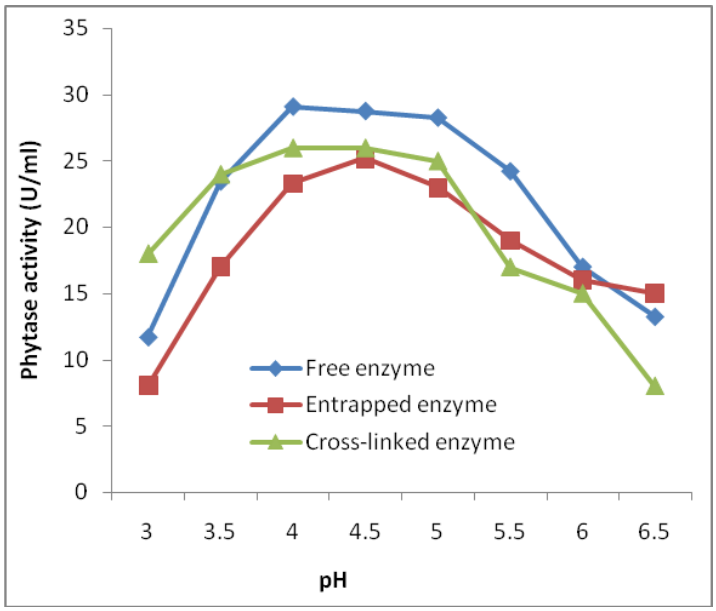

Figure 1: Effect of pH on activity of free and immobilized phytase

Generally, the bacterial phytases have optimum pH in neutral to alkaline range while for fungal phytases, optimum pH range is 2.5–6.0 and stability of phytase decreased above pH 7.5 and below pH 3.0. This wide range differences in pH optima could be due to the variation in molecular conformation or stereo-specificity of the phytase enzyme from variable sources [6]. Lassen et al. (2001) also investigated pH profile of fungal phytase from *Peniophora lycii* (phy A) and found phytase activity optimum within the pH range of 4.0-4.5 [25]. However, phytase produced from *Aspergillus niger* CFR335 either under solid state or submerged fermentation showed phytase activity at pH 4.5 only [26]. Purified phytase from *Aspergillus niger van* Teighem exhibited maximum activity at highly acidic exhibiting pH 2.5 [6]. The optimum pH for the immobilized *Aspergillus ficuum* phytase was not much different from that of the intact enzyme [27]. Liu et al. (1999) observed that the activities of free phytase were more dependent on pH than the activities of the immobilized one. However, the optimum enzyme activity was obtained at pH 5.15 from both free and immobilized phytases [12].

Influence of temperature on the enzyme activity

The temperature dependence of the phytase activity was investigated in the temperature range from 30-70°C and the results are shown in Fig.2. The crude phytase from *Aspergillus heteromorphus* was active over a temperature range of 50°C to 65°C and optimally active at 65°C, hence can be considered as thermophilic enzyme. The immobilized phytase showed optimum enzyme activity at 45°C and decreased sharply on further increase in temperature (Fig. 2). The reason is obvious that the gel could not withstand the temperature above 45°C.

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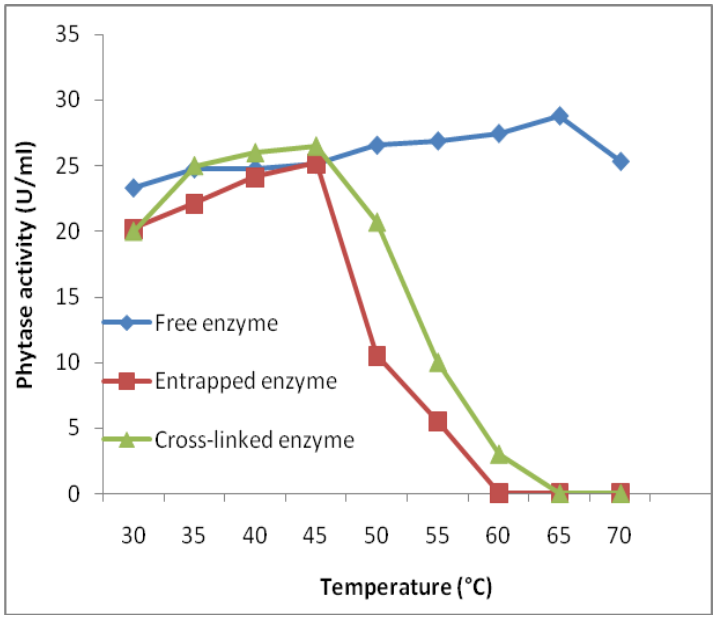

Figure 2: Effect of temperature on activity of free and immobilized phytase

The higher temperature optima of 65°C was observed for phytase covalently immobilized on Fractogel TSK HW-75F, as compared to the free phytase (58°C) from *Aspergillus niger* NRRL 3135 [28]. Liu et al. (1999) also reported enhanced temperature tolerance by the phytase produced by *Aspergillus ficcum* on immobilization by cross linking method. The optimum temperature of immobilized phytase was reported to be 58°C while for free phytase was 50°C [12]. In general, free phytases show high activity in the temperature range of 50–70°C and optimum temperature is mostly between 45°C to 60°C for microbial phytases [6].

Thermal stability

The effect of the temperature on the stability of the enzyme is shown in Fig. 3. The crude enzyme was found to be stable at 65°C for 30 min. retaining about 65% of original activity. The immobilized phytase preparations exhibited higher thermal stability at their optimum temperature. The carrageenan-entrapped and cross-linked phytase were stable at 45°C for about 90 min. with 78% and 85% residual activity respectively (Fig. 4).

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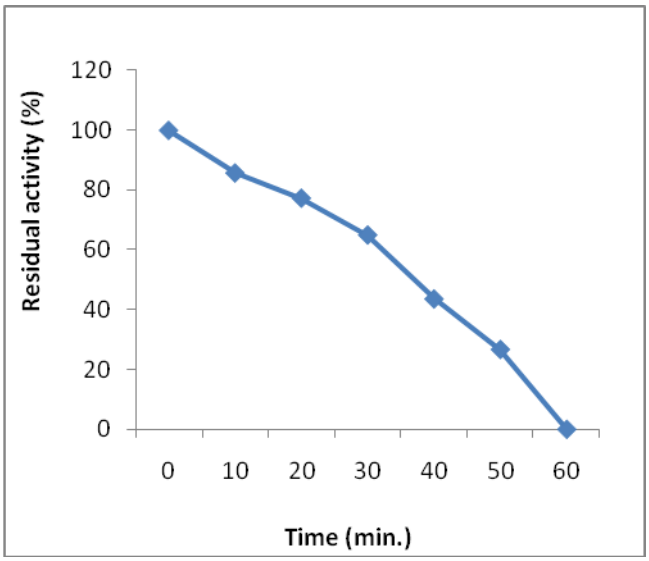

Figure 3: Thermal stability of free phytase at 65°C

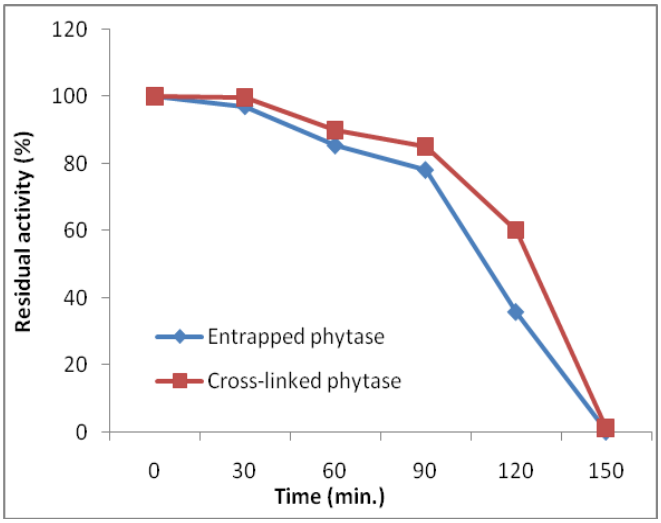

Figure 4: Thermal stability of immobilized phytase at 45°C

Aspergillus niger NCIM 563 produced two different extracellular phytase (Phy I and Phy II). The thermal stability of these phytases was investigated and found that, after 65°C for 1.5 h, Phy I retained 40% activity while Phy II retained 55% of its original activity [29].Greiner et al. (2013) demonstrated the enhancement in thermal stability as a consequence of covalent immobilization of phytase on Fe_3O_4 magnetic nanoparticles. The immobilized phytases from rye, *Aspergillus niger* and *Escherichia albertii* exhibited maximum catalytic activity at 60°C, 65°C and 70°C respectively [30].

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Reusability and Storage stability

Reusability and storage stability are of considerable importance for various applications of biocatalysts with a commercial point of view. An increased stability can make the immobilized enzyme more advantageous than its free counterpart. Enzymes can easily lose their catalytic activity and denatured, thus, careful storage and handling are essential [31]. The stability of immobilized phytase on repeated use was examined by measuring the activity for the hydrolysis of phytate at 45°C (Fig. 5). The carragenan entrapped enzyme retained 80% of its initial activity after 4 cycles of reuse, while crosslinked phytase retained 80% of its initial activity after 7 cycles. Celem *et al.* (2009) reported high operational stability for phytase immobilized on Sepabead EC-EP. The immobilized phytase retained 42% of its initial activity after 21 cycles of reuse at 37°C and 44% of its initial activity after 7 cycles of reuse at 60°C [31].

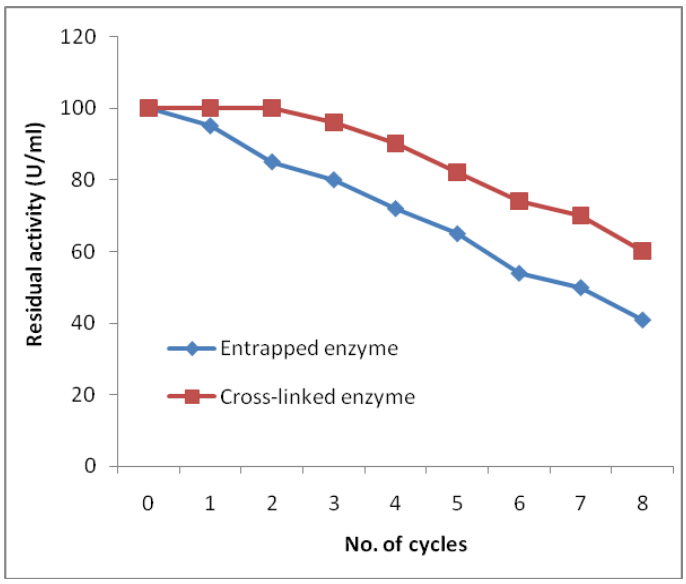

Figure 5: Operational stability of immobilized phytase

The carragenan entrapped phytase retained 70% of the original activity while cross-linked phytase retained 80% activity on storage at 4°C after 7 days. The free phytase however could retain 80% activity upto 4 days and thereafter decreased sharply with only 40% remaining activity after 7 days (Fig. 6). The enzyme immobilization has stabilization effect which varies with the immobilization method employed. Since, the enzyme in solution is not stable on storage and the activity is gradually decreased overtime. Mckelvie et al. (1995) utilized immobilized phytase for the determination of phytase hydrolysable phosphorus present in natural waters. The immobilized enzyme exhibited good operational and storage stability over a period of time [32].

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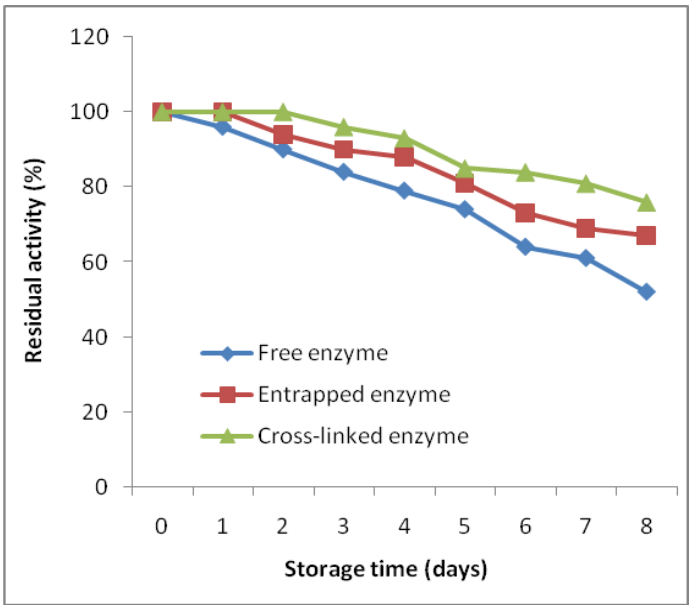

Figure 6: Storage stability of free and immobilized phytase

CONCLUSION AND FUTURE PERSPECTIVES

Phytases are gaining attention for their potentials in human nutrition and health, animal feed industry and aquaculture. The phytate hydrolyzing ability of phytase is also utilized for industrial applications like production of phytate free soy protein, soybean milk in food industry, production of *myo*-inositol phosphates and their derivatives used as potential drugs, enzyme stabilizers etc. In this study, the comparative study of biochemical characterization of free and immobilized phytase produced from *Aspergillus heteromorphus* MTCC 10685 is reported. The immobilized phytase exhibited high immobilization yield, operational stability and appreciable thermal stability which are desirable for its industrial applications. However, to obtain better and alternative source of phytases, there is an ongoing interest in screening new organisms producing novel and efficient phytases with the ultimate aim to produce this enzyme to cost effective level and establish the suitability for its industrial application.

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REFERENCES

[1] Mullaney EJ and Ullah AHJ. Biochem Biophys Res Commun 2003; 312: 179-184.
[2] Singh B and Satyanaryana T. J Sci Ind Res 2010; 69: 411-414.
[3] Vohra A and Satyanarayana T. Crit Rev Biotechnol 2003; 23: 29-60.

[4] Pandey A, Szakacs G, Soccol CR, Rodriguez-Leon JA and Soccol VT. Bioresour Technol 2001; 77: 203-214.
[5] Liu B, Rafiq A, Tzeng Y and Rob A. Enz Microb Technol 1998; 22: 415-424.
[6] Vats P and Banerjee UC. Enzyme Microb Technol 2004; 35: 3-14.
[7] Datta S, Christena LR and Rajaram YRS. Biotechnol 2013; 3: 1-9.
[8] Norouzian D. Iran J Biotech 2003; 1: 197-206.
[9] Singh B and Satyanarayana T. J Appl Microbiol 2008; 105: 1858-1865.
[10] D'Souza SF. Curr Sci 1999; 77: 69–79.
[11] Dischinger HC and Ullah AHJ.
[12] Enzyme Microb Technol 1999; 25: 517-521.
[13] Quan CS, Fan SD and Ohta Y. Appl Microbiol Biotechnol 2003; 62:41-47.
[14] In M, Kim K and Oh N. Biotechnol Bioprocess Eng 2007; 12: 348-353.
[15] Singh B and Satyanarayana T. Bioresour Technol 2008b; 99: 2824-2830.
[16] Blackburn DM, Jorquera M, Gianfreda L, Rao M, Greiner R, Garrido E and Mora ML. Bioresour Technol 2011; 102: 9360-9367.
[17] Lata S, Rastogi S, Kapoor A and Imran M. Int J Adv Biotechnol Res 2013; 4: 224-235.
[18] Fiske CH and Subbarow Y. J Biol Chem 1925; 66: 376-400.
[19] Bae HD, Yanke LJ and Cheng KJ. J Microbiol Methods 1999; 39:17-22.
[20] Kennedy JF, Kalogerakis B and Cabral JMS. Enzyme Microb Technol 1984; 6:127-31.
[21] Ullah A and Cummins B. Ann N Y Acad Sci 1988; 542: 102-106.
[22] Greiner R and Sajidan A. Brazil Arch Biol Technol 2008; 51: 235-246.
[23] Greiner R and Konietzny U. J Biotechnol 1996; 48: 153-159.
[24] Cho EA, Kim EJ and Pan JG. Enzyme Microb Technol 2011; 49: 66-71.
[25] Lassen SF, Breinholt J, Ostergaard PR, Brugger R and Bischoff A. Appl Environ Microbiol 2001; 67:4701-4707.
[26] Gunashree BS and Venkateswaran G. J Ind Microbiol Biotechnol 2008; 35:1587-1596.
[27] Ullah AHJ and Phillippy BQ. Prep Biochem 1988; 18: 483-489.
[28] Ullah AHJ and Cummins BJ. Biotechnol Appl Biochem 1987; 9: 380-388.
[29] Soni SK, Magdum A and Khire JM. World J Microbiol Biotechnol 2010; 26: 2009–2018.
[30] Greiner R, Konietzny U, Blackburn DM and Jorquera MA. Bioresour Technol 2013; 142:375-383.
[31] Celem EB and Onal S. J Mol Catal B: Enzyme 2009; 61: 150-156.
[32] Mckelvie ID, Hart BT, Cardwell TJ and Cattrall RW. Anal Chim Acta 1995; 316: 277–289.