### Chapter V

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5.1 Introduction

5.1.1 Phytase stability

Stability is an important parameter as discussed in literature review II and III, which codetermines the economic feasibility of applying an enzyme in an industrial process. The increasing interest in applying enzymes in industrial processes has spurred the search for biocatalysts with new or improved properties [Kirk et al., 2002]. Due to the unique capacity of enzymes to catalyze reactions with high velocity and unmet specificity under a variety of conditions, the development of new biocatalytic processes is principally feasible and potentially profitable. Most phytases from plants and microorganisms start to lose activity around 55–60 °C [Phillippy, 2002]. For example, the Tm of A. niger phyA phytase is 63.3 °C and the denaturation is associated with an irreversible conformational change with loss of 70% to 80% of the activity.

5.1.2 History and current trends

To improve the enzyme’s activity and stability, various methods being explored include the enzyme immobilization [on solid support, sol–gel, or CLEA], physical or covalent attachment to PEG, rinsing with n-propanol methods [PREP and EPRP], water-in-ionic liquids microemulsions and the design of enzyme-compatible ionic solvents. [Koeller and wong, 2001]. The most efficient stabilization strategies are often based on the simultaneous use of all these three methods. Although there are many examples of enzymes that have been stabilized by introduction of only one or two mutations and despite many successful efforts to understand the structural basis of protein stability, there is still no universal strategy to stabilize any protein by a limited number of rationally designed mutations [Schmid et al, 2001].

5.1.3 Enzyme immobilization

Enzyme immobilization entails attaching or incorporating enzyme molecules onto or into larger structures, including immobilization on surfaces, attachment within porous solids, or encapsulation in polymer, sol–gel, or composite materials [Kim et al., 2005]. Especially in the case of multipoint attachment, immobilization reduces protein unfolding and hence improves stability. These conventional enzyme immobilization approaches often result in a final immobilization of micrometer scale, and the mass of support compared to that of the enzyme is often quite large. Enzyme immobilization can provide increased resistance to changes in
conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again. The main task of enzyme immobilization is to select a suitable immobilization method to design an immobilized biocatalyst which can meet not only the catalytic needs [expressed as productivity, space–time yield, stability and selectivity] but also the noncatalytic needs [e.g. separation, control, down-streaming process] of a given application. [Cao., 2005]. The advantages of using immobilized enzymes are: reusability, continuous use, less labour intensive, saving in capital cost, minimum reaction time, less chance of contamination in products, more stability, improved process control and high enzyme : substrate ratio. There are mainly five different techniques for immobilization of enzyme: Adsorption, covalent bonding, entrapment, copolymerization or cross-linking, and encapsulation.

5.1.4 Workings with various nanoparticles for increasing the perceptive skill of enzyme actions

Particles having one or more dimensions of the order of 100nm or less are generally known as nanoparticles. Nanoparticles are sized between 1 and 100 nanometers. Nanoparticles may or may not exhibit size-related properties that differ significantly from those observed in fine particles or bulk materials. The transition from microparticles to nanoparticles can lead to a number of changes in physical properties. Two of major factor in this are the increase in the ratio of surface area to volume, and the size of particle moving into the realm where quantum effects predominate. [Holister et al., 2003]. The recent developments of nanotechnology in synthesizing biocompatible and functionalized magnetic nanoparticles have numerous novel applications in biomedicine as well as diagnosis. Especially, Fe₃O₄ ferrites magnetic nanoparticles have been rising as a significant useful material due to their specific properties such as superparamagnetic, non toxic and small size, etc. Magnetic nanoparticles are used to coat several surfactants to anti-aggregation which was caused by magnetic dipole – dipole attractions between particles. [Dung et al., 2009] Magnetic particles are used as the support materials and they can be easily separated from the reaction medium and stabilized in a fluidized reactor by applying a magnetic field. The most popular applications of magnetic carrier technology are bioaffinity chromatography, wastewater treatment, immobilization of
enzymes or other biomolecules and the preparation of immunological assays. [Holister et al., 2003].

5.1.5 Specialized coating for easy separation
It has been demonstrated that enzymes attached on magnetic iron oxide nanoparticles can be easily recovered using a magnet and recycled for iterative uses. Separation of magnetic nanoparticles coated enzyme from the reaction mixture magnet was used for easy separation. Magnet was coated with some membrane so it’s very easy to remove membrane and so separation of nanoparticles. According to enzyme type some buffer was used for the washing of the enzyme. It is very simple and easy method for separation of the magnetic nanoparticles coated enzyme.

5.1.6 Effect of immobilization and coating on enzyme
Immobilization and coating of enzyme makes enzyme more stable, while the activity of enzyme could be decreased due to the some reasons. Thus, immobilization and coating of enzyme have some benefits and some drawbacks.

5.1.6.1 Benefits
• Enzyme immobilization and coating increase the enzyme stability.
• They make enzyme for repeated uses and also easy to recycle.
• So, that continuous use of enzyme can be possible.
• Save the cost of the product.
• They provide easy separation of enzyme from the reaction mixture.
• Due to the immobilization and coating enzyme can act in the high or low pH, high temperature, and high salt concentration.
• They also allow enzymes to be held in place throughout the reaction.
• Better availability of active sites of enzyme due to the immobilization.
• They improve high enzyme: substrate ratio.
• Shaking condition can’t affect the enzyme activity.

5.1.6.2 Drawbacks
• Immobilization and coating means additional cost.
• Loss of enzyme activity during preparation.
• Activity yields may be low due to exposure of the enzyme to toxic reagent.
• Active site may be modified through the chemical reactions used to immobilization and coating.

5.1.7 Present study

The principal objective of our research was to develop the effective strategies for antifouling coating of phytase. Thermostability and acid tolerance are to important advantageous features which commercial phytase should have, as many phytase enzymes are available in the market [novozyme, magzyme etc.], the major drawback of all these enzymes is inactivation during feed pelleting process, and instability at stomach pH 2. The study was focused on developing the pH and temperature stable enzyme coatings, than can be easily separable from the reaction mixture. Present study was conceptualized by using the basic work of Kim et al, [2005] on development of hierarchical ordered mesoporous silica structure. In our study we developed this kind of highly ordered structure by using biopolymer carboxy methyl-cellulose and high methoxy pectin, the silica framework sequentially grown the template of this complex polymer and enzymes molecules are entrapped in this highly porous structure, the process parameter was optimized and the enzyme was tailored as per the need.

![Diagram of the proposed antifouling coating process](image)

**Figure 5.1** Pictorial presentation of present study
5.1.8 Objective of present study

✓ Synthesis of hierarchical ordered mesoporous structure and its characterization
✓ Synthesis of magnetic nanoparticle and its characterization
✓ Crosslinking of enzyme with nanoparticles and standardization of process
✓ Development of co-adsorption process of enzyme in meso-cellular pores
✓ Catalytic profiling of coated and free enzyme
✓ Study on effect of various parameters like temperature, pH, inhibitors and incubation periods
✓ Comparison of this coating technology with other commercially available coated enzymes
5.2. Materials and Methods

5.2.1 Nanoparticles synthesis

The established methods of magnetic nanoparticles synthesis include co-precipitation of salts by the addition of a base under inert atmosphere at room temperature. The size, shape, and composition of the magnetic nanoparticles very much depend on the type of salts used (e.g. chlorides, sulphates, nitrates), the Fe\(^{2+}/Fe^{3+}\) ratio, the reaction temperature and the pH value. For synthesis of nanoparticles there are many methods are used. For magnetic nanoparticles we used two different methods. In the first method we used FeSO\(_4\) (0.01 M) and FeCl\(_3\) (0.02 M). Mixed both solution in the same amount and then stirred it for 20 minutes. Add 25\% ammonium hydroxide (0.08 M) till the pH reached 9 to 11. Centrifuge the solution and supernatant was discarded. Dry the pellet and pellet obtained were magnetic nanoparticles of Fe\(_3\)O\(_4\). In the second method, 1 M FeCl\(_3\) (10 ml) mixed with 1 M FeSO\(_4\) (5 ml) solution in a flask under nitrogen atmosphere. Ammonium hydroxide solution (about 30 ml) was slowly added with vigorous stirring until a pH reached between 11 and 12. Continuous stirring was done for another 30 minutes. The solution colour could be seen to alter from orange to black, leading to black Fe\(_3\)O\(_4\) nanoparticles. Applying a permanent external magnet isolated the magnetic nanoparticles. The supernatant was decanted and discarded. Deionized and degassed water was then added to wash the particles thoroughly and was repeated for 5 times to remove excess ions and salts. Finally particles were washed with ethanol twice and stored as dispersion in ethanol, and stored. Before the use, dry Fe\(_3\)O\(_4\) nanoparticles\textsuperscript{[Kim et.al, 2005]}.

5.2.2 Purity analysis of nanoparticles by X-ray powder diffraction (XRD), Transmission electron microscopy (TEM), thermo gravimetric analysis (TGA).

To analyze the purity of nanoparticles x-ray diffraction method was used. X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analyzed material is finely ground, homogenized, and average bulk composition is determined. The XRD technique takes a sample of the material and places a powdered sample in a holder, then the sample is illuminated with x-rays of a fixed wave-length and the intensity of the reflected radiation is recorded using a goniometer. This data is then analyzed for the
reflection angle to calculate the inter-atomic spacing (D value in Angstrom units - \(10^{-8}\) cm). The intensity (I) is measured to discriminate (using I ratios) the various D spacings and the results are interpreted to identify possible matches. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law \((n\lambda=2d \sin \theta)\). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. By scanning the sample through a range of \(2\theta\) angles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings. Typically, this is achieved by comparison of d-spacings with standard reference patterns. (Moecher, and David, 2004) (XRD Instrument of BRUKER–D2 PHASER was used.).

Synthesized nanoparticles were also characterized with TGA. Thermo gravimetric Analysis (TGA) measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. Measurements are used primarily to determine the composition of materials and to predict their thermal stability at temperatures up to 1000°C.

For TEM analysis 2mg of nanoparticles were first washed with acetone, and then sample was charged on TEM grid by capillary and allowed to dry for 60 min. Transmission Electron Microscopy was used to analyze the prepared sample.

5.2.3 Synthesis of mesoporous silica (HHMS) by using natural and artificial polymer as template

The synthesis of polymeric silica was carried out by using reported method of Lee et al.,[2005].
5.2.3.1 Synthesis of mesoporous silica by Pluronic p123 (BASF)

Pluronic P-123 is triblock polymer was purchased from BASF corporation. Typical synthesis procedure for polymeric silica is as follows: 9.7 g of pluronic P-123 and 4.48 mL acetic acid was dissolved in 200 mL water. The solution was heated to 60°C and maintained at that temperature for more than 1 h. Sixteen milliliters sodium silicate (27% SiO2, 14% NaOH) diluted with 200 mL of water was poured into the prepared solution with vigorous stirring. The pH of the synthetic solution was 6.3 to 6.4 pH. The solution was further heated to 60°C and aged at that temperature for 20 h, followed by hydrothermal treatment at 100°C for 24 h. Calcination of filtered materials at 300°C generated polymeric silica [Kim et al., 2007].

5.2.3.2 Synthesis of mesoporous silica by using carboxy methyl cellulose (CMC) and high methoxy pectin

Nanoporous biopolymer was prepared by modification in the method used by Lee et al. [2008] 20 g of CMC (water soluble) was first dissolved 400 ml of 1.6M HCl at 35 °C. About 5 g of tri methyl benzene (TMB) was added as pore expander in polymer solution, and stirred for 1 h. The 20.25 g of triethoxy silicate, (Himedia) was added as the substratum for silica. After the mixture was stirred for 1 h, incubated at 35°C for 24 h, and aged at 100 °C for 24 h, the solids were collected by filtration and dried in air and aged at 100 °C for 24 h in hot air oven. The resulting powder was calcinated at 300°C for 4 h. Tem, SEM, TGA, BET and XRD analysis characterized the synthesized structure.
5.2.4 Optimization of process parameters

For developing an efficient silica coating over enzyme and nanoparticles, the concentration of nanoparticles, HHMS, linking agent (glutaraldehyde) was optimized before setting up an actual experiment, all the data were taken in triplicate and their mean was calculated for optimization, verification studies were also conducted to check the efficiency of statistically optimized results.

5.2.4.1 Physical adsorption of enzyme on HHMS pores and standardization for glutaraldehyde concentration for development of cross-linked enzyme aggregates

The preparation of cross-linked enzyme aggregates (CLEAs) in HMMS requires a two-step process. The first step involves the adsorption of enzymes into HMMS by mixing at 200RPM for 3h at 55 °C at 50mg/ml concentration, which proceeds with a high degree of enzyme loading within a short time of 1 hour (Lee et al, 2008). The second step involves glutaraldehyde (GA) treatment, which results in the crosslinking of enzyme molecules to create aggregates within the pores of HMMS. This approach is designed to yield stable enzyme activity by preventing leaching, since the enzyme aggregates created in the larger mesocellular pores (37 nm) are not expected to leach out through the smaller mesoporous channels (13nm). GA crosslinking is performed promptly after the enzyme adsorption, in order to enhance the high enzyme loading. Glutaraldehyde is a bi-functional agent, to standardize the glutaraldehyde concentration, various concentrations of 0.1%, 0.2%, 0.3% was considered for designing an optimization experiment. Standard assay of phytase (2.5U/ml) was performed and the enzyme activity was measured at each step in the presence of glutaraldehyde (0.1%, 0.2%, 0.3% respectively) and HHMS (10mg/ml) at 540 nm. It was found that at 0.1% concentration of glutaraldehyde, the enzyme showed optimum activity. Therefore all other experiments were conducted by using 0.1% glutaraldehyde concentration.

5.2.4.2 Cross-linking of enzyme by glutaraldehyde

In enzyme immobilization method, cross-linking reaction is very essential for coating of enzyme. Cross-linking reaction between enzyme molecules is an attractive strategy because it affords stable catalysts with high retention of activity. Glutaraldehyde is used as bifunctional cross-linking agent. Glutaraldehyde helps the enzyme to cross-linking with nanoparticles. For
cross linking 10 µg of interacting enzyme, in 50 mM Sodium Acetate buffer (pH 4.5) with 10 µl of 0.1% freshly prepared solution of glutaraldehyde in a total volume of 100 µl were treated for 5-10 minutes at 37 ºC.

5.2.5 Co-adsorption of enzyme and Nanoparticles in HMMS pores

Kim et al, (2005) had reported a method of co-adsorption of chymotrypsin and NP on HMMS, was used with modifications. Magnetite nanoparticles in hexane (0.8 mL; 10mg/ml) and Phytase in hexane and sodium acetate buffer, pH 4.5 (0.5 mL; 10mg/ml) were mixed well and aged for overnight, enzyme activity was measured by using the method reported by Gibson and Ullah (1988), and HMMS (10 mg) was added for the co-adsorption of Phytase and magnetite nanoparticles. The mixture was incubated at room temperature by shaking (250 rpm). After 30 min incubation, the sample was dried at room temperature until the hexane was completely evaporated. Glutaraldehyde solution 0.3% treatment, followed by Tris-capping (Tris: Tris(hydroxymethyl aminomethane) of unreacted aldehyde groups. After Tris- sample were further washed under shaking at 250 rpm, followed by application of a magnetic field. The supernatant of mixture sample after the centrifugation is colorless and transparent and contains no Fe this indicates the efficiency of adsorption process that magnetite nanoparticles do not leach out of HMMS under vigorous shaking (250 rpm). The supernatant was analyzed for phytase activity to check the leaching of phytase during the ageing and processing (data not shown), absence of phytase activity in supernatant indicate the efficient adsorption of enzyme in HHMS. The phytase activity (by adding 0.02 mg/ml phytate) and absorption at A_{280} was also measured from dried powder, dried powder was also analyzed by TEM to confirm the presence of nanoparticles inside the pore.

5.2.6 Analysis of enzyme activity of free and silica coated phytase

Enzyme activity assay of free phytase, HHMS adsorbed phytase, and NP+ phytase co-adsorbed on HHMS was performed, their Kcat and Km value was measured in duplicates and their catalytic specificity (Kcat/Km) were calculated.

5.2.7 Parameter optimization of free and coated phytase and evaluation of stability profile

Stability study of Phytase enzyme was studied by using various parameters. In this experiment we studied the stability of phytase at different substrate concentrations, high temperatures, and different pH, in shaking, against protein degrading enzyme, its reusability and after long time incubation at static condition. Phytase assay was performed with method reported by Heinonen and Lahti [1981].

5.2.7.1 Effect of substrate concentration on coated and uncoated phytase

Phytase assay is performed by using various concentration of phytic acid, Aspergillus derived
phytase was assayed by adding 44.1 mM Phytic Acid, pH 2.5 at 37°C (w/v)\[\text{Heinonen and Lahti, 1981}\]. While in our previous experiments (Chapter 4) we found our phytase was given maximum catalytic specificity between the substrate range 4.82 to 12.82 mM. To check and standardize the substrate concentration (phytase) for coated phytase, various substrate concentrations in the range 4.82 mM, 6.82 mM, 8.82 mM, 10.82 mM, 12.82 and 14.82 mM were taken. The standard assay of phytase was performed with all three concentrations separately.

5.2.7.2 Study against high temperature
The optimum temperature of our purified *Saccharomyces boularadii* purified phytase is 50°C. To study the stability of phytase at various temperatures, a range of different temperatures including the optimum temperature were used, 37°C, 55°C, 75°C and 100°C. Phytase activity was measured using standard assay procedure.

5.2.7.3 Study against different pH
Phytase gives maximum activity at acidic pH 4.5. In standard assay of phytase, the pH of buffer was 4.5. For study the stability, we studied the activity of phytase at various pH (pH 0.5, 2.5, 4.5, 6.5, 8.5, 10.5, and 12.5).

5.2.7.4 Stability at shaking condition
Stability of free and HMMS coated enzyme were analyzed at the shaking condition at around 1000-1500 (non-magnetic stirrer) RPM at regular interval of time like 1 hr, 2 hr, 3 hr, and after incubation phytase activity of samples were measured by the standard assay. The results were compared and analyzed.

5.2.7.5 Stability against protease
Effect of protease on free and coated phytase was studied, coated and free phytase were incubated with 10U/ml protease for one hour and the activity of phytase was measured.

5.2.7.6 Reusability
The enzyme was separated from the reaction mixture with the use of strong magnetic field. The recovered enzymes were again analyzed for its enzyme activity by using the same set of reagent and protocol. The same kind of experiments were performed for three times and with every washing step the enzyme activity assay was performed with recovered pellet and spent out liquid medium.

5.2.7.7 Stability at static condition after 2.5 years without freezing
The enzymes have very short shelf-life, one of the aim of our study was to increase shelf-life,
uncoated enzymes was inactivated within one hour after storage at room temperature, therefore we want to evaluate the efficiency of coating in increasing the shelf-life at room temperature. The stability was analyzed after each 365 days of storage at room temperature.
5.3 Results and Discussions

5.3.1 Characterization of synthesized NP

Synthesis of Fe₃O₄ particles were conducted by co-precipitation of Fe[II] and Fe[III] in the ratio of 1:2 in the presence of NH₄OH. The dark brown coloured nanoparticles were chemically synthesized and subjected for the XRD analysis. XRD analysis of nanoparticles revealed the presence of Fe₃O₄ nanoparticles at the 2θ value = 30.163. And the gamma phase of Fe₂O₃ at the 2θ value = 63.131. The purified nanoparticles were analyzed by XRD, which shows the highly crystalline structure of alpha Fe₃O₄. The result was analyzed by using the standard database provided with the instrument, overlapping the peak with standard, the characteristic peaks of Fe₃O₄ were observed [Fig. 5.3a]. Figure 5.3 a showing the pattern of Fe₃O₄ and Fe₂O₃ NP, the first peak at 2θ value = 30.163 is the characteristic diffraction angle of Fe₃O₄, the second characteristic peak was observed at the 2θ value = 63.131 is of Fe₂O₃. Figure 5.3 b, representing the TEM image of Fe Nanoparticles, average size of synthesized nanoparticles is 10nm, with rectangle shape. The synthesized NP appeared dark brown in color [Figure.5.4].

![Figure 5.3a XRD analysis of NP](image1)

![5.3b TEM analysis of magnetic nanoparticle](image2)
Figure 5.4 characteristic dark brown color of Fe₃O₄

Thermo gravimetric analysis of [Figure.5.5] of nanoparticles shows that, weight loss in three steps between the ranges of 22-600 °C. Loss at first step indicates removal of water, while second step indicate the loss of solvent preparations and at third step loss shows the conversion of Fe₃O₄ to Fe₂O₃. Thus such data supports the properties of magnetic nanoparticles. The thermogravitometric analysis of each individual component was performed to check the weight loss obtained by the individual components, in final preparation the presence of each components was confirmed by this individual level weight loss.
5.3.2 Characterization of HHMS

HHMS is depicted with highly ordered porous structure, in order to use it as host for enzyme and nanoparticles, it is important to measure the pore volume and pore size of synthesized HHMS. Synthesized dried powder was first analyzed by nitrogen isotherm [BET analysis] to measure the size of porous structure and volume. Scanning electron microscopic image and XRD analysis of HMMS [Figure 5.6a and 5.6 b] shows that the synthesis of highly ordered structure of silica, while representative TEM image shown that average ≈10-nm-sized small ordered pores are associated with larger ≈ 50-nm-sized mesocellular pores. The synthesized HHMS structure was also compared with previously reported structure [Kim et al, 2005], the result shown that slight deviation in pore size.

Nitrogen adsorption/desorption isotherms [BET] analysis confirms the results obtained by TEM images [Figure 5.7a,b,c]. The corresponding pore-size distribution obtained from the analysis of the data of relative pressure [P/P₀] Vs. volume [cc/g] shown in Figure 5.7. The nitrogen isotherm shows two major capillary condensation steps at relatively high pressures of
Figure 5.6a XRD analysis of HHMS

Figure 5.6b SEM analysis of HHMS

0.894 P/P₀ results from adsorption in the ≈10.14-nm-sized mesopores, while the other at 0.9–1.0 P/P₀ is from adsorption in the ≈40-nm sized cellular mesopores. The BET surface area and single point total pore volume of HMMS are 2.237e-01 cc/g. While TGA analysis was indicate that 3.02 mg i.e. 19% of weight loss in four steps, indicates the loss of CO₂ and H₂O from acetic acid and other components. Nearly at 55°C there is no loss of weight with temperature indicates the silicates formed. All the experiments were conducted with HHMS synthesized by tri-block polymer Pluronic p123.
Figure 5.7 TEM images of porous structure of HHMS, mesocellular pores are clearly visible in image a,b,c, images a,b,c were taken from three different scales.

In figure [5.7] the red arrow indicates the presence of mesocellular pores on synthesized structures.

The thermogram of HHMS, shows the first change between room temperature and 100°C, total 4.5% weight loss was observed at this first stage, that is ascribed to removal of physically adsorbed and structural water. At higher temperatures, TGA curves yield the decomposition pattern of HHMS. TGA curves of HHMS revealed degradation between 650°C and 750°C, reflecting breakdown of ZS groups on the silica surface, in agreement with earlier reports [Dong et al., 2012].
Figure 5.8 BET analysis of HHMS

Figure 5.9 TGA analysis of HHMS.
5.3.3 Characterization of phytase-NP-HHMS complex

HHMS was shown to be as good host material, the presence of nanoparticles in HHMS pore was confirmed by TEM analysis [Figure 5.10], as it is difficult to obtain TEM image of proteins, the presence of protein with complex was confirmed by phytase activity assay, and absorption at $A_{280}$. The step-by-step analysis of phytase activity shown that the vigorous processing and addition glutaraldehyde, was not decreasing phytase activity. Kim et al [2005] has developed this approach by using chymotrypsin as model enzyme, their studies shown that drastic decrease in enzyme activity in this ternary complex, with contrast to that result in our study we did not found any great loss in enzyme activity, that may be due to the removal of heating steps, or because of different nature of enzymes. The complex was further characterized by TGA analysis to confirm the presence of each component [NP, phytase, GA and HHMS], [Figure 5.11].
A sample was analyzed by thermo gravimetric analysis. The TGA of composite of HMMS, MNP and glutaraldehyde was carried out from 22°C – 600°C at 10°C/minute where the major
loss is corresponding to HMMS that is 3% next weight loss 0.7%, 1.25%, 0.4% which correspond to weight loss of phytase, glutaraldehyde and the remaining mass was of NP and unburned carbon. The results of TGA analysis confirm the interpretation of TEM image, i.e. the presence of enzyme and nanoparticle in dried powder of HHMS.

5.3.4.1 Optimization of glutaraldehyde concentration

Mixing phytase carried out optimization of glutaraldehyde concentration, no significant difference was observed with increasing glutaraldehyde concentration, very negligible difference was observed with increasing concentration of GA [Figure. 5.12], and therefore 0.1% glutaraldehyde concentration was used for further experiments. Moreover, only glutaraldehyde gives the absorbance when measured by phytase assay that indicates the P-impurities in glutaraldehyde preparation, therefore glutaraldehyde linked enzyme had slight higher activity compared to free phytase added, however 2.5 U/ml of phytase was mixed with glutaraldehyde and assayed, even some activity loss was observed in free phytase, that may be because of the presence of any inhibitors in reagent preparations. To remove the P-impurities of glutaraldehyde, glutaraldehyde was purified through ion exchange resins.

![Figure 5.12 Optimization of glutaraldehyde concentration](image-url)
5.3.4.2 Optimization of substrate concentration for uncoated (free) and coated (HHMS +NP+phytase)

With increasing substrate concentration [Figure 5.13], enzyme activity was increased. Result shows maximum phytase activity was observed at 10.82 mM concentration of sodium phytate, therefore 10.82mM phytic concentration was used for both coated and uncoated enzymes. Obtained data were processed and Vmax and Km and Kcat was calculated by using double reciprocal plot, the results are summarized in Table 5.1.

![Figure 5.13 optimization of substrate concentration](image)

Table 5.1 Comparisons of free and coated enzyme activity (U/ml)

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<td>0.885</td>
<td>1.005</td>
<td>0.885</td>
<td>0.880</td>
</tr>
</tbody>
</table>

5.3.4.3: Temperature stability profile

Thermal stability profile of coated [Figure 5.14], free and commercially available coated phytase, shown that our coating technique can withstanded better compared to others, moreover the enzyme remain active at 100°C for two hours, however activity decreases upto twofold. Megzyme and novozyme both are commercially available enzymes[2.5 U/ml ]were used for
this study. However, in this experimental conditions this enzyme has proved inefficient compared to our uncoated and coated enzymes.

![Enzyme activity profile](image)

**Figure 5.14** temperature stability profile of coated and uncoated phytase

### 5.3.4.4 Quantification of enzyme activity after magnetic separation

Magnetic nanoparticles are incorporated with enzyme for efficient separation of enzyme from reaction mixture to reuse it. The enzymes were separated from reaction mixture by applying the external magnetic field [Figure 5.15], supernatant was transferred and analyzed for phytase activity, in all the experiment no detectable phytase activity was found. The magnetically separated enzyme was washed with distilled water and transferred in to other reaction mixture to check its phytase activity [Figure. 5.16], these steps are repeated three times, gradual decrease in enzyme activity was observed, this problem still required a efficient solution, after 3rd wash two fold decrease in enzyme activity was observed. It is extremely difficult to figure out the single factor responsible for loss of activity. There are many factors, nanoparticles and its magnetic property, enzyme inactivation under the magnetic field etc.
Figure 5.15 separation of coated enzyme by applying external magnetic field

![Graph showing enzyme activity](Figure 5.16)

Figure 5.16 effect of magnetic separation on enzyme activity

5.3.4.5 pH stability profile of phytase

Phytase enzyme were prepared in sodium acetate buffer of respective pH, was incubated for 4hr. Uncoated enzyme was found pH labile and almost no activity was observed while coated enzyme was less pH stable compared to commercially available enzymes[Figure. 5.17]. The result indicate that there is enough room for improvement in the technology to increase the pH stability upto pH 2., by surveying the literature, further modifications in the technology are needful, such are addition of protein stabilizing agent, incorporation of unnatural amino acids etc.
5.3.4.6 Stability against proteases

The major application of phytase is as poultry probiotic enzyme, which digest phytate and increase the availability of micronutrients to monogastric animals. As stomach contains variety of proteases, the supplemented enzyme with feed must be active in the presence of stomach pH. Enormous efforts were made to increase the pH stability [Granier et al, 2006], but very few reports are found for protease stability of enzyme. This developed technology may partially solve this problem, silica coating may decrease the chances of direct contact of protease to phytase enzyme, as protease are large molecular weight proteins it can not easily enter inside the mesocellular pores like phytic acid. The result shows that coated enzyme is completely stable in the presence of protease while others are not. Commercially available enzymes are also failed in presence of protease enzyme, however activity of coated enzyme was decreased [Figure 5.18].
Figure 5.18 Stabilization profile of phytase in presence of protease
Conclusions

In conclusion, a novel approach of antifouling coating of phytase is developed. We have developed a unique approach for multifunctional nanocomposites of enzyme phytase and magnetic nanoparticles in polymeric silica cage; this approach employs a simple, two-step process that involves the co-adsorption of enzyme and magnetic nanoparticles into polymeric silica followed by glutaraldehyde treatments. Cross-linking enzymes within mesocellular pores of a uniquely designed mesoporous material successfully retained the resulting enzyme aggregates, retained in the pores, providing high loading capacity. Using this ship-in-a-bottle approach, greatly improved stability was achieved with model enzymes, phytase. Since this ship-in-a-bottle approach of cross-linked enzyme aggregates [CLEAs] in polymeric silica is very simple and effective for enzyme stabilization. Biological polymeric silica was synthesized using a single biological non-toxic polymeric agent under neutral conditions. Most of the porous materials reported so far are meso–macroporous or micro–macroporous materials. This biological polymeric silica is very easy to synthesize in laboratory and not very expensive. While mostly all the mesoporous polymers synthesis needs very specific chemicals and they are expensive. In addition, biological polymeric silica has two advantages over other mesoporous materials. First, the overall synthetic process is very cost-effective because inexpensive sodium silicate is employed as the silica source and the synthesis is conducted under mild, neutral conditions. Secondly, the synthetic procedure using a single biological polymer is much simpler than those employed for the synthesis of other mesoporous materials.

These nanocomposites, termed Magnetic-CLEAs, are proven to be magnetically separable, highly loaded with enzymes, stable under harsh shaking conditions like high temperature, high pH, shaking condition, and recyclable for repeated use with negligible loss of enzyme activity. It offers great potential to expand to any other enzyme and any other nanostructure in the matrix for the development of stable enzyme system in many enzyme-catalyzed reactions.