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Immobilization of Alkaline Protease enzyme from *Pseudomonas aeruginosa*on Surface functionalized Magnetic Iron Oxide Nanoparticles.

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ABSTRACT

Protease is an enzyme which has a wide range of applications in various fields. Extracellular protease was produced from *Pseudomonas aeruginosa* and its enzymatic activity was determined before and after each purification step. Protease enzyme immobilized on Magnetic iron oxide nanoparticles prepared by co-precipitation method. Presence of Nanoparticles, its size and morphology was characterized by UV-Visbile spectra, XRD, and DLS methods. The protease enzyme was bound to magnetic nanoparticles via surface transformation technique including amine functioned Nano composite formation. Successful binding of protease onto the particles was confirmed by FTIR analysis. The stability of the immobilized enzyme increased in comparison with the free enzyme. This study showed that the stability of the protease was enhanced by immobilization to the magnetic nanoparticles, able to perform formidable role under broader temperature and pH ranges.

Keywords: Protease, Super paramagnetic Nanoparticle, Nano composite, Immobilization, Activity and Stability

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INTRODUCTION

In a current scenario, Enzymes are widely used as an alternative chemical catalyst for various industrial application and have a huge impact in developing an ecofriendly technology.Enzymes have certain properties that makes them more valuable for use them in the industrial application such as high specificity, requires mild reaction condition, low toxicity and sensitivity[1], [2]. Protease is one of the most important enzymes that account for 60% of total enzyme sales worldwide [3]. In addition to peptide synthesis in an non aqueous media, protease were able to hydrolyze the peptide bond in aqueous media [4].Approximately 40% of total global enzyme sales and production is of alkaline protease, as they were tend to find too much application in industrial sectors such as detergent, pharmaceutical, leather, diary, silk etc[5][6]. They tend to find many application is because of, they alkaline protease enzyme can quite able to handle extreme harsh processing condition, and their high stability at particular pH and stability enhances their role in cleaving the peptide bond [3], [7], [8]. Their activity can be increased to a formidable level with the help of certain support material for immobilization and most importantly increasing the reusability of the enzyme[9], [10]. The most effective way of immobilizing the biocatalyst is the use of the nanostructured material with the help of crosslinkers to immobilize the enzyme, because they have certain advantage is that, they can provide high surface area with large lading capacity of enzyme on nanostructured materials with minimum mass transfer resistance [11].Various functional nanomaterials such as semiconductors, metal and magnetic materials have high values in both scientific and technological aspect[12]–[14]. While there are so many ways for the preparation of nanoparticles, best method will depend upon the solubility of the particle under a range of different condition [15].Magnetic nanoparticles have superparamagnetic properties, and because of this magnetic nanoparticles finds immense application in the medicinal field especially in the diagnosis of cancer [16], [17]. The use of MNPs extremely provide an inert environment, low toxicity, and surface modification properties. Magnetic field susceptibility, have revealed a perfect mechanism for the removal of the magnetic nps that been immobilized with the enzyme and it increases reusability value with enhanced catalytic value, low diffusional problem and low operational cost[18]. Magnetic nanoparticles tend to agglomerate due to dipole-dipole interactions and can reduce the biocompatibility value, inorder to overcome these kind of drawbacks functionalization is been done and then crosslinker is added for efficient immobilization of enzyme[19]. In the present study protease was produced and isolated from the pseudomonas aeruginosa, were partially purified with the help of ammonium sulphate precipitation and dialysis process.The partially purified enzyme is immobilized onto iron oxide nanoparticles were amine functionalized inorder to prevent the agglomeration during the separation process and glutaraldehyde is used as a cross linker to immobilize the enzyme and further applications is carried out.

MATERIALS AND METHODS

Materials:

Pseudomonas aeruginosa was available in our microbiology laboratory. Ammonium sulphate (Chemical grade), Dialysis bag, Ferric chloride ($Fecl_3$), Ferrous sulphate ($FeSO_4$), Ammonium hydroxide (NH_4OH) and Glutaraldehyde 25% (w/v) was obtained from our biochemistry lab.3-Amino propyltriethoxysilane (APTES, 98% purity) was purchased from TCL chemicals, chennai.

Enzyme Production:

Microorganism and growth condition

Pseudomonas aeruginosa, a bacterial strain was used for the production and isolation of protease enzyme. The bacterial strain was cultured in nutrient media for 48 hrs at 37°C in an orbital shaker as per the procedure of Masi et al [20].

Partial Purification of Protease

Cell free culture filtrate was prepared by centrifuging the culture at 10,000 rpm for 48 hrs and the supernatant was collected, as it were enriched with extracellular protease. Partial purification of protease was done by ammonium sulphate precipitation with different saturation levels (20% - 80%). The precipitated protease was dissolved in 0.1M glycine-NaOH buffer at alkaline pH of dialysis bag to remove the salt from the

precipitated protein. The enzyme activity and protein concentration was estimated using the procedure of Beg et al [21], [7].

Estimation of protease activity and protein concentration

Protease activity was estimated with the slight modification of universal protease assay [22] .The diluted enzyme solution of nearly 100µl is added to 1ml of 1% casein in 0.1M glycine-NaOH buffer of pH 9 was incubated at 45ºC for 15 mins and the reaction was terminated with the addition of 5ml of trichloroacetic acid (5%) . The mixture was filtered after 30 mins and 2ml of filtrate was then added to the composition containing 5ml of sodium carbonate (0.4M) and 1 ml of Folin-Ciocalteau reagent. Absorbance was measured at 660nm. Concentration of L-tyrosine released during the Course of reaction was determined by comparing the absorbance value with the standard curve of L-tyrosine constructed in a similar manner. It was stated that one unit of protease activity is defined as the amount of enzyme required to release 1 µg of tyrosine per ml per minute under the above assay condition. Total protein content of the partially purified filtrate were measured according to the [23], by using bovine serum albumin as a standard substrate [7].The isolated and purified protease was used to determine its molecular weight using SDS-PAGE.

Synthesis of iron oxide nanoparticles

Iron oxide particles were prepared and modified with the amine functionalized group by the method of reza et al [24], Teng-gen Hu [25]. Ferric chloride and Ferrous sulphate was taken in the molar ratio of 2:1 in 25 ml of deionized water, were heated upto 50°C for 10 mins. The solution was titrated with the ammonium hydroxide of 25 ml, added in a drop wise manner with the help of burette and were mixed in an magnetic stirrer in a continuous mixed flow pattern. The magnetite i.e Fe_3O_4 was separated with the help of magnets, was washed several times with water to obtain a alkaline pH and dried in an hot air over at 100°C for overnight [26].

Functionalization of iron oxide nanoparticles

Immobilization of alkaline protease

Alkaline protease enzyme was immobilized onto amine functionalized nanoparticles as per the procedure of panek et al [7], Teng-gen Hu et al [9]. 50 mg of amine functionalized nanoparticles were mixed with 5 ml of 4% glutaraldehyde solution for 3 hrs under vigorous shaking. 5 ml of enzyme solution was added to the above solution and vigorously stirred for another 3 hrs. The immobilized enzyme on iron oxide nanoparticles were removed by magnetic decantation process and washed with phosphate buffer to attain the alkaline pH.

Characterization of MNPs and Immobilized MNPs

The synthesized iron oxide nps was been characterized with the help of UV-Visible spectra (300-900 nm). The surface modification and enzyme linked to the crosslinker were detected with the help of Fourier Transform Infrared Spectroscopy (400-4000 cm^{-1}). The surface morphology of the magneticnps was detected using SEM and the mean size distribution of MNPs was obtained using DLS. The morphology and phase change of the iron oxide nps is detected using XRD and pattern were determined at high angles (5-80°).

RESULT AND DISCUSSION

Enzyme production and its molecular weight determination

Protease enzyme is isolated from the 48 hrs culture of Pseudomonas aeruginosa. The isolated protease enzyme activity and its concentration is determined before and after purification using universal protease assay[22] and lowry's method[28]. The specific enzyme activity, yield and recovery of protease enzyme was determined for each step of the purification process in **Table 1.**The molecular weight of the alkaline protease is measured using SDS-PAGE and found to be 50 kDa as in **Figure 1**, its been in close association with the result of protease from pseudomonas aeruginosa of 55 kDa[20].

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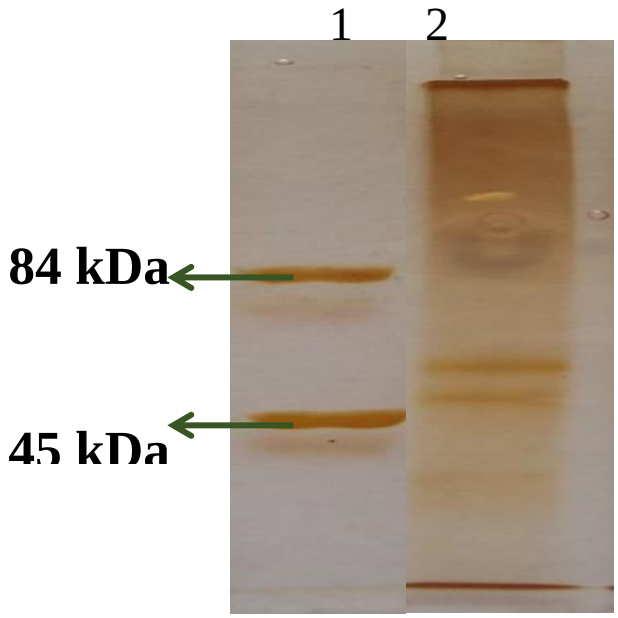
Table 1: Determination of Yield and Purification fold

Process	Protein Concentration (mg)	Enzyme activity (U/ml)	Yield (%)	Specific enzyme activity (U/mlmg)	Purification fold
Culture filtrate	1.47	1.90	100	1.29	1
Ammonium sulphate precipitation	1.2	1.65	86.8	1.37	1.06
Dialysis	1.05	1.5	78	1.42	1.10

50 kDa



Lane 1: Protein marker -15µl

Lane 2: Test sample - 30µl

Fig.1 SDS-PAGE Molecular Weight Determination

Characterization of MNPs and Immobilized MNPs:

Optical properties of Fe_2O_3

Optical and photocatalytic activity of iron oxide nps can be determined with the help of UV-Visible spectra. On absorption of radiant energy by the molecules or atoms cause shift in the position of electron and undergo transitional change[29].Optical properties of Fe_2O_3npswas detected using UV-Visible spectra (300-900 nm), but the data were tend to be collected from 400-900 nm due to abundance of noise absorbance below 400 nm. The absorbance attains a high value in a wavelength of 400 nm, which is due to charge transfer spectra. Similar results were observed by cherepy et al [30], Cornell &schwartman[31], lyon et al[32], most common result for iron oxide optical properties are show at 270nm [33], 370 nm [34] respectively.Bandgap energy is calculated on the basis of equation (i) for iron oxide nps and it was found to be 2.48, according to following equation

E_{bg} =1240/ lambda mx (eV) (i). E_{bg} band gap energyand lambda max is the maximum wavelength of magnetic nanoparticle.

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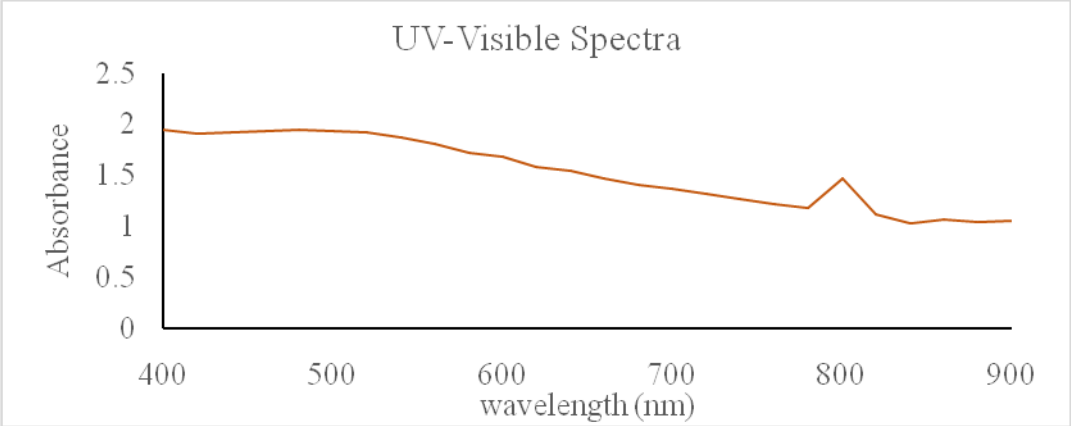

Fig 2: UV-Visible spectra XRD analysis of Magnetic nanoparticles

X-ray scattering analysis gives a detailed information regarding the crystallographic structure, chemical composition, and physical properties of materials and thinfilms.X-ray diffraction can be used to determine which iron oxide compounds are present in NPs by calculating or comparing with the standard value of lattice parameters, crystal structures and crystallinity [29]. The phase of the sample was determined with the help of XRD and several pattern were determined at high angles of (5-80°).The sample showed the major characteristic peaks for as grown crystalline metallic iron at 21.20 (111), 30.39 (220), 35.83 (311), 53.53 (422), 57.33 (511), 62.83 (440) shown in **fig 3** and the patterns been compared with the XRD of hematite (JCPDS data PDF number 39-1346).Diffraction pattern indicates that our nps is crystalline in nature and our synthesized powder were iron oxide [35]. The average size of the synthesize were determined from the FWHM of the XRD peaks using scherrer equation and the results are indicated in **Table 2**.

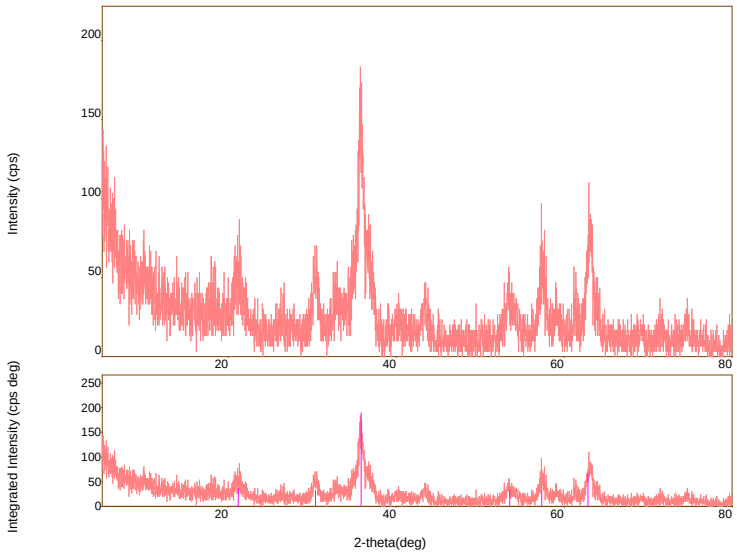

Figure 3: X-ray Diffraction analysis

2-theta (deg)	d (ang.)	Height (cps)	Int. I(cps·deg)	FWHM(deg)	Size
21.20(13)	4.19(2)	18(8)	24(3)	1.12(13)	75(9)
30.39(10)	2.938(10)	24(9)	22(2)	0.74(9)	116(14)
35.83(8)	2.504(5)	65(15)	127(5)	1.56(7)	56(3)
53.53(12)	1.711(3)	17(7)	22.6(16)	1.26(9)	74(5)
57.33(8)	1.606(2)	24(9)	21.2(16)	0.82(7)	116(10)
62.83(8)	1.4778(16)	39(11)	44(3)	1.01(6)	97(6)

Table 2 Peak list of XRD analysis of iron oxide nps

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Dynamic Light Scattering

Dynamic light scattering (DLS) uses a light source that emits through a solution containing particles and measures the amount of light reflected from the particles. The software paired with the device allows for complex calculations to relate the amount of light to the size and distribution of particles in the solution. As the particles move within the solution there is a slight time difference, and therefore light intensity difference, which is measured by the device. The results from the DLS allow for graphical readings showing the size of the particles based on the intensity reading, the size by volume of solution, and the size by number of particles. The determination of the diffusion coefficient of the nanoparticles in solution gives access to the hydrodynamic radius of a corresponding sphere and the polydispersity of the colloidal solution [38]. Magnetic nanoparticles were produced by the co-precipitation of $FeCl_3$& $FeCl_2$ under alkaline condition. These nanoparticles were analyzed by Dynamic Light Scattering particle size analysis spectroscopy the size of the particles were summarized and **Fig. 3** shows the results and the average size of the particles were found to be 50nm

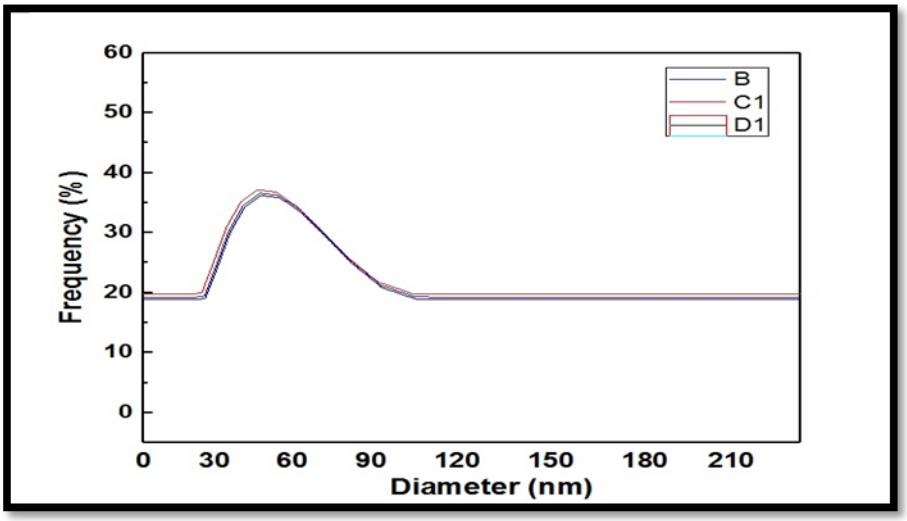

Fig 4: Dynamic Light Scattering of iron oxide Nanoparticles

FTIR analysis of MNPs, amine functionalized MNPs, and Protease immobilized MNPs

The synthesized, amine functionalized and protease immobilized iron nanoparticles were characterized by FTIR and its was found to be 631 cm^{-1}(Fe-O stretching)**Fig.5** for bare nps, 1041 cm^{-1}(Fe-O-Si linkage), 890 cm^{-1} (Si-O) stretching vibration **Fig. 5** for amine functionalized nps, protease immobilizedwas confirmed at 1644 cm^{-1}(CO=NH) **Fig. 5s**, these similar peaks and stretching were observed in other FTIR interpretation results ofFuntionalized MNPs [15] and Protease immobilized MNPs [26]. In **Fig.5** of free MNPs 1700-3600 cm^{-1} due to C=O stretching [33]. In **Fig.5** of functionalized MNPs 1041 cm^{-1} absorption band of Si-O-Si stretching vibration [36] was observed.Protease immobilized onto iron oxide nanoparticle with the help of crosslinker is confirmed by absorption band at 1644 cm^{-1}[36] with respect to the amide bond present in free protease **Fig.6** 1670 cm^{-1}[26]due to the stretching vibration of carbonyl groups in protease. The APTES linked to the MNPs is confirmed on the basis of absorption band at 1644 and 3362 cm^{-1} NH_2 bending mode of free NH_2 and N-H stretching vibration, Confirms the presence of APTES [37]. The broad spectrum at 3400 cm^{-1} is due to O-H stretching in **Fig.5,6**.

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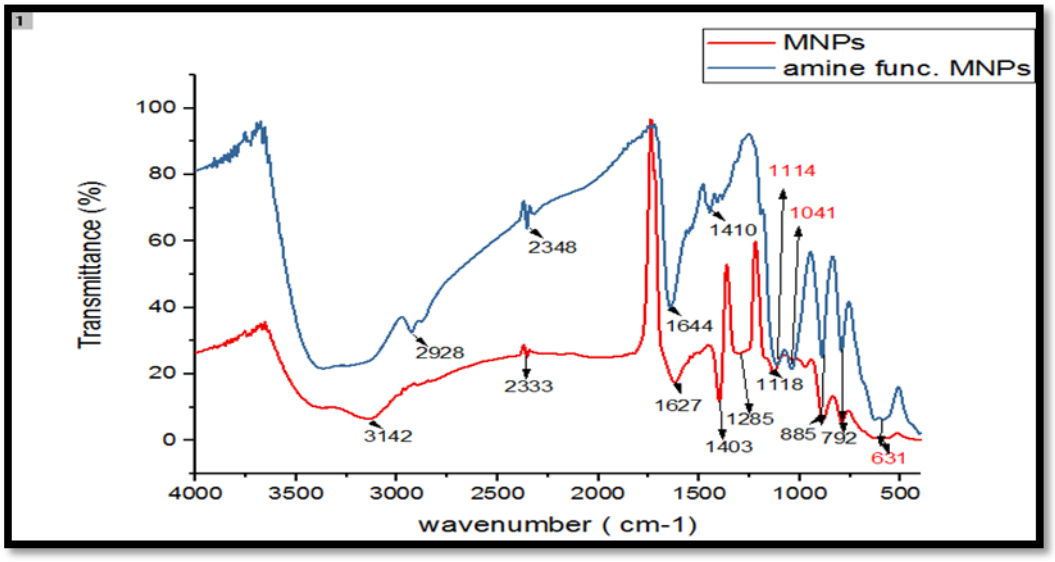

Fig 5: FTIR analysis of Iron Oxide MNPs and amine functionalized iron oxide MNPs

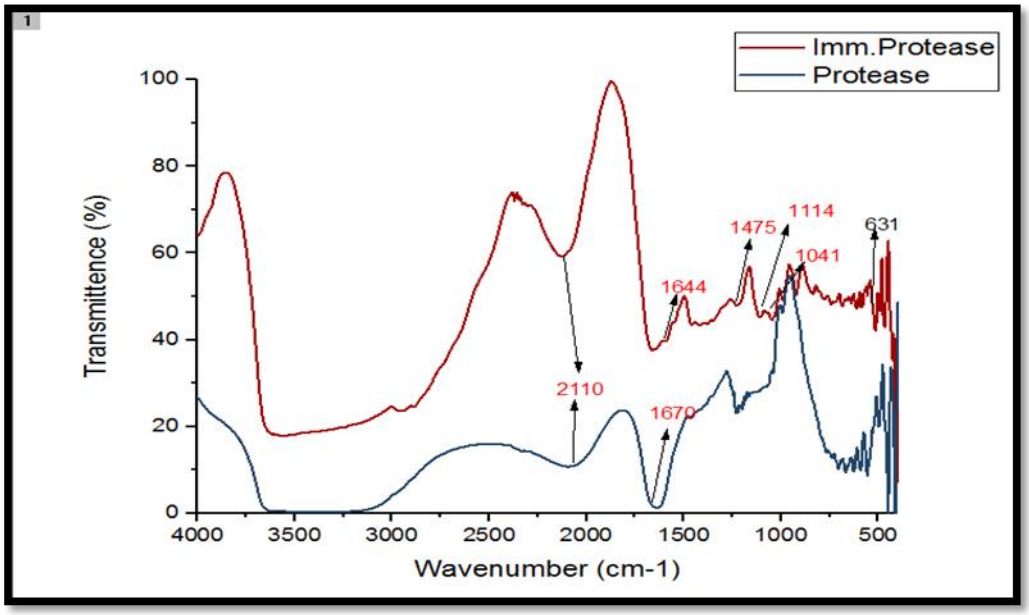

Fig 6: FTIR analysis of Free Protease and Immobilized protease

Characteristics of Free and Immobilized Alkaline Protease

Optimal pH and Temperature. As shown in **Fig.7 and 8** the highest activity of free enzyme and immobilized alkaline protease displayed at pH 7 and pH 9, which was much higher than that of the free enzyme, indicating that the immobilized alkaline protease was more resistant to alkaline conditions. It implied that covalent bonds or hydrogen bonds formed during the process of cross-linking made the immobilized enzyme more stable and have a stronger pH tolerance. The optimal temperature for the free enzyme was 55 °C while it shifted to 65 °C for the immobilized enzyme. It may be due to the covalent bond formation between proteins or protein and support caused by glutaraldehyde, which might increase the conformational inflexibility of enzyme and prevent it from distortion or damage by heat exchange[39]. This improvement also may be caused by a mass of covalent cross linking between enzyme and amino-functionalized Fe_3O_4 nanoparticles

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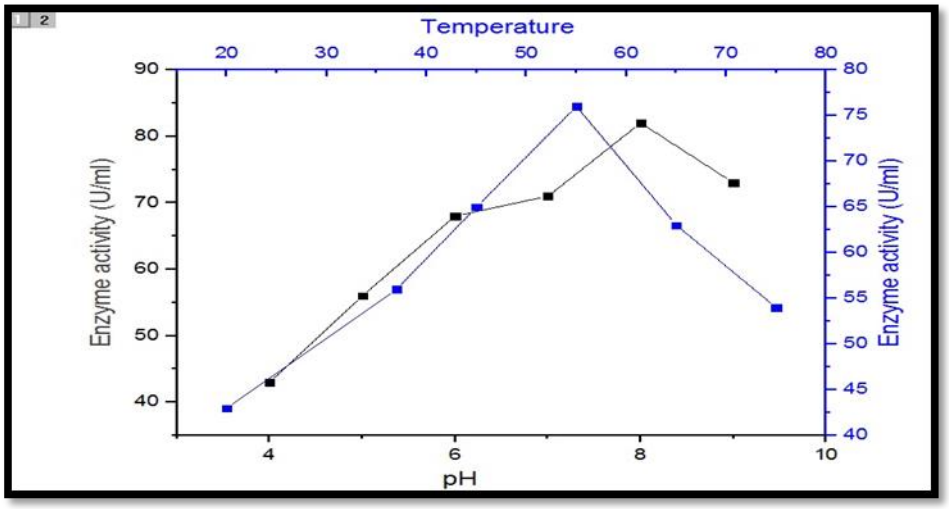

Fig 7: Optimum pH and Temperature

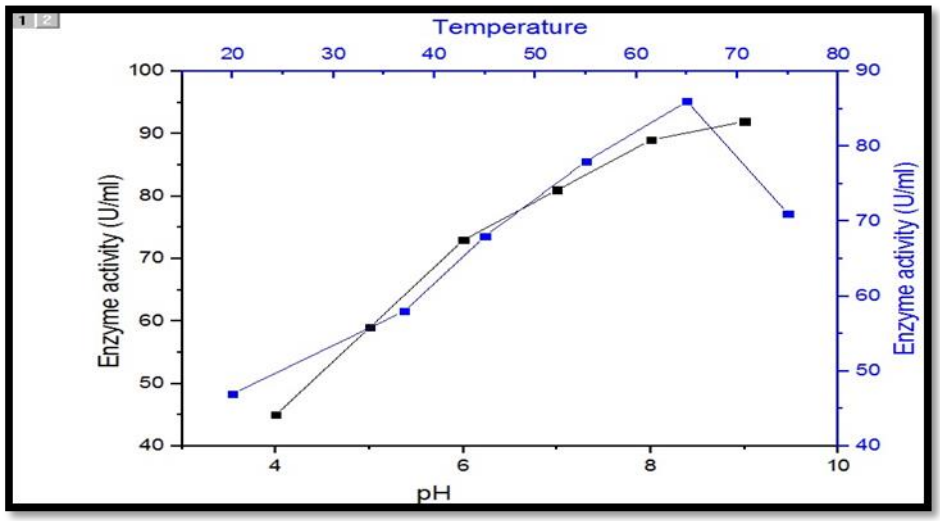

Fig 8: Optimum pH and Temperature of Immobilized Protease

CONCLUSION

The immobilization of protease with MNPs was confirmed by Fourier Transform Infrared Spectroscopy. The characterization of nanoparticles was determined by UV-Visible spectra, XRD anlaysis and DLS methods. The average size of Magnetic nanoparticles 50 nm respectively. The activity of the enzyme was also confirmed by the Universal protease activity method. Alkaline protease was successfully immobilized onto amino functioned magnetic nanoparticles, which solved the problems of enzyme recycling, and substrate and product separation. The immobilized enzyme possessed greater pH and thermal stabilities. This study showed that the immobilization of protease enzyme via surface transformation on to super paramagnetic nanoparticles gives greater stability, this was remarkably so at higher pH.

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