J Biochem Tech (2014) 5(4): 838-845
ISSN: 0974-2328

Immobilization of tropizyme-P on amino-functionalized magnetic nanoparticles for fruit juice clarification

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Received: 17 June 2014 / Received in revised form: 25 December 2014, Accepted: 25 December 2014,
Published online: 25 December 2014

Abstract

Nowadays nanoparticles are widely used as a key tool for enzyme immobilization. Tropizyme-P, a unique pectolytic enzyme was successfully immobilized on amino functionalized magnetic nanoparticles (AMNPs) using glutaraldehyde as a cross-linking agent at 15 mM concentration and 4h cross-linking time. The average size of the synthesized AMNPs was found below 80 nm by particle size analysis. The binding of tropizyme-P on nanoparticles was confirmed by FTIR spectroscopy. SEM analysis revealed that there was no significance difference in the size of nanoparticles after tropizyme-P immobilization. XRD results showed no phase change in nanoparticles after enzyme immobilization. Physical parameters viz. pH and temperature were optimized. The pH was found to be same but there was shift in optimum temperature of immobilized tropizyme-P by 5ºC with more thermal stability than free one. The kinetic studies revealed an increase in V_{max} of the immobilized enzyme. Reusability of immobilized tropizyme-P was found to retain up to 85% of initial activity after sixth cycles of reuse.

Keywords: tropizyme-P; magnetic nanoparticles; glutaraldehyde; immobilization; optimization

Introduction

Pectin substances are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1-4) linkage and widely found in the primary cell walls and middle lamella of higher plants (Kashyap *et al.* 2001). During mechanical crushing of pectin-rich fruits like apple, a fruit juice with high viscosity is produced which remains bound to the pulp in the form of a jellified mass and it is difficult to extract this juice by pressing or using other mechanical methods like filtration or centrifugation.

The raw press juice is rich in insoluble particles, which are mainly made up of pectic substances (Ceci and Lozano 1998). The use of pectinase is promising approach to achieve maximum fruit juice clarification as it degrades pectins directly through the cleavage of glycosidic linkages. During pectinase treatment of fruit pulp, monomeric sugars are released followed by pressing in continuous filters or rotary presses to extract the juice and the juice is separated from remaining solid debris by centrifugation (Grassin and Fauquembergue 1996; Kashyap et al. 2001). But using enzymes in soluble form the reuse of enzymes is restricted which ultimately increases the overall processing cost (Talekar et al. 2013a; Xue and Woodley 2012).

Hence the use of immobilized form of enzyme is an alternative way to overcome this major problem as it leads to ease in handling, relieve separation of enzymes from the reaction mixture at any time and reuse, possible increase in thermal and pH stability and a low product cost (Ansari and Husain 2012; Husain 2010; Lei et al. 2002; Wang 2006). But during immobilization of enzymes the support provided should be biocompatible providing an inert environment retaining native structure of proteins, with no loss of activity (Mitchell *et al.* 2002).

Therefore nowadays use of magnetic nanoparticles (MNPs) have been extensively studied for enzyme immobilization (Gupta and Gupta 2005; Muthulingam *et al.* 2013; Panek *et al.* 2012; Selim *et al.* 2007). The high surface area to volume ratio provided by the magnetic nanoparticles favors high binding capacity and catalytic specificity. Also the use of MNPs extensively provides an inert environment, low toxicity and surface modification properties (Ibrahim *et al.* 2013; Jiang *et al.* 2009). In addition, magnetic field susceptibility revealed a mechanism for efficient recovery of the enzyme complex thereby preventing the enzyme contamination of the final product. Enzyme stability is maximized with nanoscaled support with the additional advantages of possible modulation

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of the catalytic specificity, lower transfer resistance to solve the diffusion problem and lower operational cost (Bruno *et al*. 2005).

Tropizyme-P, a unique pectolytic enzyme is capable of effectively degrading tropical fruit pulp. It lowers the viscosity, increases juice recovery and enhances the degree of concentration of juice/pulp. No report about the immobilization of tropizyme-P on to magnetic nanoparticles exists. In the present study, tropizyme-P was successfully immobilized by covalent attachment on magnetic nanoparticles using glutaraldehyde as a cross-linking agent. The optimization of immobilization, pH and temperature has been subsequently studied. Moreover thermal stability, kinetic parameters and reusability of immobilized biocatalyst was compared with the free form of enzyme.

Materials and methods

Chemicals

Tropizyme-P was procured from Enzymes India Pvt. Ltd (Chennai, India). Sodium hydroxide pellets (NaOH) was purchased from Merck Specialties (Mumbai, India). APTES (3-aminopropyltriethoxysilane), and D-(+) galacturonic acid were purchased from Sigma Aldrich (Bangalore, India). Ferric chloride hexa hydrate ($FeCl_3.6H_2O$), ferrous chloride tetra hydrate ($FeCl_2.4H_2O$) and DNS (3,5-dinitrosalicylic acid) were the product of HiMedia Lab. Pvt. Ltd (Mumbai, India).Glutaraldehyde 25% (w/v), pectin was obtained from Sd Fine Chem Ltd. (SDFCL Mumbai, India). All the other chemicals used were of analytical grade with highest purity.

Synthesis of magnetic nanoparticles

Magnetic nano particles MNPs were synthesized by chemical co-precipitation method (Karaoglu E. 2013). $FeCl_2 \cdot 4H_2O$ (0.5 M) and $FeCl_3 \cdot 6H_2O$ (1.0 M) (molar ratio 1:2) were dissolved in deionized water at a final concentration of 0.3 M. HCL (1.0 M, 1.825 mL) was added to increase the ionic strength and known amount of sodium hydroxide pallets were added until a black precipitate was formed. The resultant mixture was continuously stirred to increase the pH of solution at constant value (pH 12). The precipitating mixture was then heated at 90°C for 30 min with continuous stirring and cooled to room temperature. The precipitating mixture was then washed with deionized water and with aqueous ethanol several times until pH 7. Finally, the resultant precipitates were dried on hot plate at 80°C for 1h. MNPs were scrubbed and stored at room temperature.

Preparation of amino functionalized magnetic nanoparticles (AMNPs)

MNPs (0.62 g) were dispersed in 22.5 mL of ethanol and water solution. This solution was then sonicated for 30 min for getting homogeneous suspension. APTES (2.5 mL) was added to the suspension drop wise. The solution was refluxed for 3 h at 50±1°C and with vigorous mechanical stirring (1000 rpm). The APTES modified particles were centrifuged at 8000 rpm for 10 min followed by washing with double-distilled water (3 times) and ethanol once. The step of APTES coating was repeated for 2-3 times to ensure the coating. The particles were then dried in oven at 50±1°C for 3h and stored.

Immobilization of tropizyme-P on amino functionalized magnetic nanoparticles

Tropizyme-P was immobilized on nanoparticles by using glutaraldehyde (GA) as cross-linker as per the method previously described by Panek *et al*. 2012. AMNPs (1g) was taken in phosphate buffer (0.1 M, pH 5.0) and sonicated for 10 min. Then 1 mL of 25% glutaraldehyde was added in the solution and kept for stirring at 30±1°C for 12h the particles were then separated and washed with deionized water 3 times. Enzyme solution (10 mL, 25 mg/mL) was added and the resulting solution was stirred for 20 h at 30°C. The enzyme immobilized nanoparticles were then removed from the solution and washed with deionized water several times. During each wash the amount of protein content in the supernatant was estimated by using Bradford method (Bradford, 1976). The immobilized amount of protein was calculated by subtracting the protein recovered in the supernatant from the protein subjected to immobilization. In order to achieve good immobilization efficiency and catalytic activity the concentration of GA (5,10,15,20,25,30,35 and 40 mM), cross-linking time (1,2,3,4,5,6,7 and 8h) at constant tropizyme-P (10 mg) were optimized. Also the amount of tropizyme-P loading (50-500 µg) on MNPs required to achieve highest activity recovery were optimized at optimized glutaraldehyde concentration and cross-linking time (Muthulingam et al. 2013).

Physicochemical characterization of MNPs and immobilized MNPs

The mean size distribution of MNPs was obtained using particle size analyzer (DLS), Malvern, ZS 90, (UK) Instrument. The surface modification and tropizyme-P binding were confirmed by Fourier transform infrared (FT-IR) spectroscopy (Perkin Elmer spectrum one) using Nujal mull method in the range of 400–4000 cm^{-1}. The surface morphology of the magnetic nanoparticles and immobilized tropizyme-P MNPs were studied by SEM using FESEM, S4800 Type-II (Hitachi High Technologies Corporation Tokyo, Japan). The absorbances were measured on Agilent UV-double beam spectrophotometer. The morphological and phase changes of the nanoparticles were determined by X-ray diffraction (XRD) measurement using XRD D8 Advance (Bruker Axs GnbH, Berlin, Germany) in the receiving slit operation mode with a single Cu Kα radiation (λ= 0.154 nm) and the XRD patterns were recorded at high angles (10–80°).

Enzyme activities

Tropizyme-P activity was measured using pectin as a substrate (Dekker 1977; Bailey and Pessa 1990). Tropizyme-P (1 ml) was added to 1.0 mL of pectin solution (0.1 M acetate buffer, 1.0 mg/mL of pectin). Pectin containing test tubes were then incubated at optimum conditions for 1h. The activity of immobilized tropizyme-P was calculated in same way except it was removed magnetically before the addition of DNSA reagent. One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1µmol of reducing sugar (estimated as galacturonic acid) per minute at 37°C. The amount of reducing sugar formed will be estimated by the dinitrosalicyclic acid method (Miller 1959).

Optimization of pH and Temperature

The pH and temperature of free and immobilized tropizyme-P was optimized at different ranges. The range for pH optimization was kept from 3 to 8 and that of temperature was 30 to 80°C. The best optimum pH and temperature of free and immobilized tropizyme-P was then determined by estimating the relative activity of free and immobilized tropizyme-P.

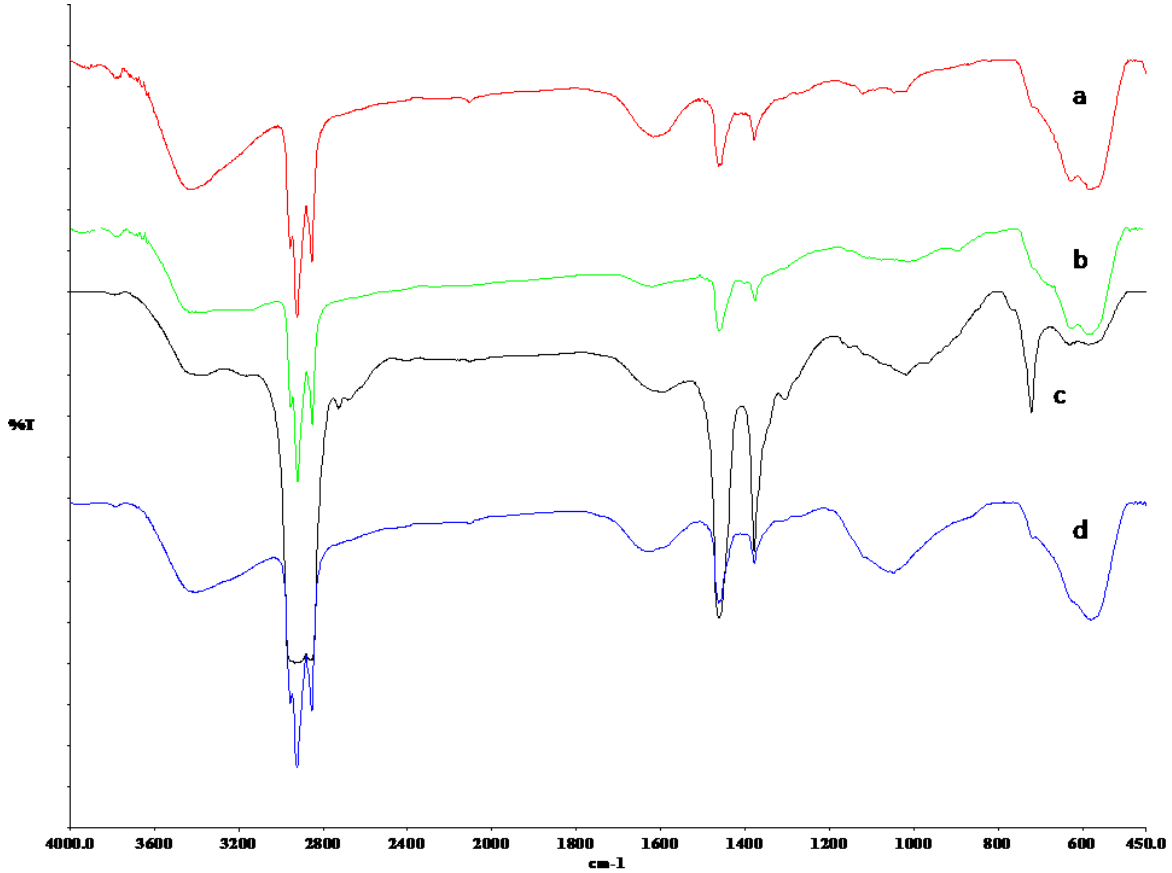

Figure 1: FT-IR spectra of (a) MNPs (b) APTES coated MNPs (c) Glutaraldehyde activated MNPs (d) tropizyme-P immobilized MNPs

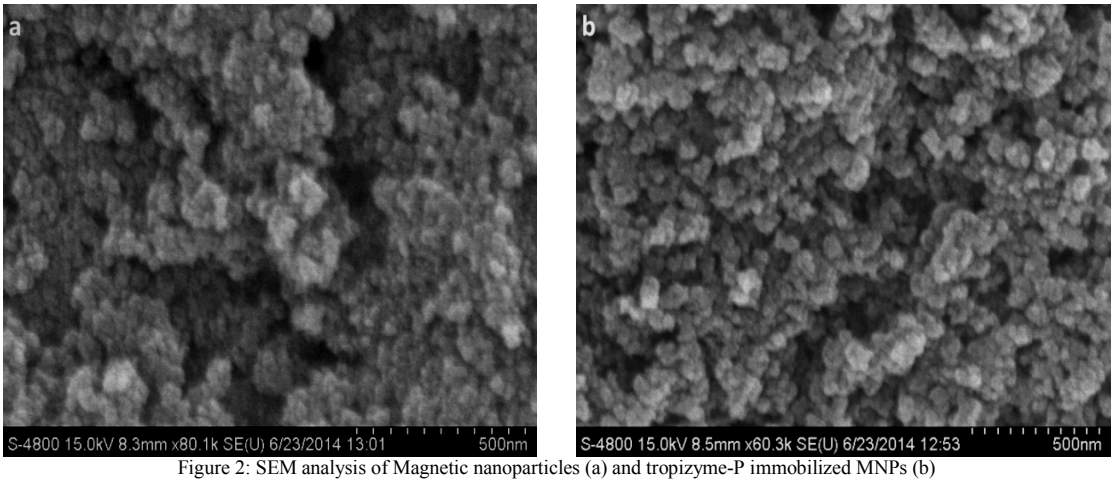

Figure 2: SEM analysis of Magnetic nanoparticles (a) and tropizyme-P immobilized MNPs (b)

Determination of kinetic parameter

Kinetic parameters of free and immobilized tropizyme-P were determined using different concentrations of pectin solution in the range of 0.1–5.0 mg/mL in 0.1 M acetate buffer at optimum conditions respectively. Tropizyme-P was used both in free and immobilized form. K_m and V_{max} values of free and immobilized tropizyme-P were calculated from non linear regression fitting of the initial reaction rates corresponding to different pectin concentrations by Graph Pad Prism software (Talekar *et al.* 2013a).

Thermal stability studies

The thermal stability of immobilized tropizyme-P was studied by incubating immobilized tropizyme-P in 0.1 M acetate buffer (pH 5.0) without substrate at 55, 65 and 75°C respectively and collecting the samples at 30 min intervals for a total of 180 min and determining the activity in immobilized tropizyme-P as given in standard assay (Talekar *et al.* 2012). The residual activity of tropizyme-P at each temperature was determined by taking the activity at 0 min as 100%.

Reusability of immobilized biocatalyst

The reusability of tropizyme-P in immobilized biocatalyst was evaluated, subjecting to depectinization reaction with 1% (w/v) pectin under optimal conditions. The reaction was carried out for specified time. The immobilized biocatalyst was then separated magnetically, washed with buffer two times and then suspended again in a fresh substrate to measure enzyme activity. The residual activity of tropizyme-P was calculated by taking the enzyme activity of the first cycle as 100% (Ibrahim *et al.* 2013).

Results and discussion

FT-IR analysis

The appearance of absorption peak at 586 cm^{-1} confirms the presence of Fe-O bond in MNPs (Fig.1a). The characteristic peak at 1016 cm^{-1} shows the presence of Fe-O-Si covalent bond (Fig.1b). Presence of glutaraldehyde appears at peak value of 1610 cm^{-1} (Fig.1c). After enzyme immobilization the formation of amide bond appears at peak value of ~1630 cm^{-1} (Karaoglu E. 2013) which confirms the successful binding of tropizyme-P on MNPs (Fig.1d).

SEM analysis

The SEM images of the MNPs (a) and tropizyme-P immobilized nanoparticles (b) are shown in Figure 2a and 2b. Results demonstrated that magnetic nanoparticles and tropizyme-P immobilized MNPs have particle sizes below 80 nm. Though the size distribution for all samples was found to be wide in particle size analysis as shown in Figure S1 (Supplementary Document). Moreover, the size of the particles was not significantly increased after tropizyme-P immobilization and was found to be spherical shaped Fig.2 (b).

XRD analysis

XRD pattern of MNPs and tropizyme-P immobilized MNPs is shown in Figure S2 (Supplementary document). Five characteristic peaks occurred at 2θ of 30.24, 35.63, 43.28, 57.27, 62.92 and their indices 220, 311, 400, 511, 440 for MNPs were almost similar to tropizyme-P immobilized MNPs which imply that there was no phase change in MNPs after enzyme immobilization (Muthulingam *et al.* 2013). The sharp diffraction peaks in both XRD patterns clearly indicates that spinel magnetite product is well defined crystallites, without impurity diffraction peaks, which showed synthesized magnetite nanoparticles in pure phase (Ibrahim *et al.* 2013).

Effect of glutaraldehyde concentration and cross-linking time

To enhance and optimize tropizyme-P immobilization by covalent attachment on magnetic nanoparticles, the effect of glutaraldehyde concentration and cross-linking time was investigated, and the results are shown in Figure 3a and 3b. From results it was found that the activity recovery of tropizyme-P increased with increasing glutaraldehyde concentration, reaching maximum value (97.26 %) at 15 mM glutaraldehyde and further increase in glutaraldehyde concentration had no significant effect on activity recovery. As depicted from figure 3a the maximum activity recovery was observed at 15mM glutaraldehyde concentration.

Chui and Wan, 1997 indicated that enzymatic activity was proportional to the concentration of glutaraldehyde. However, at higher glutaraldehyde concentration exceeding 30 mM the enzyme activity recovery decreased considerably by 52.23% of the maximal value. Extended concentration of this coupling agent is not suitable as it may cause conformation changes in active site leading to enzyme denaturation (Gallego *et al*. 2005). Similar results were obtained by Chang and Juang, (2007) and Pan et al. (2009). Moreover, Talekar *et al.* (2013a) discussed that decrease in activity recovery at higher glutaraldehyde concentration might be due to rigidification of the enzyme molecules occurring due to excessive cross-linking at higher glutaraldehyde concentrations which results in a loss of the enzyme's flexibility that is essential for its activity. Rigidification creates steric hindrance which prevents the pectin from reaching the active site of enzyme.

Cross-linking time is another important factor which is the time required to recover maximum activity of enzymes in immobilized form during cross-linking. By increasing cross-linking time activity recovery of enzymes was increased. Maximum percent recovery (96.13%) of tropizyme-P used for immobilization was obtained at 4 h cross-linking time as shown in Figure 3b. Cross-linking time less than 4 h showed reduced relative activity which could be because of incomplete cross-linking at lower linking time period. Cross-linking reactions with cross-linking time greater than 4h resulted in decrease in activity recovery of tropizyme-P in immobilized MNPs. This result implied that extended cross-linking time restricts enzyme flexibility abolishing enzyme activity due to more intensive cross-linking (Talekar *et al.* 2013b). Also cross-linking time below 4h showed lower activity recovery, which may be due to insufficient cross-linking time for glutaraldehyde with magnetic nanoparticles and enzyme molecules. The resultant tropizyme-P cross-linked to magnetic nanoparticles can be easily separated from reaction mixture using external magnet (Fig. S3 Supplementary document).

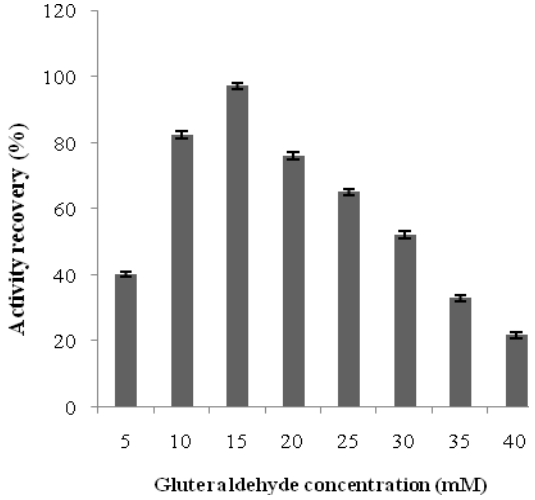

Figure 3a: Effect of glutaraldehyde concentration on activity recovery of tropizyme-P immobilized MNPs. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Effect of tropizyme-P addition on % immobilization

The maximum percentage of immobilized tropizyme-P was found to be around 85% and decreased gradually with more tropizyme-P addition on MNPs. The weight ratio and percentage relative activity increased with increase in initial amount of tropizyme-P for immobilization onto MNPs as shown in Table 1. Similar results were observed by Muthulingam *et al*. (2013). The maximum percentage relative

activity was observed at 200 µg. Further increase in the amount of tropizyme-P showed less relative activity. Ibrahim *et al.* (2013) stated that the resultant decrease in relative activity may be due to saturation of the MNPs surface with excess of enzymes which in turn causes a steric hindrance among the enzyme molecules thereby blocking the active sites over MNPs, thus making the non availability of the binding sites for the enzymes towards substrate.

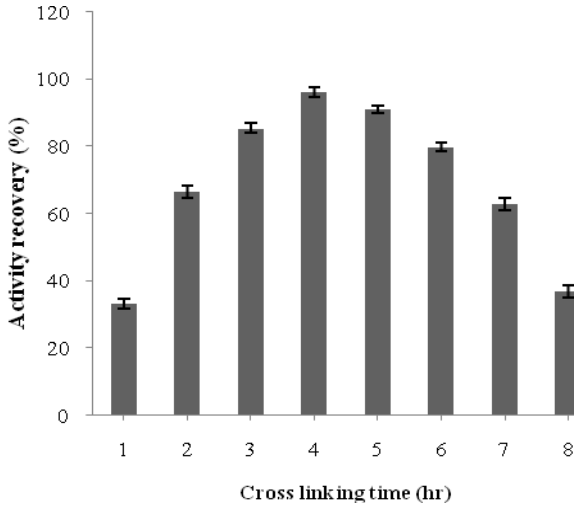

Figure 3b: Effect of cross-linking time on activity recovery of tropizyme-P immobilized MNPs. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Table 1. Effect of initial amount of tropizyme-P on percentage immobilization, weight ratio and percentage relative activity: MNPs=10.0 mg, pH 4.5 and temperature=50°C.

Tropizyme-P added (µg)	Tropizyme-P immobilized (%)	Weight ratio (mg bound tropizyme-P/mg MNPs)×10^{-3}	Relative activity (%)
50	85.6	8.5	47
150	65.2	14.7	85
200	47.9	15.8	100
250	32.5	16.7	88
300	24.8	17.5	79
400	20.1	17.7	72
500	18.7	17.3	69

Effect of pH and temperature

The pH stability for both the free and immobilized tropizyme-P was studied in different range of pH (3.0–8.0) at 50±1°C as shown in the Figure 4a. Optimum pH is the pH at which the free and immobilized enzyme gives maximum activity. Figure 4a shows that optimum pH for both free and immobilized tropizyme-P was same at pH 5.0. Results also showed that immobilized tropizyme-P shows better percentage relative activity than that of the free enzyme. The reason behind this may be that in acidic region the magnetic nanoparticles provide greater affinity for the tropizyme-P to act against the substrate provided. Also the immobilization of enzyme on magnetic nanoparticles provides a large surface area for the enzyme so as to give better activity than that of the free enzyme (Muthulingam *et al.* 2013).

The optimum temperature is the temperature at which enzyme molecules possess greater kinetic energy, thereby increasing the possibilities of impact between the enzyme and the substrate. The thermal stability of the free and immobilized enzyme was studied in different range (30–80°C) at pH 5.0 as shown in Figure 4b. Results showed that there was shift in optimum temperature of immobilized tropizyme-P (55±1°C) than that of the free enzyme (50±1°C). Also there was increase in percentage relative activity of immobilized tropizyme-P over free form at high temperatures. The reason behind this may be due to an existence of a strong covalent bond between the nanoparticles and the enzyme molecules (Kim *et al.* 2006 and Muthulingam *et al.* 2013) due to which, the rigidity was increased and the particle protects the tropizyme-P from the unconditional disturbances. Another potential reason for this was given by Ibrahim *et al.* (2013) and Song *et al.* (2012), that there may be a "vicinal effect" enhanced by the immobilized enzyme which might favor the arrangement of the enzyme through H-bonds that may contribute to the improved thermal stability of immobilized tropizyme-P. However, with the temperature exceeding the optimum limit (>50°C for free tropizyme-P and >55°C for immobilized tropizyme-P), the tropizyme-P starts to degrade due to the breaking of the bonds thereby resulting in the loss of active sites.

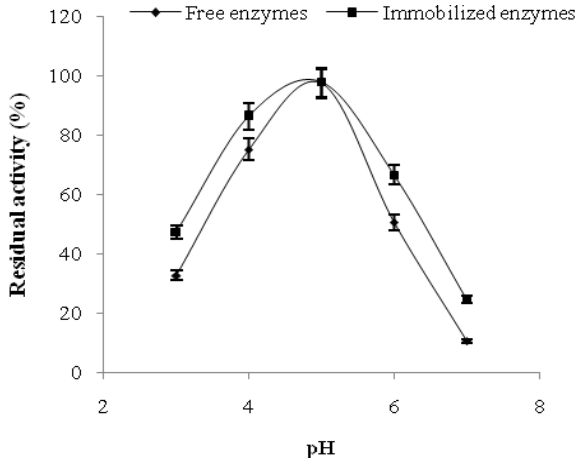

Figure 4a: Effect of pH on relative activity of free enzymes and tropizyme-P immobilized MNPs. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

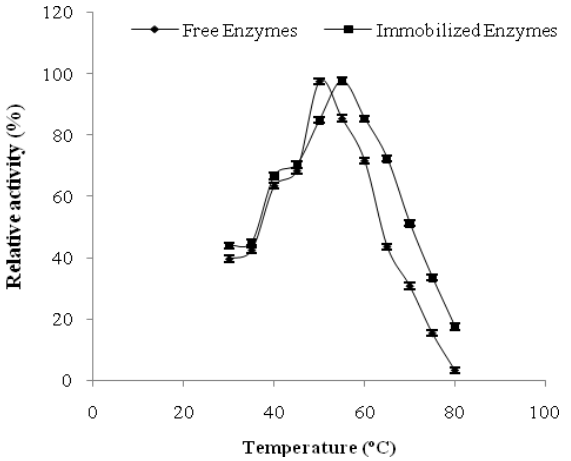

Figure 4b: Effect of temperature on relative activity of free enzymes and tropizyme-P immobilized MNPs.The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Kinetic analysis

Kinetic parameters of free and immobilized tropizyme-P were determined as shown in table 2. The rate of conversion of product was calculated by varying the concentration of pectin. Lineweaver-Burk plot was fitted by using graph pad prism software to estimate V_{max} and K_m. It was found that V_{max} for immobilized enzyme was increased than that of the free enzyme, which indicates increase in the rate of substrate conversion and inline results were reported by Muthulingam *et al.* 2013 while there was no significance difference in K_m value of both free and immobilized form of enzyme (Figure S4 and S5 supplementary document).

Table 2. Kinetic parameters for free and immobilized tropizyme-P: pectin-0.1 to 5.0 mg/mL of 0.1M acetate buffer (Optimum conditions).

Tropizyme-P	V_{max} (µmol/min)	K_m (mg/ml)
Free	4.5454	0.561818
Immobilized	5.7372	0.550775

Thermal stability

Thermal stabilities of the free and immobilized tropizyme-P were studied by measuring the residual activities of the tropizyme-P after incubation in acetate buffer (0.1 M, pH 5.0) at 55, 65 and 75°C respectively and collecting the samples at 30 min interval for a total of 180 min with continuous shaking. Figure 5 (a, b and c) shows the comparison of thermal stability for both free and immobilized tropizyme-P in acetate buffer (0.1 M, pH 5.0). The residual activity of immobilized tropizyme-P decreased less and more slowly than the free one. At 55°C, immobilized MNPs retained 97.66 % activity for at least 180 min of storage time. Also immobilized MNPs retained more than 80% and 65% of their initial activity for at least

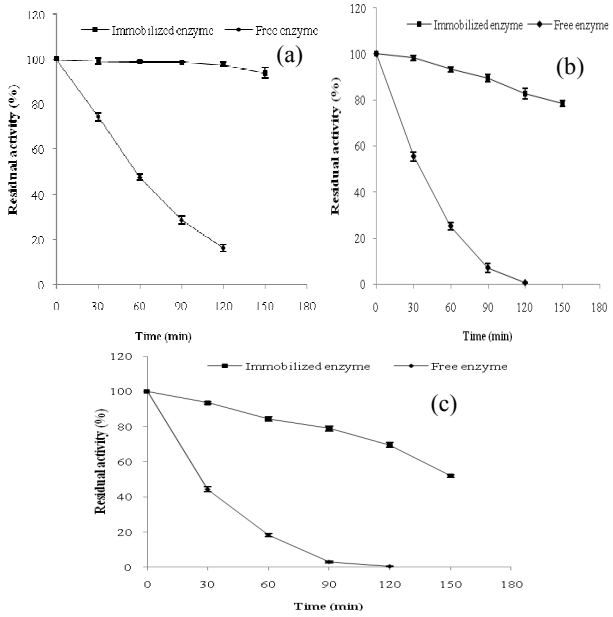

Figure 5: Thermal stability of free and immobilized tropizyme-P at (a) 55°C, (b) 65°C and (c) 75°C. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

180 min of incubation time at 65°C and 75°C respectively, whereas residual activity of free tropizyme-P was reduced to 16% and almost lost its activity at 55°C, 65°C and 75°C for 120 min incubation time. These results indicated that the thermal stability of immobilized tropizyme-P on MNPs was much better than that of free enzyme. This improvement in the thermal stability of immobilized tropizyme-P might be due to the covalent cross-linking between enzyme molecules and amino functionalized magnetite nanoparticles. The formation of covalent bonds gives effective conformational stabilization of tropizyme-P in MNPs hence requiring more energy to break down this active conformation than free enzyme (Dong *et al.* 2010, Park *et al.* 2010, Talekar *et al.* 2012 and Wang *et al.* 2010).

Reusability study

From economic point of view the reusability of immobilized tropizyme-P was studied. Figure 6 shows the residual activity of immobilized tropizyme-P upto 10 cycles. It was found that the residual activity of tropizyme-P was good upto sixth cycle (above 85%) and decreased gradually upto tenth cycle (about 50% of the initial activity). The decrease in the enzyme activity may be caused due to several reasons like protein denaturation and leaching of enzyme molecules due to repeated use which may be because of weakening of bond between enzyme molecules and magnetic nanoparticles.

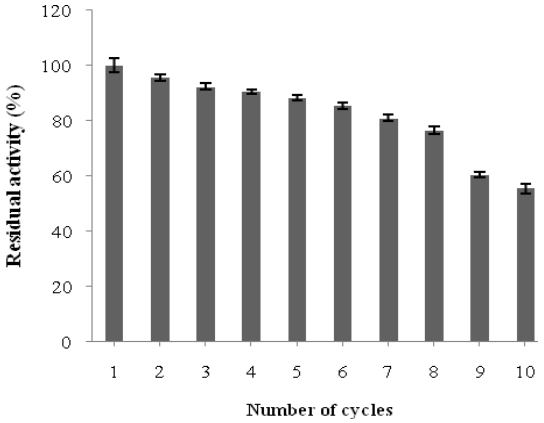

Figure 6: Reusability of immobilized tropizyme-P on MNPs. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Conclusion

From results it was demonstrated that successful binding of tropizyme-P on magnetic nanoparticles can be carried using glutaraldehyde as a cross-linking agent. SEM analysis showed that the size of MNPs did not alter significantly after tropizyme-P immobilization. XRD analyses showed there was no phase difference in MNPs after enzyme immobilization and found to be crystalline in structure. Optimization study showed that immobilized tropizyme-P was more stable for pH and temperature when compared with the free form. Kinetic analysis confirmed that there was increase in the rate of conversion of substrate after enzyme immobilization. Study of reusability showed that residual activity of immobilized enzymes was retained 85% upto sixth repeated cycle thereafter activity was declined upto 10th cycle retaining 50% of initial activity

Acknowledgement

The authors gratefully thank to SSBT's College of Engineering and Technology, Jalgaon, Maharashtra, India for giving permission to use all facilities to undertake this research work.

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Appendix 1

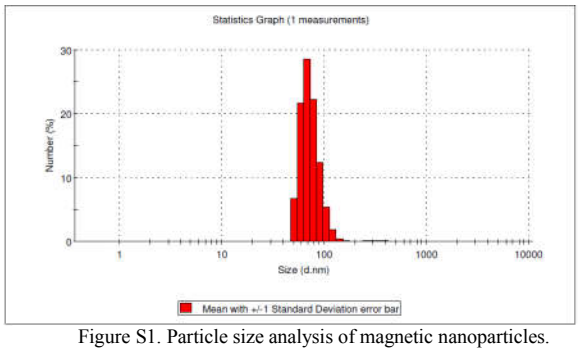

Figure S1. Particle size analysis of magnetic nanoparticles.

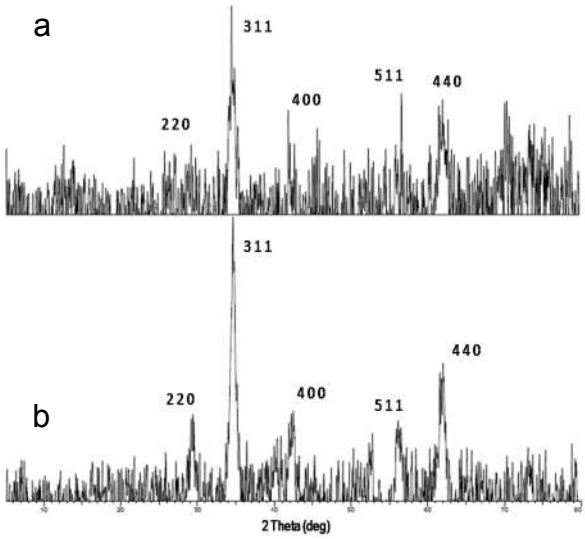

Figure S2. XRD Pattern of magnetic nanoparticles (a) and tropizyme-P immobilized MNPs (b)

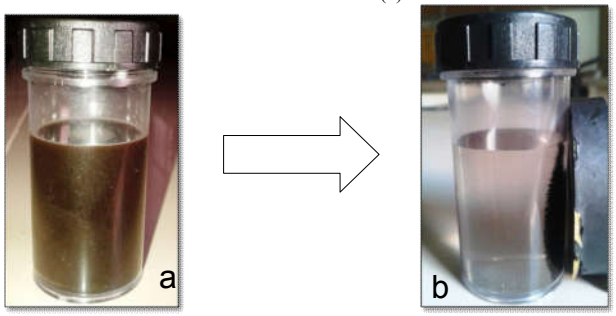

Figure S3. Dispersability and magnetic affinity of immobilized tropizyme-P in aqueous solution (a) and to external magnetic field (b).

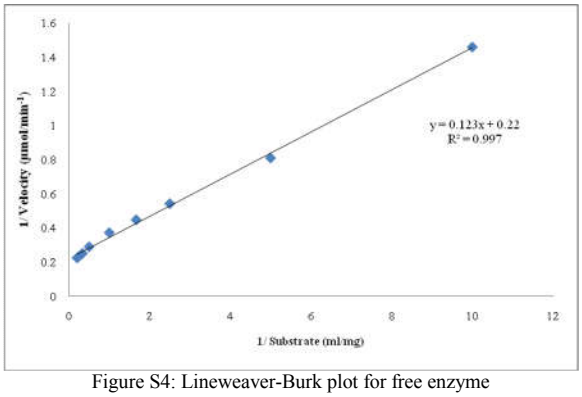

Figure S4: Lineweaver-Burk plot for free enzyme

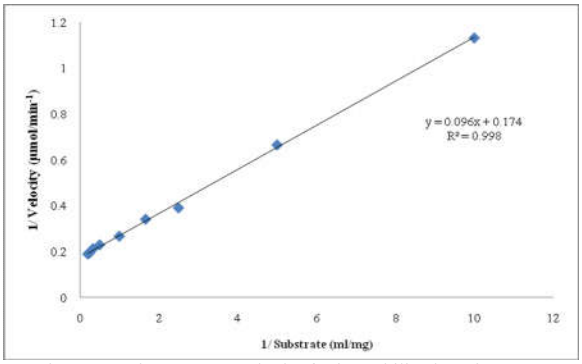

Figure S5: Lineweaver-Burk plot for immobilized tropizyme-p.