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Modification and Immobilization of Bacterial Phytase.

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ABSTRACT

Phytase (myo-inositol hexakisphosphate phosphohydrolase, E.C.3.1.3.8) was isolated and purified from *E. coli* and *S. aureus*. The enzyme was purified to homogeneity by ammonium sulphate (80 %), DEAE-Cellulose and Sephadex G- 200. The final specific activities were 403 and 355 Umg^{-1} protein, respectively. Phytase was immobilized successfully on alginate and chitosan. $CaCl_2$ increased the immobilization efficiency to 82% at 0.3 mM. The immobilized enzyme retained 38% and 43% of its activity from *E coli* and *S aureus* after five cycles. The chitosan-immobilized and alginate –immobilized enzyme showed 28 % and 45 %, respectively of its activity after 30 days at 25°C. The enzyme from both sources was desorbed by SDS, however desorptivity was stronger with alginate-immobilized phytase than chitosan- immobilized enzyme.

Keywords: Phytase, *E. coli*, *S aureus*, Purification, Modification, Immobilization.

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INTRODUCTION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisdihydrogenphosphate) is an organic phosphorus compound found in legumes, cereals, and oilseed crops which represents a major source of nutrients for the animals.

Phytic acid is an anti-nutrient component in plant-derived feed and food because it forms complexes with amino acids and proteins **[1, 2]**. Phytic acid acts as an excellent chelator of cations such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} [3, 4, 5].

The phosphate of phytic acid cannot be metabolized by mono-gastric animals such as poultry, pig and fish. This is due to lack of phytase and thus participates in the problem of phosphorus pollution in areas where there is an livestock production [6].

Phytases catalyze the splitting of phytic acid or phytate to inositol phosphate esters and inorganic phosphate [7, 8]. Phytase is produced by animals, plants, bacteria, and fungi. Phytase producing microorganisms are mostly present in rhizospheric soil of crop plants [9].

Phytase is used as an additive in animal feed as diets of mono-gastric animals. Also, it is used in processing and manufacturing of food for human consumption [7]. Degradation of phytic acid by phytase does not produce carcinogenic toxic by-products [10]. A thermostable phytase is applied in degradation of phytic acid in pulp and paper processing.

Calcium alginate beads are used as carriers in the entrapment of biocatalysts [11, 12]. The advantages of this method are low cost, high porosity and simplicity of preparation. However, this compound has some advantages such as large pore size, biomolecule leakage and biocompatibility [11, 13]. Chitosan beads are also used for enzymes immobilization and they are cheap and effective [14].

The aims of the present work were: firstly to isolate, purify and study the biochemical characteristics of phytase from *E. coli* and *S. aureus*. Secondly, to immobilize phytase on calcium alginate and chitosan for future biotechnological application.

MATERIAL AND METHODS

Experimental organisms

The experimental strains which used in this study were *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538). They were obtained from the American Type Culture Collection (ATCC, Minnesota, USA).

Growth medium

The following media were used for isolation and identification of the strain.

1-Tryptic soy broth (TSB) was obtained from Sigma- Aldrich. The medium contained tryptone 17.0 g, soytone, 3.0 g, glucose (= dextrose) 2.5 g, sodium chloride 5.0 g, and dipotassium phosphate 2.5 dissolved in 1000 ml distilled water. The final pH was 7.3 at 25 °C.
2-Reasoner's 2A agar (R2A) [15]. This medium contained 0.5 g casamino acids, 0.5 g proteose peptone , 0.5 g dextrose, 0.5 g soluble starch, 0.5 g yeast extract, 0.3 g dipotassium phosphate, 0.05 g magnesium sulphate per $7H_2O$ and 15.0 g agar was dissolved in 1000 ml distilled water. The final pH was 7 at 25 °C.

Enzyme preparation

The culture filtrate of *E. coli* and *S. aureus* grown for 48 h at 37°C was collected by centrifugation at 5000 rpm and used as crude extract of phytase.

Assay of phytase enzyme

Phytase was assayed according to [16] through measuring the amount of inorganic phosphorus released from sodium phytate for 30 min at 25°C. The reaction medium contained 100 µl of enzyme extract, 3 ml 100 mM Tris-HCl (pH 7.0), 0.5 ml 2 mM sodium phytate and 0.2 ml 15 mM $CaCl_2$. The reaction was stopped by the addition of 0.5 ml 10 % (w/v) TCA. The mixture was centrifuged for 25 min at 5000 rpm for removing the precipitated protein. The resulting supernatant was analyzed for inorganic phosphate. One unit (U) of phytase represents the amount of enzyme required for production of one µmol of inorganic phosphorus per min at 25°C.

Purification of enzyme

The crude extract of phytase was brought to 80% of $(NH_4)_2SO_4$ saturation and left to stand for 60 min. The resulting precipitate was centrifuged and the supernatant was then brought to 65% saturation of $(NH_4)_2SO_4$. The precipitate which contained the enzyme activity was dissolved in 20 ml of 100 mM Tris-HCl buffer (pH 7.0) containing 15 % glycerol.

A sample (20 ml) of the above preparation was applied on a DEAE-cellulose column (30 × 2.0 cm) equilibrated with buffer A and the enzyme activity was measured. The enzyme preparation from DEAE-cellulose was loaded to Sephadex- 200 (30 × 2.0 cm) equilibrated with the same buffer (100 mM Tris-HCl buffer, pH 7.0) containing 1.0 mM dithiothreitol (DTT). Then resulting fractions, each of 1 ml, were collected and the active fractions were pooled and concentrated by dialysis. The active fractions were stored at 4°C for assay.

Sodium Dodecyl Sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE) of phytase from E. coli and S. aureus.

SDS-PAGE was carried out according to [17] for the separation of proteins. The markers used were: chemotrypsinogene (25 KDa), glyceraldehyde 3-phosphate dehydrogenase (36 KDa), ovalbumin (45 KDa) and bovine serum albumin (66 KDa).

Estimation of total protein

The total protein was estimated by the method of [18].

Determination of inorganic phosphorus

Inorganic phosphate was determined according to [19].

Chemical modification of enzyme

Modification of purified phytase with N-bromosuccinimide (NBS)

NBS was used as a reagent for tryptophanyl group [20]. NBS (2, 4, 6, 8, and 10 mM) was added to 5.0 ml of phytase enzyme in 20 mM phosphate buffer, (pH 7.0). The modified phytase was dialyzed against distilled water and assayed as described by [16].

Modification of purified phytase with butanedione (BD)

The modification was performed at room temperature according to [21, 22]. The effect of butanedione-borate solution was tested at various concentrations (1, 2, 3, 4, and 5 mM) on phytase in 100 mM sodium borate buffer (pH 8.0) to start the modification reaction. The enzyme was incubated with buffer alone served as untreated control. The enzyme activity was measured immediately afterwards.

Modification of purified phytase with N-acetylimidazole (NAI)

The method used for modification with N-acetylimidazole was of [23]. Phytase solution (5.0 ml) was mixed with 2.4 ml of 150 mM sodium phosphate buffer (pH 7.0). Samples (0.5 ml) of the same buffer solution containing 1, 2, 3, 4, and 5 mM of NAI were prepared. NAI solution was prepared freshly before used to avoid the degradation of the reagent. The reaction was allowed to proceed for 60 min at room temperature. At intervals of time aliquots were removed and diluted with water to be assayed for the enzyme activity. The absorbance was measured at 278 nm against the blank solution containing the buffer and the reagent. The reaction was stopped by drops of 1.0 N HCl and the mixture was then passed through a column (1.8 × 28 cm) of Sephadex G-200 equilibrated with water and then enzyme activity was measured.

Modification of purified phytase with N-ethylmaleimide (NEM)

The modification was carried out according to [24, 25]. Purified phytase (5.0 ml) was incubated at 25ºC in 100 mM Tris-HCl (pH 5.0), 1.0 mM EDTA and NEM (2, 4, 6, 8, and 10 mM). At appropriate time intervals (12, 24, 36, 48, 60, and 72 h) during the incubation aliquots from each concentration were withdrawn and assayed for enzyme activity.

Effect of $CaCl_2$ on immobilization of phytase on alginate

$CaCl_2$ was used in the process of immobilization with alginate at various concentrations (1, 2, 3, 4, and 5 M) and the immobilization percentage was calculated.

Reusability of immobilized phytase

The activity of immobilized phytase either on alginate or chitosan was measured through 5 cycles and the percentage of remaining activity was calculated.

Storage stability of immobilized phytase

The alginate-immobilized and chitosan-immobilized phytase was stored at 25°C followed by determination of the enzyme activity every 5 days.

Desorptivity of immobilized phytase

The immobilized phytase whether entrapped or cross-linked was treated with various concentrations of SDS (0.2, 0.4, 0.6, 0.8, 1.2, and 1.4 % w/v) followed by measuring the desorptivity.

RESULTS

Purification of phytase from E. coli and S. aureus

The purification of phytase from *E. coli* using ammonium sulphate, DEAE- cellulose and Sephadex- 200 was studied. The results are shown in Table 1. These results indicate that purification process was successful for obtaining considerable specific activity of 403 Umg^{-1} protein and 155- fold. In addition, there was a single band indicating the homogeneity of the purified enzyme.

The purification of phytase from *S. aureus* followed the same method adopted for the enzyme from *E. coli*. The results are recorded in Table 2. The specific activity for the enzyme from *S. aureus* was 355 Umg^{-1} protein with 221.9- fold. Also, the enzyme was purified to homogeneity and the molecular weight was 50 KDa and 48 KDa, respectively Fig. 1a and 1b.

Effect of N-ethylmaleimide (NEM) on phytase activities from E. coli and S. aureus.

The results in Fig 2 reveal that phytase activity from the two organisms was reduced in presence of NEM and the reduction was dependent on the NEM concentration. At 10 mM of NEM the inhibition percentage was 77.2 % for phytase activity from *E. coli*; however it was 100 % for phytase from *S. aureus*. IC_{50}

values of NEM were calculated and their values were 6.7 mM and 4.4 mM for *E. coli* and *S. aureus*, respectively.

Effect of butanedione (BD) on phytase activity from E. coli and S. aureus

BD is well known as a reagent for arginyl group of enzyme. The enzyme activity was estimated from *E. coli* and *S. aureus* under treatment with BD. The results in Fig 3 show that the activity of phytase from the two organisms under treatment was inhibited in a concentration- dependent manner. However, the inhibition percentage for *S. aureus* enzyme was higher than that observed for *E. coli* enzyme. At 5.0 mM the inhibition percentage of *E. coli* enzyme was 73.3 %, however it was 100 % for phytase from *S. aureus*.

Effect of N-acetylimidazole (NAI) on phytase activity from E. coli and S. aureus

In this experiment phytase activity was determined in presence of various concentrations of NIA. The results in Fig 4 reveal that phytase activity from both *E. coli* and *S. aureus* was inhibited in presence of NAI in a concentration- dependent manner. It was observed that the inhibition of phytase activity from *S. aureus* was stronger than that recorded for *E. coli* enzyme. In case of *E. coli* the inhibition was represented by 75.3 % at 5.0 mM but in case of *S. aureus* the enzyme activity was abolished completely. The IC_{50} values of NAI for phytase from the two organisms were 2.86 mM and 2.29 mM, respectively.

The influence of N- bromosuccinimide (NBS) on phytase from E. coli and S. aureus

NBS is well known reagent for tryptophanyl group in protein [23]. The results in Fig 5 indicate reduction of phytase activity from both *E. coli* and *S. aureus* under treatment with NBS. The reduction was dependent on the concentrations of NBS. The IC_{50} values of NBS were 5.7 mM and 3.3 mM for *E. coli* and *S. aureus*, respectively.

The effect of $CaCl_2$ on immobilization of phytase

The relation between $CaCl_2$ concentrations on the immobilization efficiency (%) was studied. The results in Fig 6 indicate that increasing $CaCl_2$ concentration resulted in an increase in immobilization efficiency up to 0.3 M after which the efficiency was reduced at 0.4 and 0.5 M.

Reusability of immobilized phytase on alginate and chitosan

The effect of reusability of phytase immobilized on alginate and chitosan was studied. The results are shown in Fig 7. These results indicate that the immobilized enzyme retained 38 % and 43 % on alginate and chitosan after the 5^{th} cycle; respectively. It was noticed that the remaining activity of immobilized enzyme either on alginate or chitosan was reduced gradually.

Storage stability of free and immobilized phytase

The storage stability of the free and immobilized phytase was determined at 25 C for 30 days. The remaining activity was expressed as relative activity of the initial immobilized enzyme. The results in Fig 8 show continuous reduction of the enzyme activity by increasing the storage time. The free enzyme expressed lower activity, however alginate- immobilized enzyme expressed higher activity then the free phytase and highest was recorded for chitosan- immobilized enzyme.

Effect of SDS on immobilized phytase

The influence of various concentrations of SDS on the immobilized enzyme either on alginate or chitosan was investigated. The results in Fig 9 reveal continuous reduction of the enzyme remaining activity particularly in alginate-immobilized enzyme compared to chitosan-immobilized enzyme.

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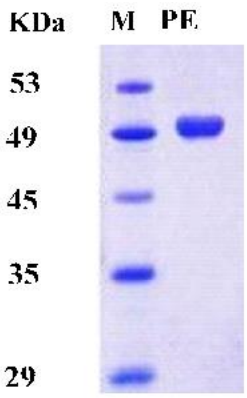

Fig. 1a: SDS-PAGE of purified phytase from *E.coli*.

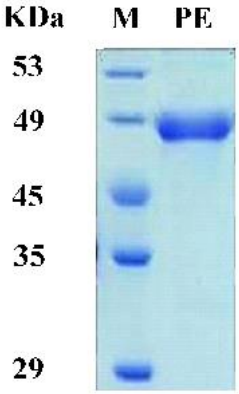

Fig. 1b: SDS-PAGE of purified phytase from *S. aureus*

M: Marker and PE: Pure Enzyme. Markers used: Glutamate DH (53 KDa), Fumarase (49 KDa), Ovalbumin (45 KDa), Lactate DH (35 KDa) and Carbonic anhydrase (29 KDa).

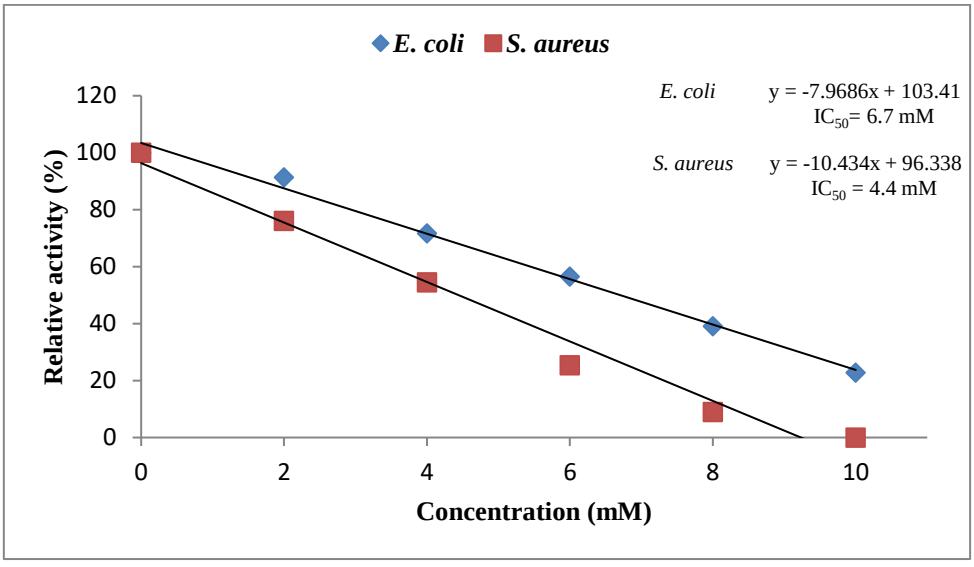

Fig. 2: Effect of N-ethylmaleimide (NEM) on phytase activity from *E. coli* and *S. aureus*.

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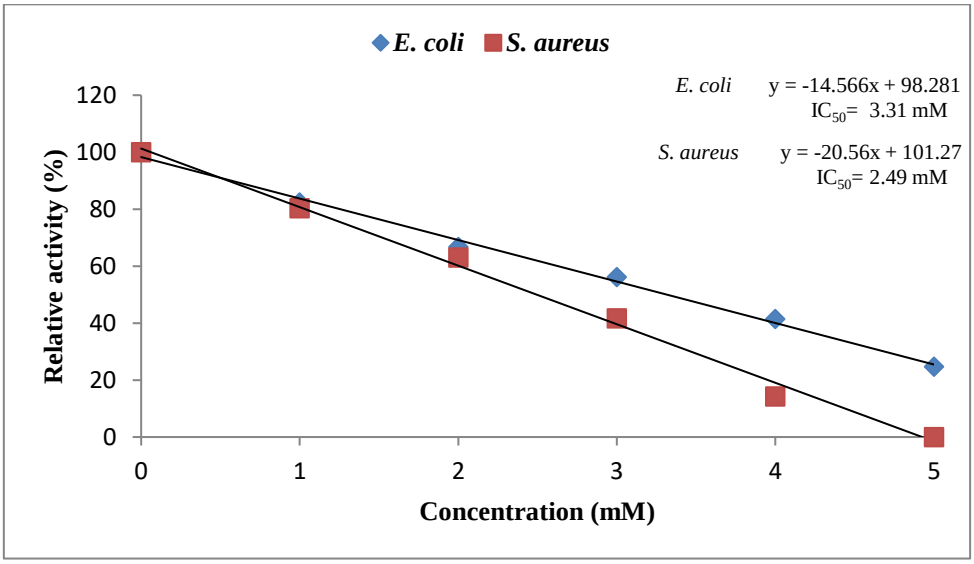

Fig. 3: Effect of butanedione (BD) on phytase activity from *E. coli* and *S. aureus*.

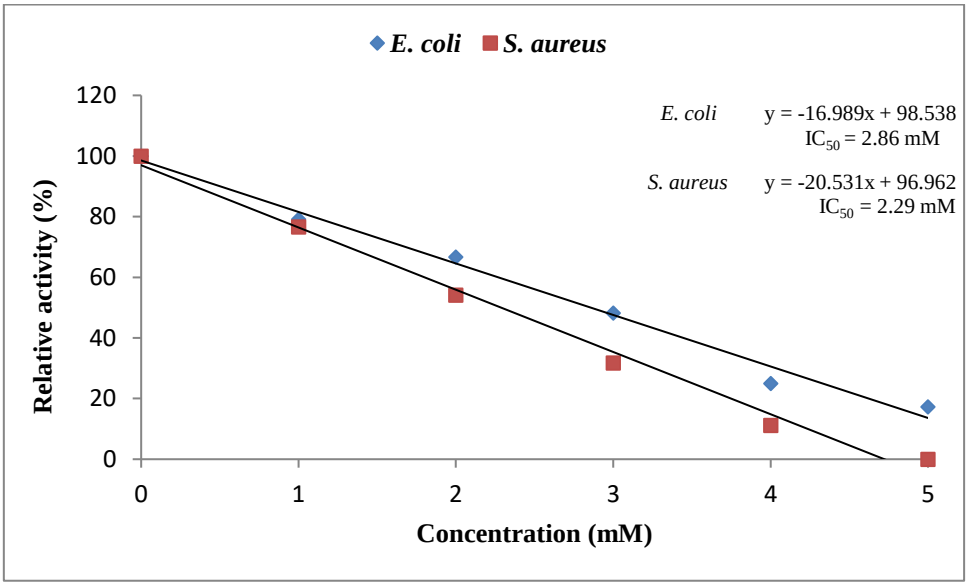

Fig. 4: Effect of N-acetyl imidazole (NAI) on phytase activity from *E. coli* and *S. aureus*.

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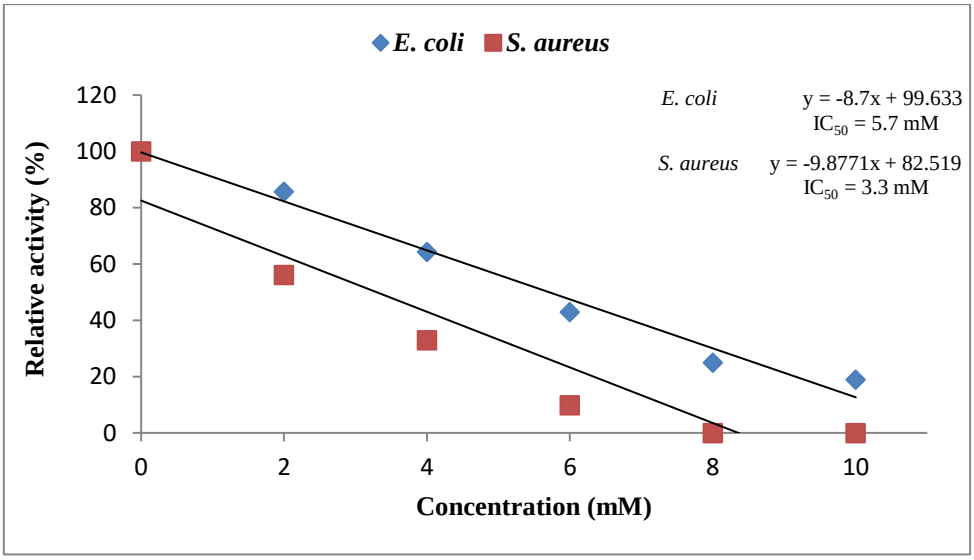

Fig. 5: Effect of N- bromosuccinimide (NBS) on phytase activity from *E. coli* and *S. aureus*.

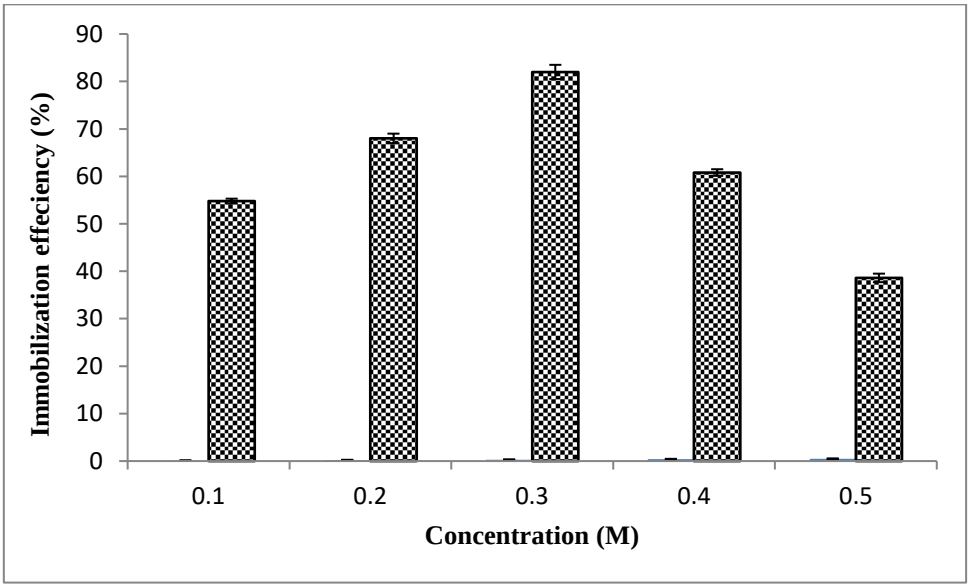

Fig. 6: Effect of $CaCl_2$ on immobilization of phytase

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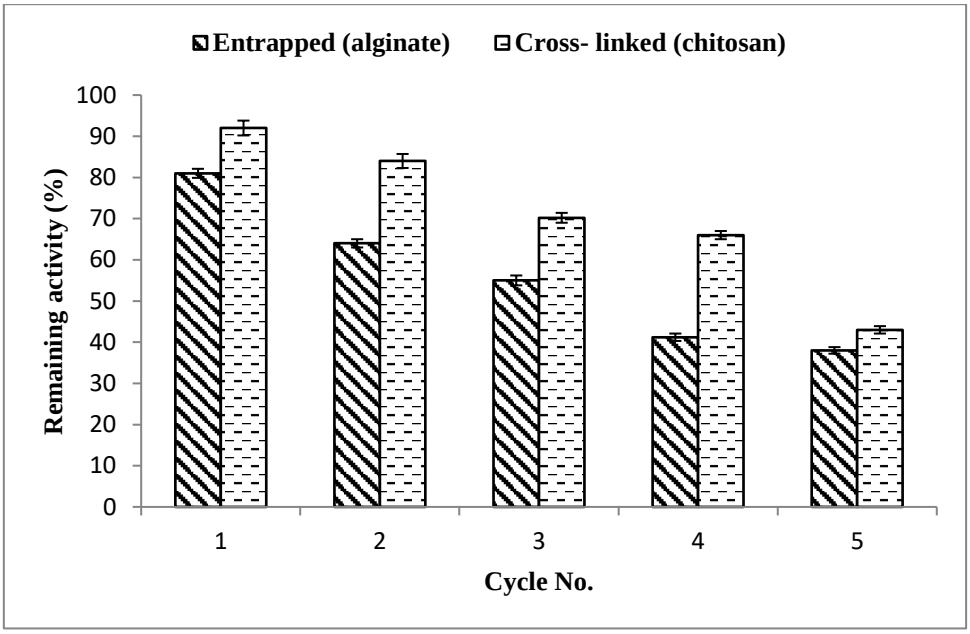

Fig. 7: Reusability of immobilized phytase on alginate and chitosan.

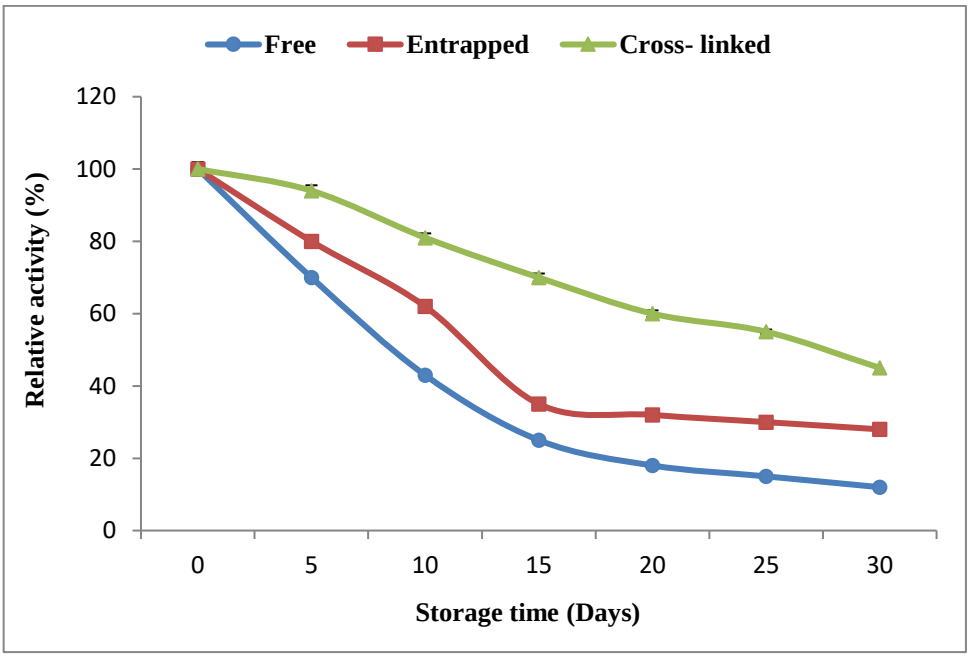

Fig. 8: Storage stability of free and immobilized phytase at 25 ºC.

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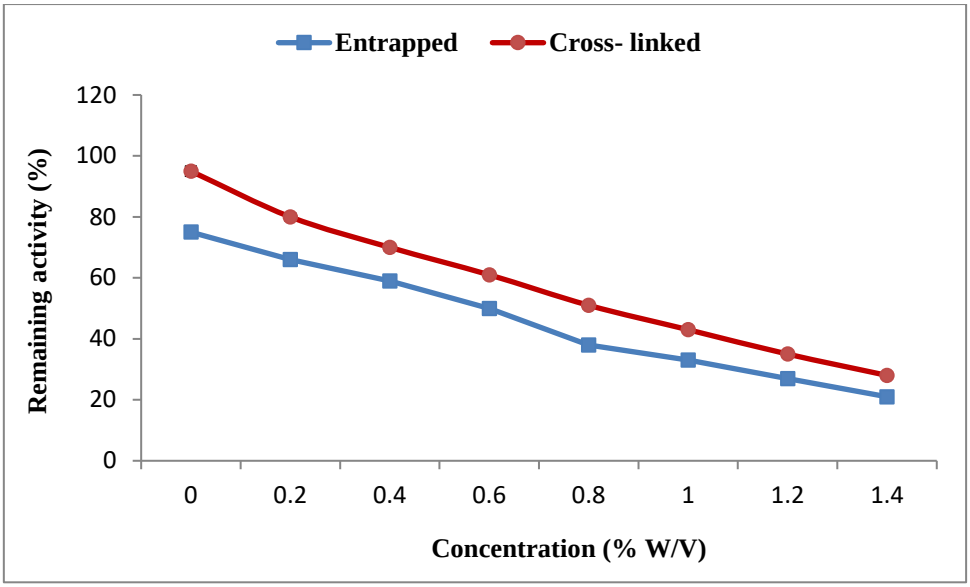

Fig. 9: Effect of SDS on immobilized phytase

Table 1: Purification of phytase from *E. coli*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1} protein)	Yield (%)	Fold of purification
Crude extract	141	362	2.6	100	1.0
$(NH_4)_2SO_4$ (40-85 %)	74.0	280	3.8	77.3	1.46
DEAE-cellulose	1.7	160	94.1	44.2	36.2
Sephadex-G200	0.3	121	403	33.4	155

Table 2: Purification of phytase from *S. aureus*

Step	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1} protein)	Yield (%)	Fold of Purification
Crude extract	160	250	1.6	100	1.0
$(NH_4)_2SO_4$ (40-85 %)	93.0	140	1.5	56.0	0.94
DEAE-cellulose	19.0	186	9.8	74.4	6.13
Sephadex-G200	0.2	71.0	355	28.4	221.9

DISCUSSION

The molecular weight of phytase was determined and was 42 KDa for *Klebsiella sp* ASR1 [26]. Also, [27] reported 38 KDa for the bacterial enzyme.

N-ethylmaleimide (NEM) and butadiene (BD) were used as a reagent for sulfhydryl group and arginyl [28]. N-acetyl imidazole (NAI) and N-bromosuccinimide (NBS) was used as tryptophanyl reagent [29]. Phytase in the present work from *E. coli* and *S. aureus* were inhibited by NEM, BD, NAI and NBS revealing that sulfhydryl, arginyl, lysyl and tryptophanyl groups are essential for the enzyme catalysis.

The chemical modification plays an important part in probing the mechanism of enzyme activity. This technique is used for identification of the individual amino acid residues which are responsible for the catalytic

properties of the entire protein. During the chemical modification experiments, changes in enzymatic specify have been reported, however it is not predicated [30].

As general, it is remarkable that the chemical modification studies could selectively target the residues specifically at the active site, as demonstrated by substrate protection. In fact, these results are valuable in determination the active group taking part for enzyme catalysis for possible regulation of the enzyme activity.

The purified phytase from *E. coli* and *S. aureus* was immobilized on alginate and chitosan. Chitosan was better than alginate as bead for immobilization. The soluble enzyme cannot be recovered from the reaction from the reaction mixtures and therefore cannot be used to catalyze more reactions, but the process of immobilization can make it feasible. The loss of some activity of the immobilized preparation in repeated use is a common phenomenon [31]. The observed decrease in the activity after immobilization is likely a result to desorption of the enzyme. After immobilization, the formation of aggregates could be lost likely because the enzyme molecules are evenly dispersed within the bead of immobilization.

The immobilized enzyme on alginate or cross-linking expressed an appreciable reusability for 5 cycles. However, the immobilized enzyme by cross-linking expressed higher activity compared to the alginate-immobilized enzyme which is consistent with the report of [32] for Mn peroxidase from *Trichoderma harziam*. In fact, the retaining of such appreciable activities after immobilization reveals the possibility of using such enzyme form in industry for reducing cost.

The present results indicate that phytase immobilized on chitosan retained 50 % of its activity after 5 cycles. These results have a significant important for industrial use of phytase. It is important to consider the enzyme stability with respect to various parameters like temperature, reusability, storage stability, etc. The soluble enzyme cannot be recovered from the reaction mixture but the process of immobilization can make it feasible. However, the loss of activity of the immobilized phytase in repeated use is a common phenomenon [31].

Studying the storage stability of free and immobilized phytase at 25ºC indicated that the immobilized enzyme expressed higher stability than the free enzyme. The immobilized enzymes expressed higher stability which is probably a result of the prevention of autolysis by immobilization. Thus, the present results indicate that the immobilized enzyme has much better storage stability than the free enzyme and the remarkable stability of the immobilized phytase system offers an attractive platform for biocatalysis in industry.

SDS is known as denaturing agent for enzyme proteins [29], treating the immobilized enzyme with SDS in the present work led to continuous reduction in enzyme activity depending on the concentration of SDS. This show that phytase is susceptible to denaturation process by SDS. In the present work SDS caused desorption of phytase. The sulphate group and alkyl chain of SDS interact with the protein via interactions with positively charged amino acid side chains as well as hydrophobic side chins of the enzyme [33]. It has been reported that SDS fully inactivated and almost unfolded tertiary as well as secondary structure of enzymes such as aminoacylase, ribulose-1,5-biphosphate carboxylase/oxygenase, fatty acid synthase and certain kinase [34].

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