Biological and Applied Sciences

Vol.60: e17160572, January-December 2017
http://dx.doi.org/10.1590/1678-4324-2017160572
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

A N I N T E R N A T I O N A L J O U R N A L

Immobilization of the Magnetic Nanoparticles with Alkaline Protease Enzyme Produced by *Enterococcus hirae* and *Pseudomonas aeruginosa* Isolated from Dairy Effluents

Chandran Masi [1]*, C.Chandramohan[2] and M.Fazil Ahmed[2].

[1]R& D Lab, Vel Tech High Tech Dr.Rangarajan Dr.Sakunthala Engineering College, Avadi, Chennai, Tamilnadu, India; [2]Anna university, Chennai, India.

ABSTRACT

Protease is an enzyme which has a wide range of applications in various fields. Extracellular protease was produced from Pseudomonas aeruginosa and Enterococcus hirae which were isolated from the effluents of diary industries. Protease immobilized with super paramagnetic nanoparticles was characterized by DLS, XRD and TEM methods in relation to their size and structure. The protease enzyme was bound to magnetic nanoparticles via surface transformation technique including Silica coated magnetic Nano composite, amine and cysteine functioned Nano composite formation. Successful binding of protease onto the particles was confirmed by TEM imaging. The maximal enzyme activity of immobilized protease was determined using universal protease assay and was found to be 105µg mL^{-1} & 290µg mL^{-1} for Pseudomonas sp. and Enterococcus sp. respectively. The immobilization capacity of protease onto nanoparticles was 6000µM/g. The stability of the immobilized enzyme increased in comparison with the free enzyme. Overall, this study showed that the stability and activity of the protease was enhanced by immobilization to the magnetic nanoparticles. This suggested that immobilized enzyme on the magnetic beads of nanoparticles could be used in an interesting range of applications, both in broader temperature and pH ranges, also permitting magnetic recovery of the enzyme for reuse or purification of the product.

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

* Author for correspondence: biochandran@yahoo.co.in

INTRODUCTION

The interest in Nano scale materials stems from the fact that new properties are acquired at this range and equally important these properties change with their size or shape. The past couple of decades have witnessed an exponential growth of activities in this field worldwide, driven both by the excitement of understanding new science and by the potential hope for applications and economic impacts. Important among these Nano scale materials are the Nano composites, in which the constituents are mixed at nanometer scale. The study of Nano composite materials requires a multidisciplinary approach with impressive technological promise, involving novel synthesis techniques and an understanding of physics and surface science [1]. Magnetic Nano composite materials are generally composed of ferromagnetic particles (grain size in nanometer scale) distributed either in a non-magnetic or magnetic matrix [2, 3].The shape, size and distribution of the magnetic particles play an important role in determining the properties of such materials [4, 5]. The unique physical properties of nanoparticles are allowing their application in many fields such as biomedicine and sensor development [6, 7, 8,] water purification [9], and environmental remediation [10, 11, 12]. Superparamagnetism of magnetic nanoparticles (MNPs) is a size-dependent property that is useful for applications requiring manipulation of MNPs by an external magnetic field. Such particles do not retain any residual magnetism once the magnetic field is removed [6]. This feature has resulted in the development of many biomolecule-nanoparticle (bio-NP) hybrids for biomedical applications in the diagnosis and localized treatment of disease [5, 6, 7, 13]. Enzymes have long been used in industry as catalysts for catabolic processes or for the production of specific chemical enantiomers. MNP-enzyme conjugates (MNP-Es) represent a specific class of bio-MNP conjugates that are of great interest for biotechnological applications where high catalytic specificity, prolonged reaction time, and in some cases the ability to recycle an expensive biocatalyst is required [14, 15, 16]. In addition, magnetic field susceptibility provides a mechanism for efficient recovery of the enzyme complex from reaction products, which is especially important in the pharmaceutical industry where enzyme contamination of the final product can cause detrimental side effects. In this study, protease which is an industrially important enzyme was produced from the bacterial species *Enterococcus hirae* and *Pseudomonas aeruginosa* isolated from the effluents of dairy industries. The protease produced from the above bacterial strains were partially purified and immobilized with the surface transformed magnetic nanoparticles [16]. Theactivity and stability of the free enzymes and immobilized enzymes were examined extensively.

MATERIALS AND METHODS

Collection of samples from dairy effluents

Samples for the isolation of bacterial species were collected from 5 dairy industry effluents in Chennai. Sterile procedures were followed during the transport of the samples and they are processed within six hours.

Isolation and screening of protease producing bacterial species

The 5 set of samples collected from the dairy effluents were serially diluted for the isolation of the bacterial strains. The serially diluted samples were inoculated using spread plate method for the growth of microbial colonies. The petri plates were incubated for 24 hours at 37°C for the growth of bacterial colonies. The morphological characters of the bacterial colonies were studied and the bacterial strains were isolated from the nutrient medium. The isolated bacterial strains were

inoculated in a suspended medium containing nutrient broth and incubated overnight for the growth of the isolated bacterial strains.The isolates of bacteria were streaked on casein agar plates and incubated for 24 hours at 37°C for the screening of protease producing bacteria. Based on the zone formation on the casein agar medium, the protease producing bacterial strains were determined and cultured separately in 100 ml of nutrient broth medium. After incubation, bacterial suspensions were centrifuged and the supernatants were collected. Casein agar plates were prepared for the screening of potential protease producing bacterial strains using well diffusion method. 1 ml of supernatant of protease producing bacterial strains was added to the well of casein agar plates. The casein agar plates were incubated for 24 hours at 37°C and then screened for the zone formation by well diffusion method to obtain the potential protease producing bacterial strains.

Identification of protease producing bacteria

The screened potential protease producing bacteria were given for biochemical identification and 16s rRNA analysis at PCBS, Pondicherry. The 16S rRNA sequences were registered at Gen Bank (NCBI, USA).

Protein and Protease estimation

The protease activity was estimated by the method described by Beg et al. [17]. After incubation, the bacterial broth was centrifuged at 5000 rpm for 20 minutes at 4^{o}C to obtain the cell free supernatant (CFS). 1 mL of CFS was added to 5 mL of 1% (w/v) casein solution in glycine-NaOH buffer of pH 10.5 and incubated for 10 minutes at 60^{o}C. The reaction was stopped by the addition of 4 mL of 5% trichloroacetic acid. To the reaction mixture 5 mL of 0.4 M Na_2CO_3 was added, followed by 0.5 mL Folins-Ciocalteu reagent and incubated at room temperature for 30 minutes. The amount of tyrosine released was determined using a UV-VIS spectrophotometer at 660 nm against the enzyme as blank. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine/mL/min under standard assay conditions. The soluble protein content of the enzyme sample was determined by Lowry's method [18] using crystalline bovine serum albumin as the standard.

Production and Partial Purification of protease

The protease production was achieved by the fermentation of the bacterial culture with 1.5 liters of sterilized medium for 48 hours using Batch Fermenter. After fermentation, the broth was collected and centrifuged to obtain the cell free supernatant which contains protease enzyme and media constituents. The obtained supernatant of bacterial culture was saturated using ammonium sulfate at different concentrations of 20%, 40%, 60% and 80% respectively. The saturated solutions were centrifuged and the pellets obtained were suspended with Tris-HCl buffer and used for dialysis. The dialyzed solutions of protease were estimated for its activity by universal protease assay.

Production of magnetic nanoparticles

The method of preparation was according to that of Massart's [19] but without the use of hydrochloric acid. 4.4 g of $FeCl_3.6H_2O$ and 1.98 g of $FeCl_2.4H_2O$ were dissolved in 61mL water devoid of air. The solution was purged with nitrogen or argon to agitate the mixture and to prevent the oxidation of Fe^{2+} ions. After 30 min of purging, 0.7M NaOH was added drop wise into the above Fe ion solution and mixed thoroughly with the help of sonicator. During the addition of NaOH, it was noticed that the solution changed color from the original brown to dark brown and then to

black. The black iron oxide product responded to a magnetic field as expected. This physical property is helpful in the separation of the particles from the liquid solution.

Preparation of silica coated magnetic nanoparticles

Direct immobilization of protease with the magnetic nanoparticles are not effective because of the surface oxidation and rejection, so the magnetic nanoparticles were subjected to Sol-gel process for the coating of Silica nanoparticles with the magnetic nanoparticles which will reduce the surface oxidation and will enhance the immobilization. 80mL of 0.125M Fe_3O_4(Iron oxide) suspension of Nano-particle was prepared by dispersing the iron oxide magnetic Nano-particles in absolute ethanol at 40°C. 4.00mL of 21% ammonia, 7.50mL of deionized water and 0.56mL of TEOS(Alfa aesar, A14965) (Tetraethyl Orthosilicate) were added in a sequence to the iron oxide mixture. After stirring vigorously with a magnetic stirrer for 2 hours, the mixture was then ultra-sonicated for 1 hour. The Nano-particles were separated from the liquid by magnetic attraction and dispersed in 30mL of ethanol. After keeping the particle suspension in 60°C water bath for 6 hours to strengthen the Si-O-Fe linkage, and the coated particles were finally separated from the liquid by a magnetic decantation method and then vacuum dried.

Preparation of amine functioned silica coated magnetic nanoparticles

Silica coated magnetic nanoparticles were dispersed in absolute ethanol at 40°C. 4ml of 20% ammonia and 1ml of APS(Alfa aesar, A11285)(3-Aminopropyltrimethoxysilane) were added in a sequence and stirred vigorously with magnetic stirrer and sonicated for 1 hour, then left for an hour undisturbed for the settling of the amine functioned magnetic nanoparticles. Then it was separated by the magnetic separation process and dried in vacuum drier.

Preparation of cysteine functioned silica coated magnetic nanoparticles

Aminated Fe_3O_4 nanoparticles (1 mg) were dispersed into dimethyl sulfoxide (DMSO, 250 μL) and sonicated for 30 min. After sonication, suberic acid bis N-hydroxysuccinimide ester (Alfa aesar, H51771-3) (DSS, 5 mg, 0.03 mmol) was added to the solution and stirred at room temperature for 1 hour. The resulting nanoparticles were washed 3 times with DMSO (100 μL) to remove excess DSS. Cysteine 3 (200 μL, 50 mM) was added to the above brown solid in Dimethyl fluoride solution and shaken at room temperature for 12 hours. The Nanoparticles were separated using a magnet and washed 3 times with deionized H_2O. Acetic anhydride (Ac_2O)/pyridine (1/2, 300 μL) was then added and the mixture was stirred at room temperature for 3 hours. Subsequently, 300 μL of 100 mM ethanolamine was added to the mixture and shaken at room temperature for 6 hours. After separation by a magnet, the nanoparticles were washed 5 times with deionized H_2O to yield the protected cysteine-functionalized magnetic nanoparticle as a brown powder. Deprotonation can be done using a cocktail of reagents comprising trifluoroacetic acid (TFA, 1 mL), triisopropylsilane (TIPS, 33 μL), and deionized H_2O (32 μL) to obtain the cysteine-functionalized magnetic nanoparticle before immobilization process.

Immobilization of the protease enzyme with magnetic nanoparticles

To immobilize protease enzyme on the cysteine-functionalized MNPs, 30 μM of protease was reacted with 5 mg of cysteine-functionalized MNPs in 1 mL of HEPES buffer while vigorously shaking at room temperature for 12 hours. The resulting enzyme-MNP (magnetic nanoparticles) complex was washed 3 times with HEPES column buffer.

RESULTS AND DISCUSSION

Production of Protease enzyme

Isolation of bacterial strains

Effluent samples of the five dairy industries were collected and serially diluted for the isolation of bacterial strains, then it was plated in nutrient agar plates and incubated for the growth of the bacterial strains. 78 strains of bacterial colonies were isolated according to differential morphological characteristics of its colonies. Then the isolated bacterial strains were individually cultured in different test tubes using nutrient broth. The isolated 78 strains were plated in casein agar plates and incubated to identify the bacterial strains which can produce protease. 12 strains of bacteria were shortlisted as protease producing and they were cultured separately in nutrient broth and incubated for 24 hours. The supernatants of the cultured strains were isolated using centrifuge and they were again plated in casein agar plate using well method and incubated for 24 hours. The zones of clearance were noticed using $HgCl_2$ precipitation method. Thus the three potential strains of bacteria were identified by well diffusion method.

Identification of bacterial strains

The isolated potential strains of bacteria for the production of protease were analyzed by biochemical identification method and 16S RNA method. The three bacterial strains were identified as *Pseudomonas aeruginosa*, *Enterococcus hirae* and *Acetinobacter sp.* Then the 16S RNA sequences were registered in Gen Bank (NCBI, USA) for accession number.

Pseudomonas aeruginosa - **KC991295**.
Enterococcus hirae - **KC991294**.
Acetinobacter sp. - **KC991293**.

Protease and protein analysis

The isolated and identified potential protease producing bacterial strains were analyzed for its protease and protein concentration in nutrient broth culture medium using the universal protease assay by Beg et al. [16] method and protein assay by Lowry et al. [17] method. **(Fig. 1)** showed that *Enterococcus hirae* produce remarkable amount of protease than the other two strains and also it can be seen from comparison of both **(Fig.1)** and **(Fig.2)** that *Enterococcus sp.* produces less amount of other protein complexes. *Pseudomonas aeruginosa* was identified as the second best strain for protease production.From the observations two strains namely ***Pseudomonas aeruginosa*** (PA) and ***Enterococcus hirae*** (EH) show maximum production of the protease with less protein concentration, so they were shortlisted as potential strains for the production of the protease. These two organisms were cultured in the nutrient broth in sterile fermenter for the bulk production of the crude protease enzyme.

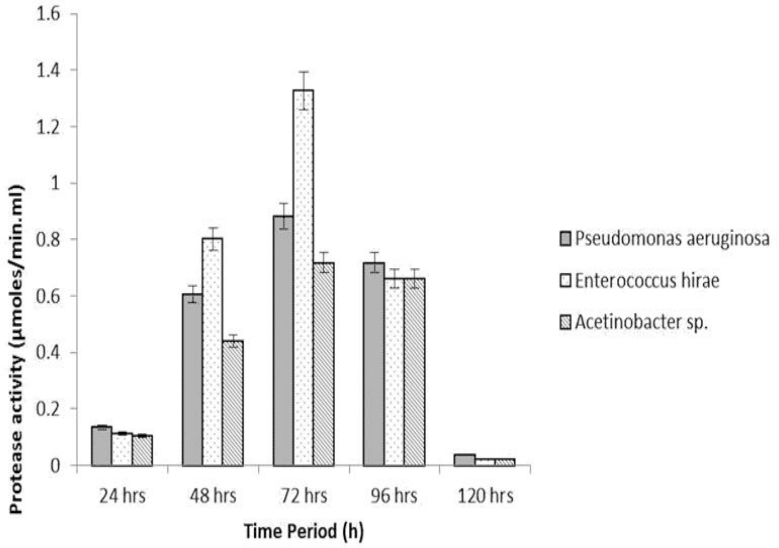

Fig. 1. Protease activity profile of the three strains which were isolated from the dairy industry

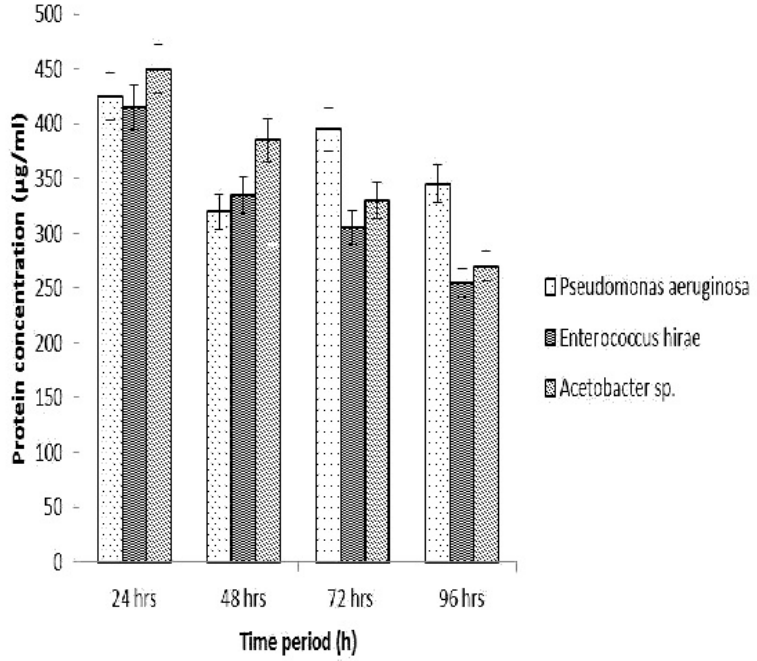

Fig. 2. Protein concentration profile of the three strains which were isolated from the dairy industry

Partial Purification of protease

The crude protease were separated from the culture by centrifugation, the supernatant which contains protease enzyme was collected and subjected to ammonium sulphate precipitation at different saturation levels. The saturated protease enzyme was partially purified by the dialysis method which will remove all the salt content in the enzyme and will help in the unsaturation of the enzyme. Fig. 3 illustrates that the 20% and 60% saturation of supernatant of *Pseudomonas aeruginosa* culture showed maximum activity indicating the production of two kinds of protease having different molecular weights. 20% saturated protease from *Pseudomonas aeruginosa* (PA) was taken for immobilization for its higher activity than 60% saturation. **(Fig. 3)** also illustrate that 60% saturation of supernatant of

Enterococcus hirae(EH) culture showed maximum activity which was also selected for immobilization.

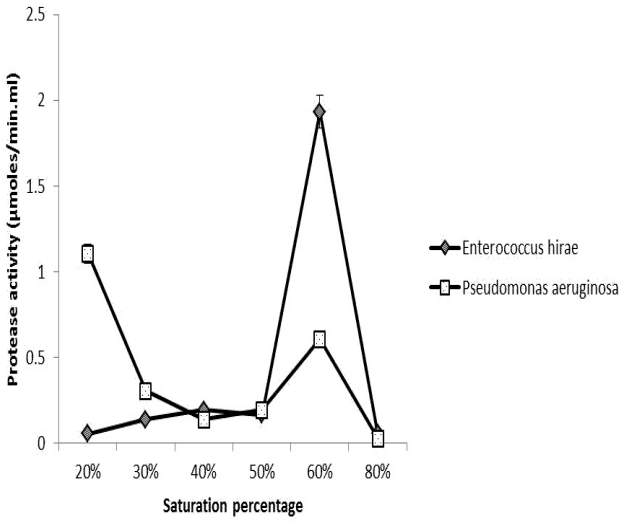

Fig. 3. Variation in the precipitation of protease by salting out method (ammonium sulphate precipitation method) between *Pseudomonas aeruginosa* and *Enterococcus hirae*

Effect of temperature and pH on the activity of the protease enzyme

The effect of temperature and the pH on the activity of the partially purified free protease were calculated and illustrated. PA-protease showed highest activity at the temperature of 60^{o}C and at the pH of 9. EH-protease showed highest activity at the temperature of 50 to 60^{o}C and at the pH of 9 **(Fig. 4 & 5)**, which indicates that both the protease enzymes are of alkaline protease type showing high activity at alkaline pH but the maximal activity of the EH-protease is very high compared to the maximal activity of PA-protease.

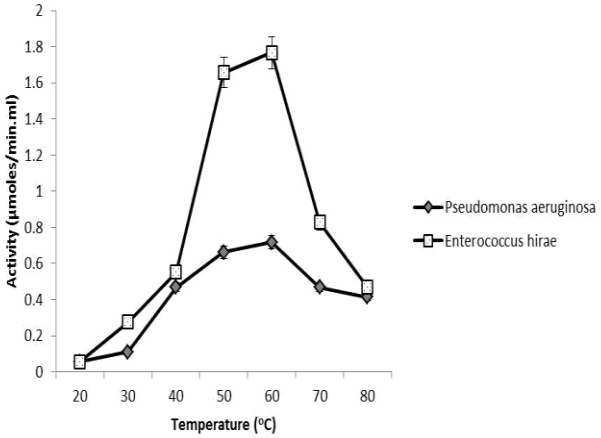

Fig. 4. Effect of temperature on the activity of the protease enzyme produced by *Pseudomonas aeruginosa* and *Enterococcus hirae*

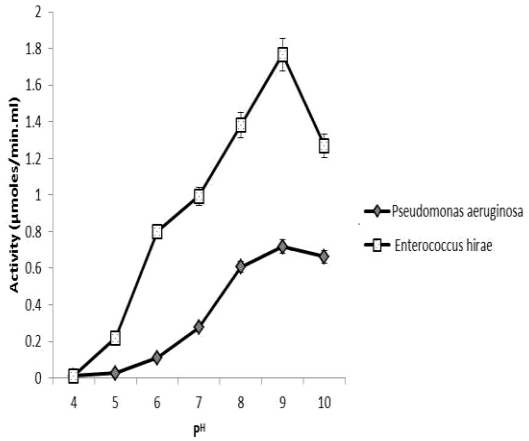

Fig. 5. Effect of pH on the activity of the protease enzyme produced by *Pseudomonas aeruginosa* and *Enterococcus hirae*

Immobilization of the magnetic nanoparticles with the partially purified protease

Direct immobilization of protease enzyme to the magnetic nanoparticles reduces the catalytic property of the protease by large folds which is mainly because of the hindrance caused by the magnetic nanoparticles on the attachment of enzymes to the substrates, to minimize this effect long chain organic compound DSS was introduced between the Nano composite of silica coated magnetic nanoparticles and protease enzyme by amine functioning with the help of APS, then the attachment of DSS with protease is facilitated by cysteine functioning. Amine is mainly used for the linkage of protease, this linkage process can play a role to escape the protease from the nucleophilic attack of cysteine, which can possibly remove the enzyme if its not properly linked . After the linkage process, amine linked magnetic nanoparticles,were coupled with the cysteine functional groups. The role of cysteine, is just to be considered because of the nucleophilic attack induced by them on the protease, increase the activity of enzyme to make the conversion of the substrate to the product at a quite high rate. Thus this study reports a method for preparation of functionalized magnetic nanoparticles and immobilized enzymes by chemical procedure. The resultants were analyzed by TEM, XRD and DLS Particle size analysis spectroscopy.

Particle size analysis

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. 6)** shows the results and the average size of the particles were found to be 786.7 d.nm.

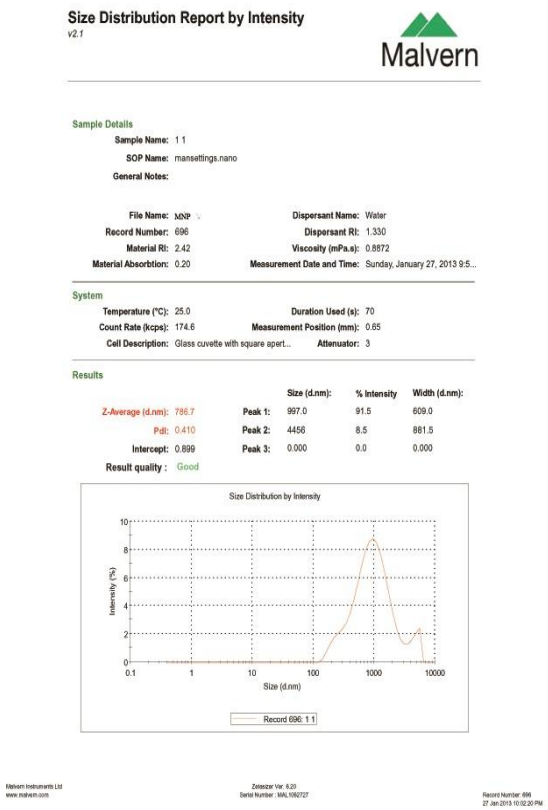

Fig. 6. Dynamic Light Scattering Particle Size Analysis Result of the Magnetic Nano Particles Produced by Coprecipitation

TEM analysis results for nanoparticles

The produced and analyzed magnetic nanoparticles were subjected to TEM analysis in TECNAI'10 Philips made Transmission electron microscope at Vetinery College, veappeary, Chennai, for the conformation of the size and shape of the magnetic nanoparticles. **(Fig. 7)** shows that magnetic nanoparticles were round in shape and it has its size ranges from 23nm to 100nm. This confirms the production of magnetic nanoparticles.

Transmission electron microscopic image illustrated in **(Fig. 8)** shows cluster of silica coated magnetic nanoparticles (A) and individual silica coated magnetic nanoparticles (B). The silica coated magnetic nanoparticles are depicted as a smaller and darker spots which has average diameter range of 17nm to 30nm. Most particles have spherical shape but some aggregation of particles can be also seen. The silica nanoparticles are attached to the magnetite surface of the magnetic nanoparticles and formed core-shell structure

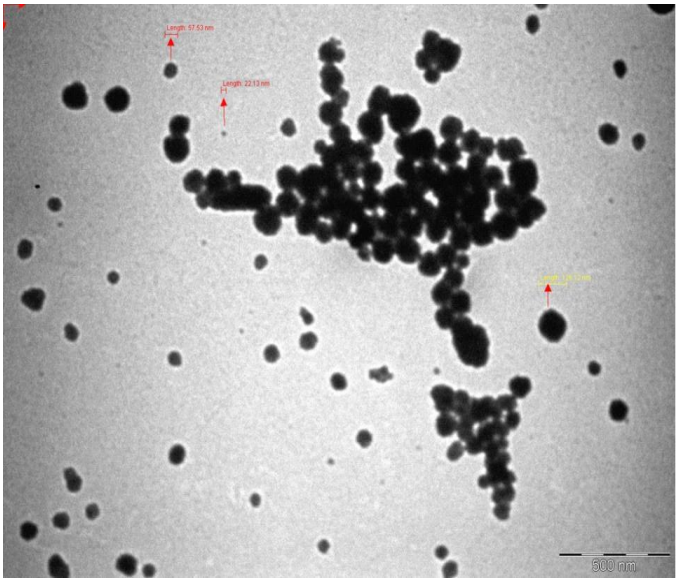

Fig. 7. Transmission Electron Microscopic Image of the Magnetic Nanoparticles

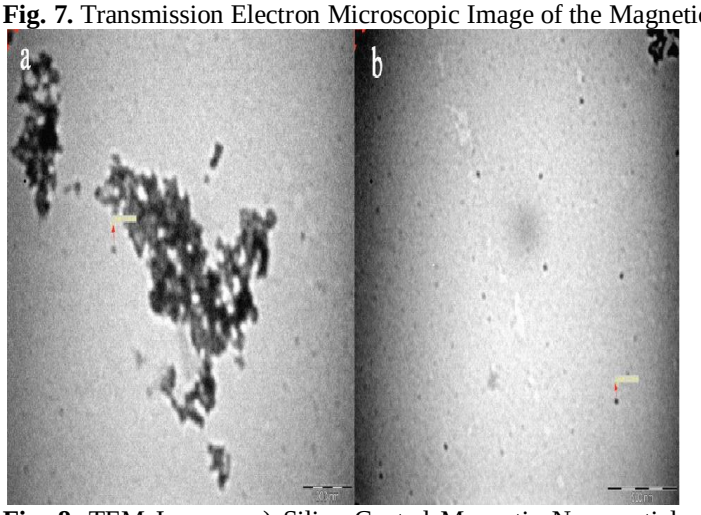

Fig. 8. TEM Images: a) Silica Coated Magnetic Nanoparticle clusters. b) Silica Coated Magnetic Nanoparticles (individual)

X-Ray Diffraction Analysis Results of Magnetic and Silica Coated Magnetic Nanoparticles

The crystalline structures of pure magnetite Fe_3O_4 and core-shell Fe_3O_4-silica nanoparticles were analyzed by XRD method at Madras University, Chennai. In B **(Fig. 9)**, series of diffraction peaks are depicted which was obtained for the pure magnetite magnetic nanoparticles. **(Fig. 10)** depicts series of diffraction peaks for silica coated magnetite magnetic nanoparticles. This indicates differences in diffract grams after coating by silica shell. **Table 1 and 2** gives the results of diffract gram and proves the difference in diffraction between magnetic nanoparticles and silica coated magnetic nanoparticles. XRD results showed the purity and also confirmed the binding of the silica nanoparticles over the magnetic nanoparticles.

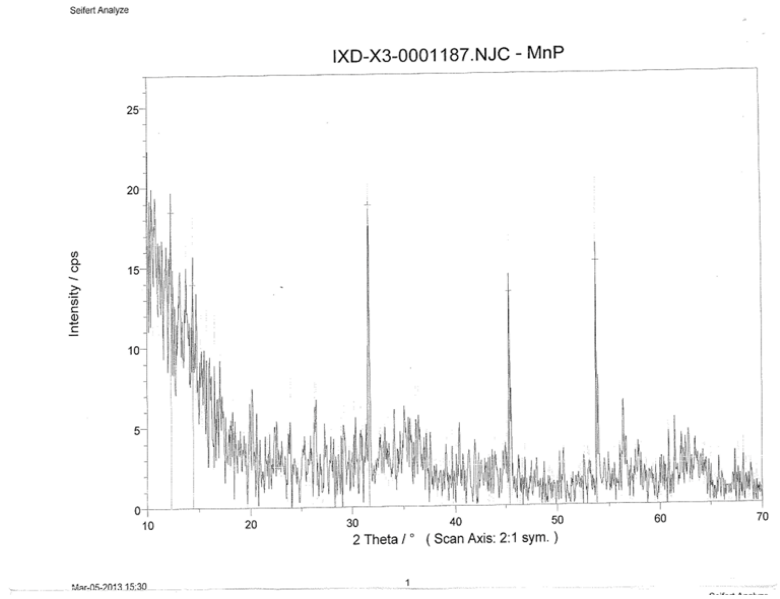

Fig. 9. X-Ray Diffraction Analysis Results of magnetic nanoparticles

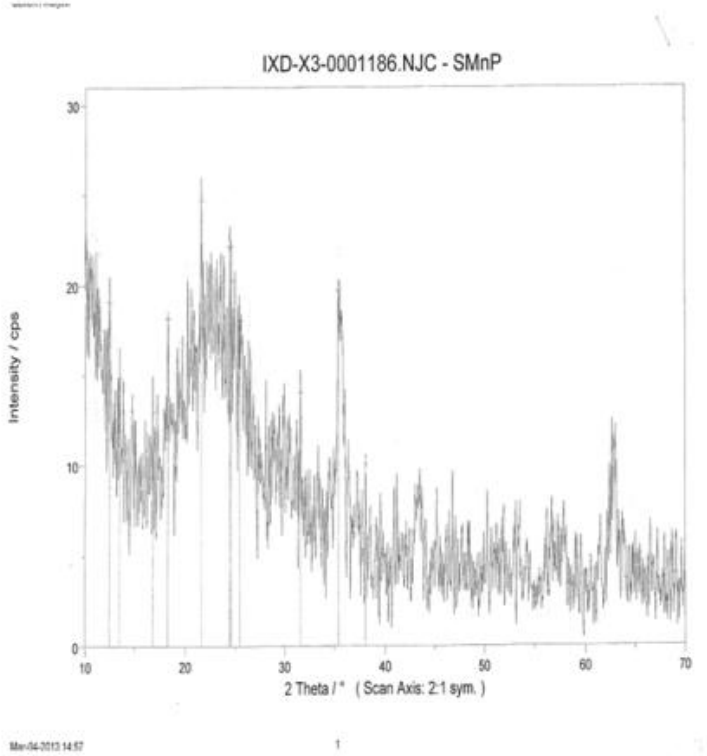

Fig. 10. X-Ray Diffraction Analysis results of Silica coated magnetic nanoparticles

Table 1: X-Ray Diffraction Analysis Results of magnetic nanoparticle

No	d_Fit(A1)	FWHM	2Θ	$\angle_{Parab}$	I_{Ref}	$\int_{Ref}$
1	8.4402	1.3720	10.4729	10.4729	79	100
2	2.8153	0.1647	31.7581	31.7581	100	20
3	2.5236	0.9608	35.5455	35.5455	92	87
4	1.9929	0.1232	45.4757	45.4757	44	6
5	1.4750	0.3411	62.9634	62.9634	70	25

Table 2: X-Ray Diffraction Analysis Results of Silica coated magnetic nanoparticles

No	d_Fit(A1)	FWHM	2Θ	$\angle_{Parab}$	I_{Ref}	$\int_{Ref}$
1	7.0822	0.6990	12.4882	12.4882	77	16
2	6.5598	0.8518	13.4873	13.4873	60	11
3	5.2722	0.2320	16.8026	16.8026	53	3
4	4.8474	1.0352	18.2872	18.2872	74	16
5	4.1106	3.3992	21.6016	21.6016	100	100
6	3.6359	0.2388	24.4627	24.4627	90	14
7	3.6109	1.1215	24.6350	24.6350	82	14
8	3.4968	3.3473	25.4520	25.4520	73	22
9	2.8317	0.2935	31.5702	31.5702	57	4
10	2.5359	0.8836	35.3670	35.3670	80	18
11	2.3634	0.1299	38.0436	38.0436	41	2

Transmission Electron Microscopic Images of immobilized protease

The immobilized protease-nanoparticles complex was analyzed by the Transmission electron microscopy and the images were examined, which shows the attachment of the silica coated cysteine functioned magnetic nanoparticles with the external surface of the protease enzyme. **(Fig. 11)** illustrates the immobilization of the magnetic nanoparticles with the protease produced from *Pseudomonas aeruginosa*. **(Fig. 12)** illustrates the immobilization of the cysteine functioned magnetic nanoparticles with the protease produced from *Enterococcus hirae*. In both the figures, magnetic nanoparticles are depicted as black spots over the surface of enzyme complex and confirms the attachment of the nanoparticles with protease.

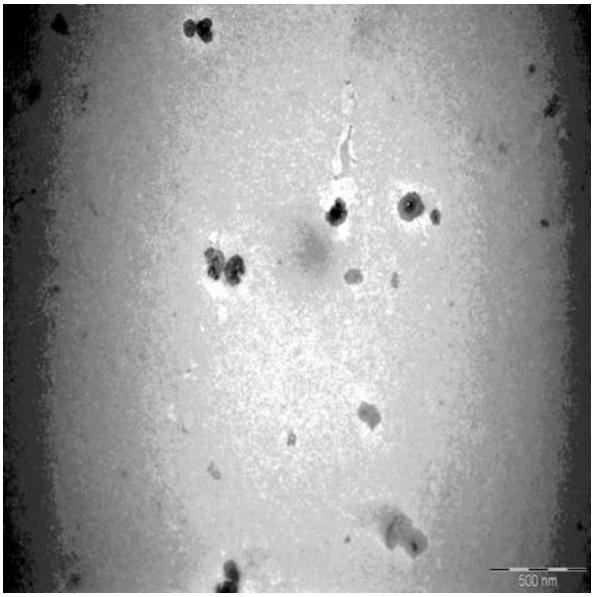

Fig. 11. Transmission Electron Microscopic Image of Protease produced by *Pseudomonas aeruginosa* immobilized With Magnetic Nanoparticles

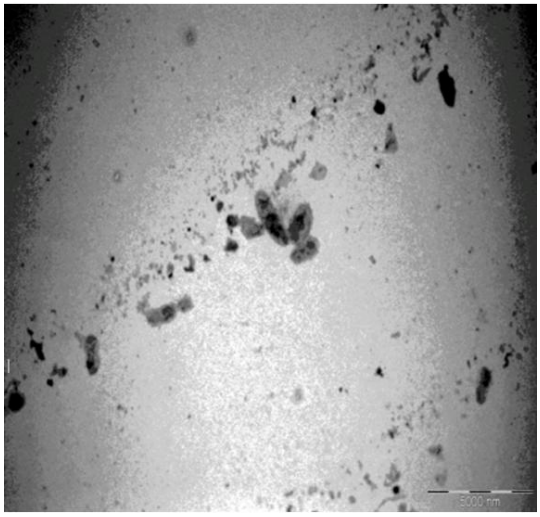

Fig. 12. Transmission Electron Microscopic Image of Protease produced by *Enterococcus hirae* immobilized With Magnetic Nanoparticles

Confirmation of immobilization by FT-IR Spectroscopy

Binding of protease with the magnetic nanoparticles were analyzed and confirmed by FT-IR Spectroscopic analysis. The raw **(Fig. 13a)**, silica coated **(Fig. 13b)** and amine functioned **(Fig. 13c)** magnetic nanoparticles were analyzed by FT-IR spectroscopy and the transmittance peaks were analyzed **(Fig. 13)**. It shows confirmation of Magnetic nanoparticles with a silica shell by the characteristic adsorption band at 1090 cm^{-1} due to silane group presence. A sample with a double coating silica-aminosilane shows the band of both materials, the band at 2943 cm^{-1} due to the stretching of C-H from methyl group ($-CH_2$, $-CH_3$), the band at 1072 cm^{-1} is due to the Si-O bond and the bands at 3309 and 1654 cm^{-1} are attributed to the aminegroup ($-NH_2$) and a new band is shown at 802 cm^{-1} due to Si-O-Si bond. The binding of protease to MNPs was also confirmed by FT-IR analysis (with raw Fe_2O_3 magnetic nanoparticles as example). **Figure 14 & 15** shows the FT-IR spectra for free and immobilized protease of *Pseudomonas aeruginosa* and *Enterococcus hirae* respectively. For PA-protease, the characteristic bands of the protein at 1627, 1539, 1124, 1060 cm^{-1}, which corresponding to the amide bands, exist in both the free PA-protease **(Fig. 14a)** and the MNP immobilized PA-protease **(Fig. 14b),** therefore, confirming the binding of PA-protease to the MNPs. In addition, strong absorption peaks at 650, 575, 523 cm^{-1}, were assigned to the Fe-O bond of raw MNPs/Fe_2O_3 and at 3332 cm^{-1} indicating amine functioned MNPs, which were present in immobilized PA-protease **(Fig. 14)**. For EH-protease, the characteristic bands of the protein at 1632, 1545, 1125, 1064 cm^{-1}, which corresponding to the amide bands, exist in both the free EH-protease **(Fig. 15a)** and the MNP immobilized EH-protease **(Fig. 15b)**, therefore, confirming the binding of EH-protease to the MNPs. In addition, strong absorption peaks at 588, 548, 522 cm^{-1}, were assigned to the Fe-O bond of raw MNPs/Fe_2O_3 and high absorbance peak at 1743 cm^{-1} indicating amine functioned nanoparticles which were present in immobilized EH-protease **(Fig. 15)**.

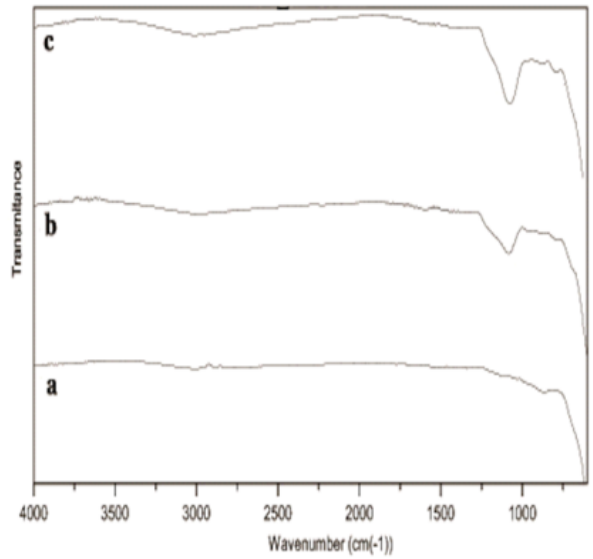

Fig. 13. FT-IR Transmittance Spectra of a) Raw magnetic nanoparticles (MNPs) b) Silica coated magnetic nanoparticles. c) Amine functioned magnetic nanoparticles.

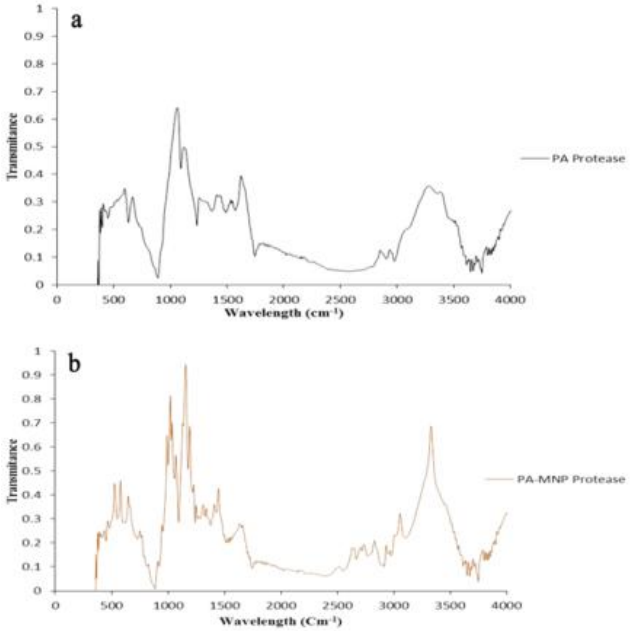

Fig. 14. FT-IR Transmittance Spectra of a) Free PA-Protease. b) Immobilized PA-Protease

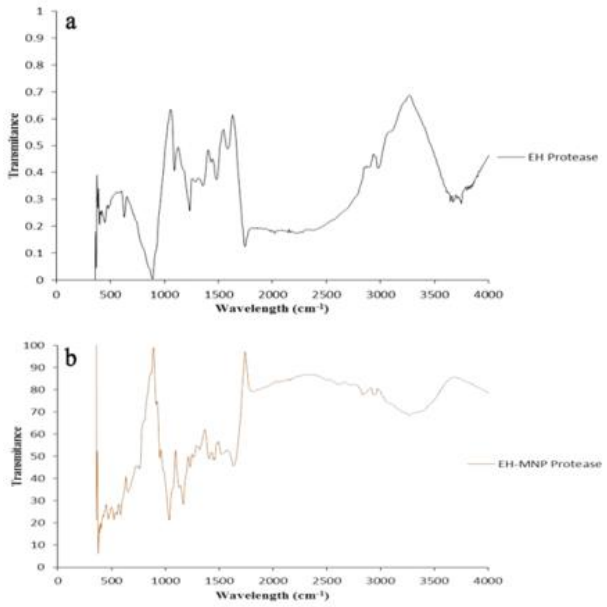

Fig.15. FT-IR Transmittance Spectra of a) Free EH-Protease. b) Immobilized EH-Protease

Activity and Stability of the immobilized protease

Effect of temperature on enzyme activity and stability

The effect of temperature on the activity of both free and immobilized protease were examined **(Fig. 8).** The free enzyme showed greater activity than the immobilized enzyme but at higher temperature of 70 to 80˚C the immobilized enzyme showed slightly greater activity for both EH & PA protease. At 37˚C, the activity of immobilized enzyme was 22% less than that of the free enzyme for PA-protease **(Fig. 16(a))** and 35% less than that of the free enzyme for EH-protease **(Fig. 16(b))**. At 50˚C and 60˚C the activity of both immobilized and free enzyme was increased. The activity of immobilized enzyme increased to some extend at high temperature 80˚C **(Fig. 16)**. Enzyme stability is important. The stability of protease enzyme was calculated over 5 hours. (**Fig. 17**: 60˚C and pH 9). The activity of immobilized enzyme was lower than free enzyme till 3 hours. Although the stability and activity of immobilized enzyme were improved at 4^{th} and 5^{th} hour. Both the EH & PA protease showed more or less same stability profile considering the immobilized protease stability but the activity profile is far more better for EH immobilized protease than the PA immobilized protease.

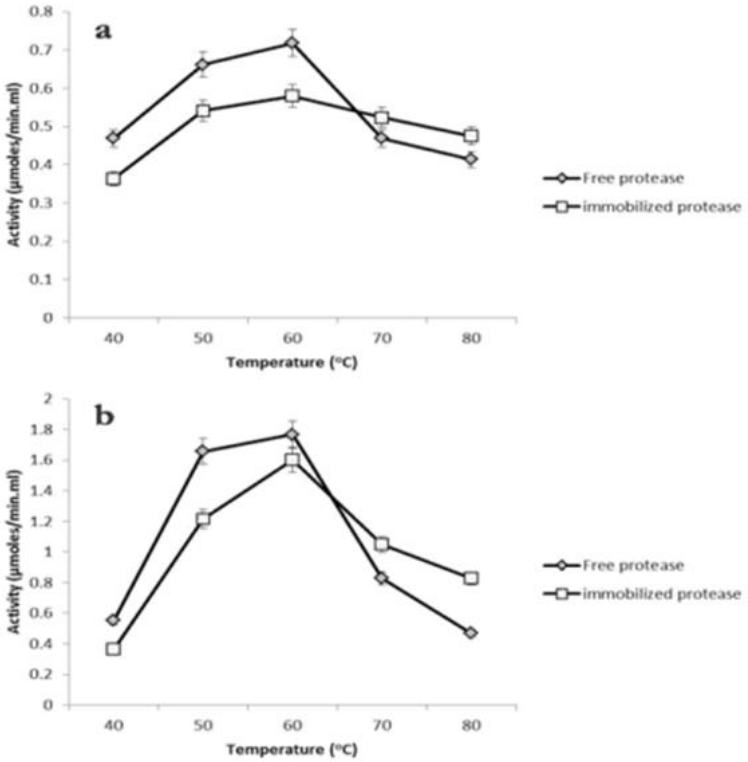

Fig.16. Comparison of activity of immobilized and free protease at various temperatures:
a) *Pseudomonas aeruginosa*-Protease. b) *Enterococcus hirae*-Protease

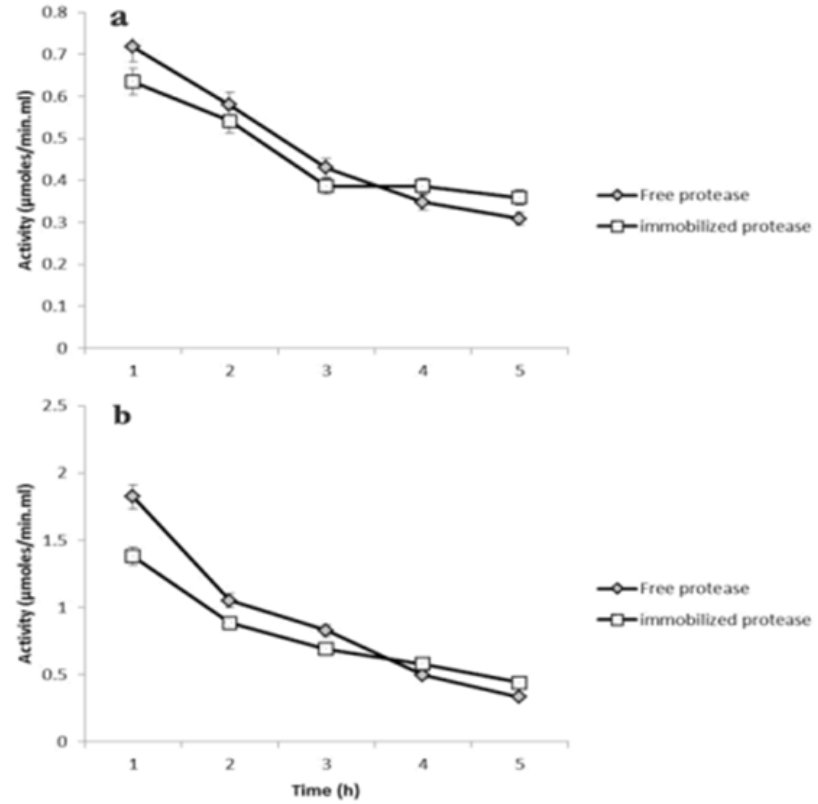

Fig.17. Comparison of stability of the immobilized and free protease: a) *Pseudomonas aeruginosa*-Protease. b) *Enterococcus hirae*-Protease

Effect of pH on enzyme activity

The effect of pH on the activity of the free and immobilized protease was examined **(Fig. 10)**. Protease activity of the immobilized enzyme was less than that of the free protease at the alkaline pH but immobilized enzyme showed a remarkable increasing activity at the acidic pH compared to the free enzyme. This change in character is mainly because of the immobilization of the magnetic nanoparticles which increases the stability of the protease enzyme at the acidic pH. Thus the activity of the immobilized protease was increased by 3 folds of the free protease for PA-protease (**Fig. 18(a)**) and by 2 folds of free protease for EH-protease (**Fig. 18(b)**) at pH 5(acidic).

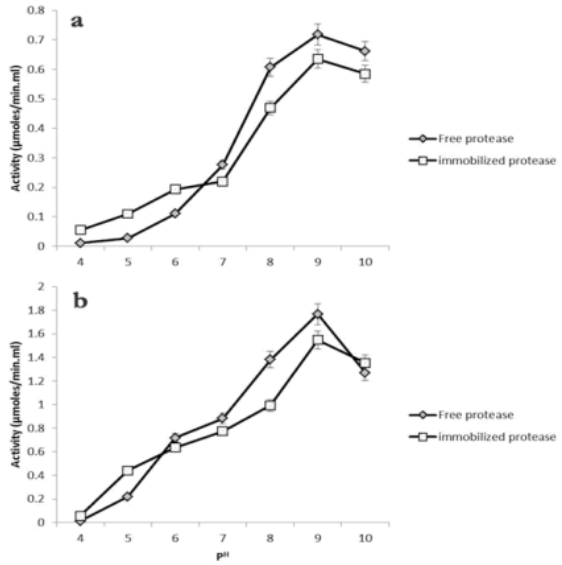

Fig. 18. Comparison of activity of the immobilized and free protease at various pH: a) *Pseudomonas aeruginosa*-Protease. b) *Enterococcus hirae*-Protease

CONCLUSIONS

The immobilization of protease with MNPs was confirmed by Transmission electron microscopy. The characterization of nanoparticles was determined by DLS particle size analysis, XRD and TEM methods. The average size of Magnetic nanoparticles and Silica Coated magnetic nanoparticles were 786.9 d.nm and 17-30 nm respectively. The activity of the enzyme was also confirmed by the Universal protease activity method. This study showed that the immobilization of protease enzyme via surface transformation on to super paramagnetic nanoparticles gives greater stability and activity, this was remarkably so at higher pH.

ACKNOWLEDGEMENTS

We heartily thank our chairman Col. Prof. Dr. Vel Sri Rangarajan, Vel Tech High Tech Engineering College, we also thank Mr. Mugesh Sankaranarayanan, Research scholar, Department of Chemical and Biomolecular Engineering, Pusan National University, for his continuous support and guidance. We thank our beloved parents for their endless support and encouragement.

REFERENCES

1. Joseph W. Krumpfer, Thomas Schuster, Markus Klapper, Klaus Müllen. Make it nano-Keep it nano. Nano Today. (2013) 8(4):417–438.
2. Georgia C. Papaefthymiou. Nanoparticle magnetism. Nano Today. (2009) 4(5):438–447.
3. S. Mahadevan, S.P. Behera1, G. Gnanaprakash, T. Jayakumar, J. Philip, B.P.C. Rao. Size distribution of magnetic iron oxide nanoparticles using Warren–Averbach XRD analysis. Journal of Physics and Chemistry of Solids (2012) 73(7):867–872
4. R. Raeisi Shahraki, M. Ebrahimi, S.A. Seyyed Ebrahimi, S.M. Masoudpanah. Structural characterization and magnetic properties of superparamagnetic zinc ferrite nanoparticles synthesized by the coprecipitation method. Journal of Magnetism and Magnetic Materials (2012) 324(22):3762–3765.
5. JitKang Lima, Sara A. Majetich. Composite magnetic–plasmonic nanoparticles for biomedicine: Manipulation and imaging. Nano Today (2013) 8(1):98–113.
6. Ito A, Shinkai M, Honda H, Kobayashi T. Medical application of functionalized magnetic nanoparticles. J. Biosci. Bioeng. (2005) 100:1–11.
7. K Niemirowicz, KH Markiewicz, AZ Wilczewska, H Car. Magnetic nanoparticles as new diagnostic tools in medicine. Advances in Medical Sciences (2012) 57(2):196–207
8. David Alcantara, Lee Josephson. Magnetic Nanoparticles for Application in Biomedical Sensing. Frontiers of Nanoscience (2012) 4:269–289
9. Ramnath Lakshmanana, Chuka Okolia, Magali Boutonnet, Sven Järås, Gunaratna K. Rajarao. Effect of magnetic iron oxide nanoparticles in surface water treatment: Trace minerals and microbes. Bioresource Technology(2013) 129:612–615
10. Xin Zhaoa, Lu Lva, Bingcai Pana, Weiming Zhanga, Shujuan Zhanga, Quanxing Zhanga. Polymer-supported nanocomposites for environmental application: A review. Chemical Engineering Journal (2011) 170(2–3):381–394.
11. Tratnyek PG, Johnson RL. Nanotechnologies for environmental cleanup. Nano Today. (2006) 1:44–48.
12. Lide Zhang, Ming Fang. Nanomaterials in pollution trace detection and environmental improvement. Nano Today. (2010) 5(2):128–142
13. Harris TJ, von Maltzahn G, Derfus AM, Ruoslahti E, Bhatia SN. Proteolytic actuation of nanoparticle selfassembly. Angew Chem. Int. Ed. Engl. (2006) 45:3161–3165.
14. Shakeel Ahmed Ansari, Qayyum Husain. Potential applications of enzymes immobilized on/in nano materials: A review. Biotechnology Advances. (2012) 30(3):512–523
15. Alcalde M, Ferrer M, Plou FJ, Ballesteros A. Environmental biocatalysis: from remediation with enzymes to novel green processes. Trends Biotechnol. (2006) 24:281–287.
16. B. Kalska-Szostkoa, M. Rogowskaa, A. Dubisa, K. Szymański. Enzymes immobilization on Fe_3O_4–gold nanoparticles. Applied Surface Science(2012) 258(7): 2783–2787.
17. Beg QK, Sahai V, Gupta R. Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. Process Biochem. (2003) 39(2):203-209.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein estimation with the Folin phenol reagent. J. Biol. Chem. (1951) 193:265-275.
19. R. Massart. IEEE Trans. Magn. Mag. (1981) 17:1247.

Received: February 03, 2016;
Accepted: July 14, 2016