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IMMOBILIZTION OF ORGANOPHOSPHORUS HYDROLASE ENZYME ON FERRIC MAGNETIC NANOPARTICLES AND INVESTIGATION OF IMMOBILIZED ENZYME STABILITY

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ABSTRACT

In the present study, organophosphorus hydrolase enzyme on Functionalized ferric magnetic nanoparticles was immobilized by the covalent binding method. The Optimized amount for parameters of mg $_{EDAC}$/mg $_{nanoparticles}$, enzyme units (U)/mg $_{nanoparticles}$, reaction time, and pH were determined to be 6.125, 0.1341, 3 h and 6.15 respectively. The amount of immobilization yield according to the enzyme activity was obtained to be 70% and also the amount of immobilized enzyme on nanoparticles was 0.25 U/mg $_{nanoparticles}$. Stability studies showed significant increase in immobilized enzyme stability at 4, 25 and 45°C. The stability of Immobilized enzyme showed a 6.3-fold increase in comparison to free enzyme at 4°C. The results demonstrated that the pH stability of the immobilized enzyme significantly increased in comparison with free enzyme. The immobilized enzyme was usable and recoverable for seven cycles. The results depicted that 80% of enzyme activity was retained after fifth cycle. FTIR test showed the covalent binding of enzyme to magnetic nanoparticles' surface and the modified enzyme magnetic nanoparticles property was superparamagnetic by vibrating sample magnetometer test.

Keywords: Magnetic nanoparticles, immobilization, organophosphorus hydrolase, stability

INTRODUCTION

Organophosphorus compounds with respect to their widespread use in agriculture, expose food resources, water and even air to the pollution. Organophosphate compounds such as insecticide parathion (*o*,*o*-diethyl-*o*-4-nitrophenyl phosphorothioate), methyl parathion and diazinon, in spite of their extreme toxicity have been used in production of plant pesticides. Compared with the potential disadvantage of conventional methods, bioremediation would appear to be more attractive because it is far less disruptive and more cost-effective (**Lei *et al.*, 2005**). Some reports have shown that organophosphorus compounds are degraded by some bacteria. *Pseudomonas diminuta* MG (**Serda *et al.*, 1985**) and a *Flavobacterium* sp. (ATCC 27551) (**Sethunathan *et al.*, 1973**) have the ability to degrade a broad spectrum of organophosphorus triesters by virtue of a constitutively expressed organophosphorus hydrolase (**Harper *et al.*, 1988**). Organophosphorus hydrolase enzyme called OPH (E.C 3.1.8.1) is able to hydrolyze the ester bond of organophosphate compounds such as parathion and paraoxon but due to the instability of this enzyme in free mode bioremediation process or its application in hydrolysis or materials detection has faced many problems. The enzyme activity is dependent on the environmental variables, so the OPH enzyme after 5 hours incubation at room temperature in the aquatic environment loses about 50% of its activity (**Obare *et al.*, 2010; Robatjazi *et al.*, 2010**). Biocatalysts immobilization processes are economically efficient and lead to development of the biological continuous processes. The unique physical properties of nanoparticles allow their application in many fields such as biomedicine (**Atanasijevic *et al.*, 2006: Gupta *et al.*, 2005; Ito *et al.*, 2005**), sensor development (**Katz et al., 2004**), water purification (**Savage *et al.*, 2005**) and environmental remediation (**Liu, 2006; Tratnyek *et al.*, 2006; Zhang, 2003**). 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 (**Gupta *et al.*, 2005; Ito *et al.*, 2005**). Using magnetic nanoparticles in biological processes and protein immobilization is considered one of the novel methods of immobilization. MNPs have found many applications in various processes due to their high specific surface area ratio, magnetic properties and special features (**Wang *et al.*, 2012**). The large surface- area-to-volume ratio of a nanoparticle allows it to serve as an efficient carrier of biomolecules. 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 (**Atanasijevic *et al.*, 2006: Gupta *et al.*, 2005; Ito *et al.*, 2005; Harris *et al.*, 2006**). MNP-enzyme conjugates (MNP-Es) represent a specific class of bio-NP conjugates that are of particular interest for biotechnological applications where high catalytic specificity, prolonged reaction time, and in some cases the ability to recycle an expensive biocatalyst is required (**Alcalde *et al.*, 2006; Swanson, 1999**). The covalent bonding and ionic bonding are two methods of MNP binding to biomolecules. Covalent binding is a method that is widely used in the biopolymers immobilization. Covalent binding is usually carried out by direct biomolecule reactive groups binding or by binding to an intermediate. The purpose of this study is to immobilize OPH enzyme on MNP covalently and immobilization optimization and evaluation of storage stability of immobilized OPH enzyme.

MATERIAL AND METHODS

Bacterial strain

Flavobacterium ATCC 27551 was obtained from Microbial Type Culture Collection (MTCC, Chandigarh, India). OPH enzyme was then isolated and purified from bacteria **(Brown, 1980).**

Magnetic nanoparticles and chemicals

Utilized magnetic nanoparticles with dextran surface cover and NH_2 agent with average size of 130nm (amino-modified magnetic nanoparticles (AMN)), *N*-(3-dimethylaminopropyl)- *N*–ethyl carbodiimide (EDAC) and 2-N-morpholino-ethanesulfonic acid (MES) buffer and parathion were purchased from Micromode, Merck and Sigma Companies respectively. All of the experimental materials in this study were of high purity and were purchased from Fluka and Merck Companies.

Immobilization of OPH enzyme on AMNs

In order to Immobilize OPH enzyme on AMNs surfaces, 20 µl of AMNs was transferred to a 2ml microtube. Ferric nanoparticles were then washed three times

with MES buffer solution and suspended in100 µl of MES buffer solution. After shaking for one minute by putting permanent magnet under the micro tube for 1-2 minute(s) AMNs were separated from the solution. Then, a 100 µl MES solution containing 0.7mg EDAC was added to the nanoparticles that had been suspended in the 100 µl MES solution and mixed with shaker for 10 minutes. A 10 µl stock enzyme solution with 2.2 U/ml activity was added to microtube containing active nanoparticles, then the microtube was incubated for 3 h at 4°C with 250 rpm. After the end of incubation, nanoparticles were washed three times with a 300 µl PBS buffer solution and suspended with a 300 µl PBS buffer solution in pH=8.

Optimization process

The optimum conditions for the covalent binding of OPH enzyme to AMNs surface were obtained using Taguchi experiment design method and Qualitek-4 software. This method allows observation and optimization of variables simultaneously. In the present study, a standard Taguchi L9 orthogonal array due to its ability to operate 4 parameters in 3 levels was chosen. Parameters and their levels for the covalent binding of OPH enzyme to AMNs are shown in Tab 1.

Table 1 Factors and level of parameters used in Taguchi experimental design.

Factor	Level 1	Level 2	Level 3
EDAC/AMN ratio(w/w)	2.45	6.126	15.313
Enzyme/AMN ratio(U/mg)	0.0894	0.1341	0.1788
Time (h)	1.5	3	4.5
pH	6	6.15	6.30

Evaluation of Immobilized enzyme stability

Free and immobilized enzyme stability was determined by using relative activity calculation. The immobilized enzyme and the samples containing free enzyme as the observer were incubated at the same condition of 4, 25, 45°C and enzyme activity in different periods was determined intermittently. The acidity stability of both immobilized enzyme and free enzyme was determined at the same condition at pH range of 4-11. The samples were incubated at 4°C for 3 h. In order to determine activity of samples, first samples were incubated at 25°C and pH=8 for 30 minutes then their activities were determined.

Analytical methods

The phosphotriesterase activity was measured on the basis of Spectrophotometric Assay method. The rate of ethyl-parathion hydrolysis was determined by measuring para-nitrophenol production (**Robatjazi *et al.*, 2010**). For this purpose, 150 µl of solution containing immobilized enzyme and 150 µl of PBS buffer at pH=8 were mixed. Then 10 µl of 50 mM $CoCl_2$ solution was added and the mixture was shaken for 30 minutes at room temperature. Next, 5 µl of 40 mM ethyl-parathion was added and p-nitrophenol production rate was determined by measuring the increase of absorbance rate at 410 nm. One unit of phosphotriesterase activity (U) was defined as the amount of enzyme required to hydrolyze 1 µmol of ethyl-parathion per minute at 30°C (as described previously in detail by **Robatjazi *et al.* (2010)**).

VSM and FTIR

Immobilized and free enzyme Sample spectrum was determined using FTIR spectroscopy. OPH enzyme was immobilized on AMNs by the determined optimized condition. Then, modified magnetic nanoparticles (enzyme-AMNs) and AMNs were freeze-dried for 24 hours at -60°C (model Alpha 2-4 LSC; Martin Christ, Osterode, Germany). The Samples spectrums were measured from 450 cm^{-1} to 4000 cm^{-1}. In order to perform the vibrating sample magnetometer (VSM) test, modified AMNs were washed twice with distilled water and then dried using freeze-drier on aluminum plates for 48 hours. VSM test measurement was carried out on magnetometer device (VSM, Kashan University, Iran) by changing the magnetic field from +8000 to -8000.

RESULTS AND DISCUSSION

Optimization experiment results are shown in Tab 2 on the basis of enzyme specific activity in mg of nanoparticles. Each parameter's effect and its level effect is shown in figure 1, according to the obtained results it can be deduced that pH parameters, enzyme(U)/mg_{AMN} ratio, mg_{EDAC}/mg_{AMN} and incubation time for enzyme covalent binding on the surface of AMNs were levels of 2, 2, 2, 2 respectively.

Results showed that 3 hours incubation time had the most positive effect on enzyme-AMN reaction. The enzyme to magnetic nanoparticle ratio and mg_{EDAC}/mg_{AMN} and pH for enzyme covalent binding on AMNs surface had 15, 45 and 65 percent effectiveness on immobilization. Immobilization process was carried out in optimized condition. Optimized condition for covalent binding by amino groups for incubation time, U/mg $_{MNP}$ ratio, mg $_{EDAC}$/mg $_{MNP}$ ratio, and pH, was determined 3 h, 0.1341, 6.125 U/mg, and 6.5 respectively.

Table 2 The results of immobilized enzyme activity in L_9 orthogonal array of the Taguchi experimental design.

Trial	Factors	Results
1	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=2.45,U_{enzyme}/mg_{AMN} ratio(U/w)=0.0894	0.038
2	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=2.45, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1341	0.076
3	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=2.45, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1788	0.032
4	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=6.126, U_{enzyme}/mg_{AMN} ratio(U/w)=0.0894	0.061
5	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=6.126, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1341	0.043
6	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=6.126, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1788	0.089
7	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=15.313, U_{enzyme}/mg_{AMN} ratio(U/w)=0.0894	0.027
8	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=15.313, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1341	0.078
9	mg $_{EDAC}$/mg_{AMN} ratio(w/w)=15.313, U_{enzyme}/mg_{AMN} ratio(U/w)=0.1788	0.048

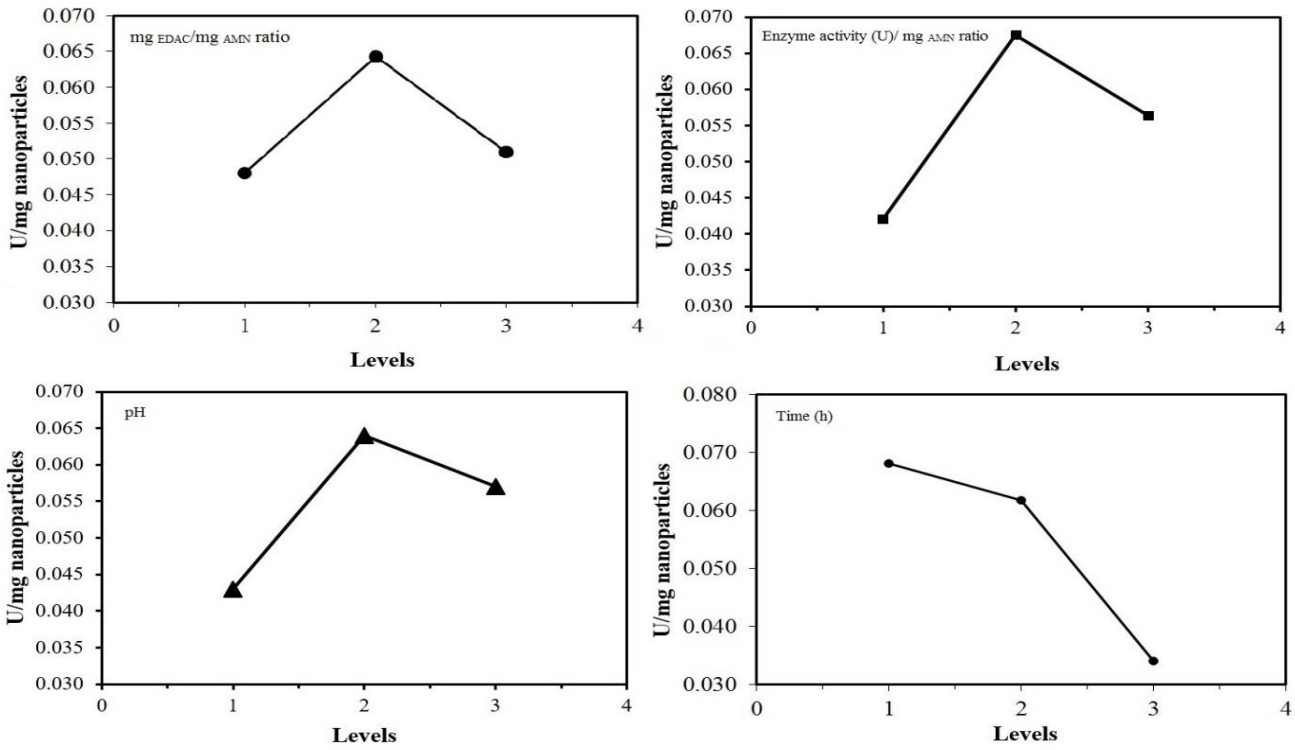

Figure 1 Average response levels for different parameters

Experiments were performed several times using the optimized conditions, and the results were in good agreement with those predicted from Taguchi experiment design. Results showed in optimized condition that amount of immobilized enzyme on nanoparticles was 0.25 U/$mg_{nanoparticles}$ with about 70% efficiency. The obtained results were in good agreement with studies performed by **Jiang *et al*. (2008)** . In a similar study by **Kuo *et al.* (2012)** immobilization efficiency was reported 58.3% for Lipase enzyme immobilization, Highest obtained activity was reported 20 unit per gram and in similar studies by **Sahoo *et al.* (2011)** , urease enzyme immobilization was reported 57% on ferric magnetic nanoparticles.

Figure 2 shows the pH stability of immobilized enzyme. The pH stability increased for the immobilized enzyme compared with the free enzyme. As the pH shifted towards the alkaline or acidic conditions, the enzyme activity of the immobilized enzyme remained higher compared with the free enzyme. This may be due to the protection of the enzyme by MNPs against extreme pH values. The remaining enzyme activity of the immobilized enzyme and the free enzyme after 8 h of incubation at pH 4 were 78% and 20% respectively.

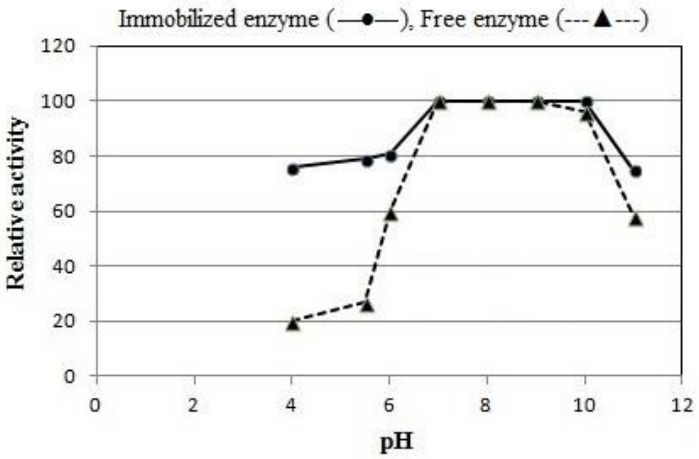

Figure 2 pH stability of immobilized enzyme

Stability studies showed significant increase in immobilized enzyme stability at 4, 25 and 45°C. The results are shown in the figure 3.The results show after 120 h of incubation at 4 °C that the relative activity has been 91% and 43% for the immobilized enzyme and free enzyme, respectively (figure 3-a). Immobilized enzyme stability was determined 6.3-fold increase in comparison to free enzyme at 4°C. A 50% reduction in the initial enzyme activity of the immobilized enzyme determined after 135 and 62 h of incubation at 25 and 45 °C, respectively, (figure 3-b, c). The results demonstrated that coupling of OPH to AMNs enhanced the enzyme thermal stability at 4, 25 and 45 °C. The Immobilized enzyme saved 87.7% of its activity for more than 10 days at 4 °C.

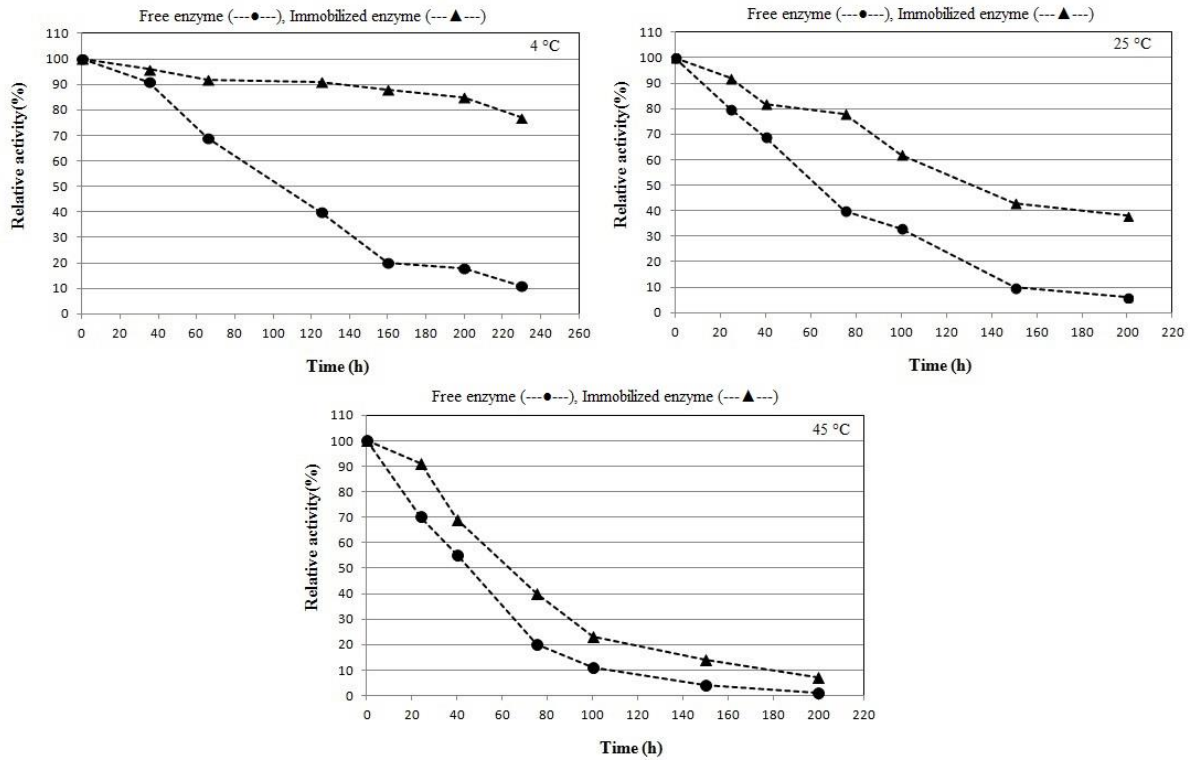

Figure 3 Enzyme thermal stability of immobilized enzyme

The immobilized enzyme was usable and recoverable for about seven cycles. The activity of immobilized enzyme was assayed up to seven cycles. The investigation depicted that 96% enzyme activity was retained after third cycle and 80% after fifth cycle. According these results, the relative enzyme activity was decreased 23% after seven cycles (figure 4).

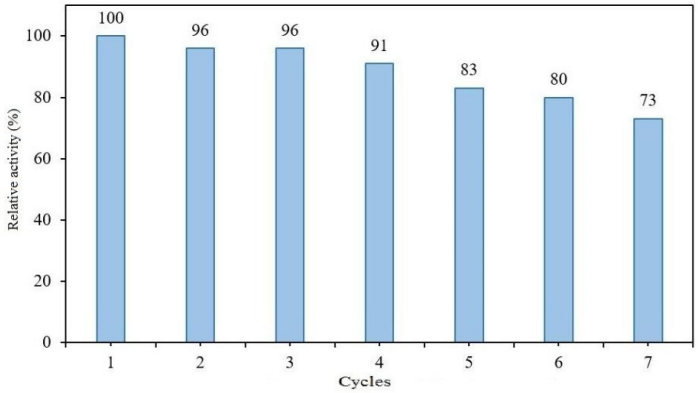

Figure 4 Relative activity of immobilized enzyme after each cycle of use

FTIR test results confirmed the covalent bonding between enzyme and magnetic nanoparticles agent groups (figure 5). On this basis, bonds are visible at 571 cm^{-1} and 1635 cm^{-1} which proves the enzyme bonding to nanoparticles such as reported by **Teste *et al.* (2010)** previously. VSM test results showed that the enzyme coated magnetic nanoparticles retain their superparamagnetic nature during the immobilization process and only about 10% of the magnetic property is decreased (figure 6).

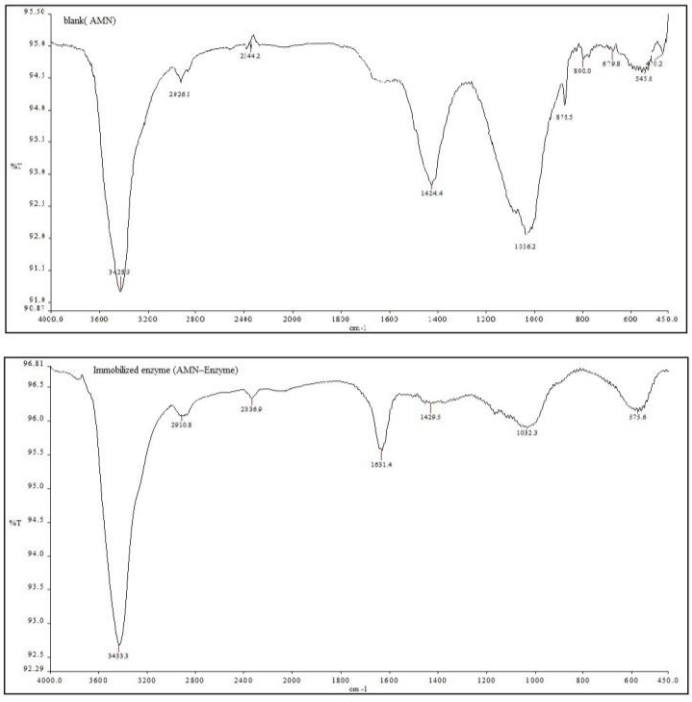

Figure 5 FTIR spectroscopy spectrum of Immobilized enzyme, a: blank (AMN), b: Immobilized enzyme (AMN–Enzyme)

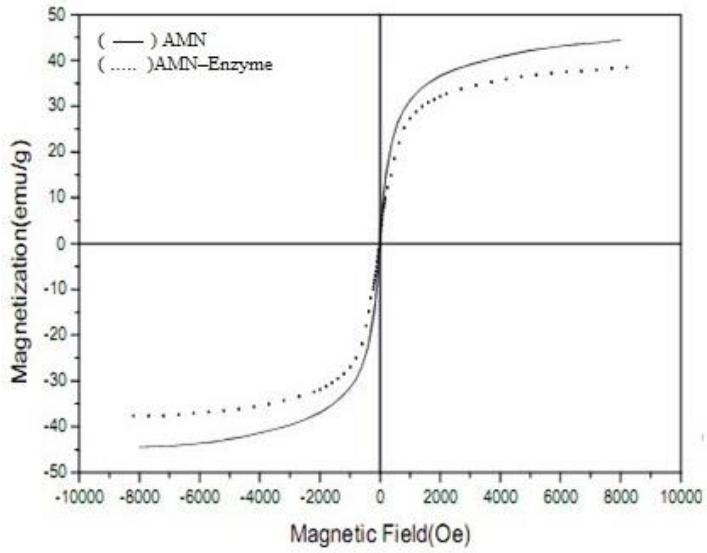

Figure 6 Magnetization curve of AMN–Enzyme

CONCLUSION

In the present study, OPH have been linked to the magnetic nanoparticles's surface by the covalent coupling method. Best conditions were determined for enzyme binding to the magnetic nanoparticle's surface. The thermal stability of immobilized enzyme on the magnetic nanoparticle's surface showed 6.3-fold, 1.4-fold and 1.1-fold increase in comparison to free enzyme after 120 h of incubation at 4, 25 and 45°C respectively. Immobilized enzyme on magnetic nanoparticle's surface can simply be separated with a magnet which leads to reuse and sequential use of enzymes. The enzyme-magnetic nanoparticles can be utilized in applications such as biodegradation, biosensing and they are also appropriate for development of nanoscale smart technologies.

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