(Supporting Information)

Heterogeneous biocatalysis by magnetic nanoparticle immobilized biomass-degrading enzymes derived from microbial cultures

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Section S-1: Materials and Instrumentation

Materials

Potassium hydrogen phosphate, Potassium dihydrogen phosphate, Nickel chloride, Iron precursors, methanol, ethanol, Ammonia solution, Hydrazine, and Potassium hydroxide were purchased from Merck™Life Science Pvt Ltd, Bengaluru, Karnataka. The protein molecular weight maker was purchased from ThermoFisher®Scientific, Mumbai, India. The pBAD bacterial expression plasmid was obtained from ThermoFisher Scientific, Waltham, USA. *Escherichiacoli* Top 10F′ cells were purchased from Life Technologies™, La Jolla, CA, USA. A total of 30 kDa cutoff size Amicon-Ultra-15 membranes were obtained from EMD Millipore, Billerica, USA. Coomassie brilliant blue R-250 from Sigma Aldrich USA.

General instrumentations and methods

Fourier transform infrared (FTIR): Spectra have been collected using a Bruker® Optics ALPHAE spectrometer with a universal Zn-Se ATR (attenuated total reflection) accessory. FTIR data are reported with a wavenumber (cm^{-1}) scale.

Scanning Electron Microscopy (SEM): Measurementswere made on a Zeiss SUPRA 55 VP scanning electron microscope and Zeiss SIGMA Scanning Electron Microscope equipped with tungsten filament as an electron source operated at 10 kV. The samples are prepared by putting a drop of nanoparticles (NPs) dispersed in methanol on a clean piece of Silicon wafer.

Transmission Electron Microscopy (TEM):TEManalyses have been performed using a UHR-FEGTEM, DST-FIST facility of IISER Kolkata at an accelerating voltage of 200 kV. The TEM samples were prepared for analysis by drop-casting the NPsamples on copper grids TEM Window (TED PELLA, Inc. 200 mesh).

Vibrating-sample magnetometer (VSM): VSM have been performed using PPMS®DynaCool®by Quantum Design (CA, USA).

ICP-OES: ICP-OES was performed using a PerkinElmer Optima 7000 DV instrument.

Dynamic light scattering (DLS): The size distribution and ζ-potential was measured with a Malvern Zetasizer® Nano ZS system. Keeping the instrument parameter configuration for solvent system water at 25°C.

Ultraviolet-visible spectroscopy (UV-vis):Data were measured using an Agilent 8453 UV-vis spectrophotometer (Agilent, Mumbai, India) at room temperature.

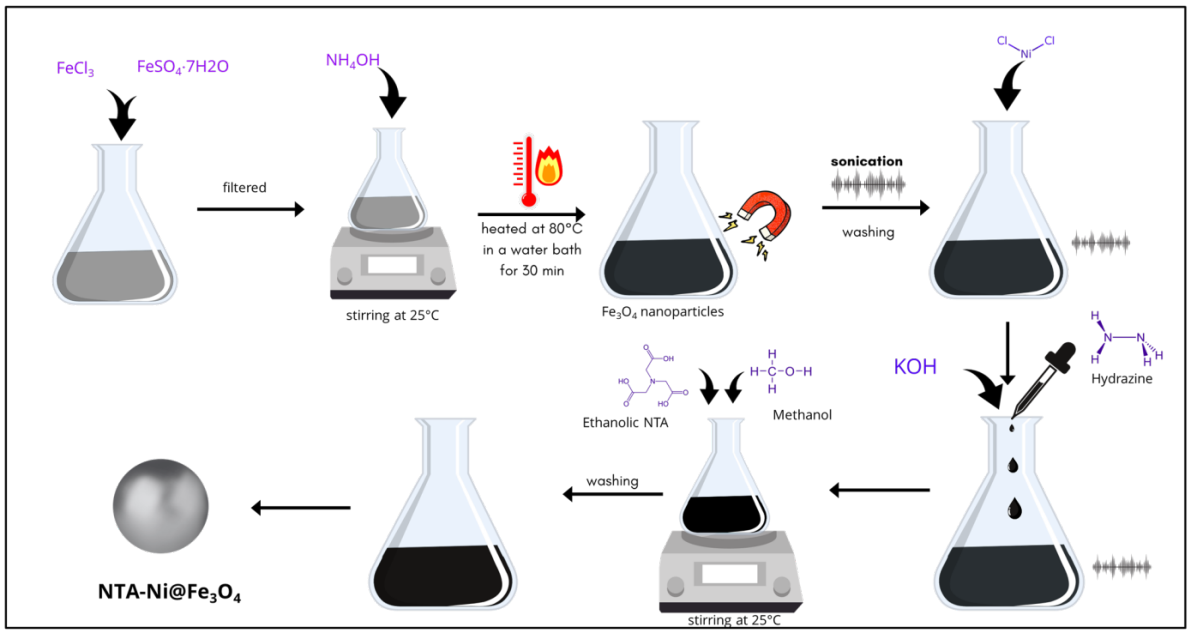
Epifluorescence microscope: Images were collected using a Nikon®ECLIPSET*i*2 epifluorescence microscope with a Nikon® DS-Qi2 coupled camera.

Plate reader: SpectraMax M2 (Molecular Devices, San Jose, USA) was used for the absorbance measurements for the assays at room temperature.

SDS-PAGE:SDS-PAGE was done according to the standard Laemmli (1970) procedure in 10% polyacrylamide gels. PageRuler™ Plus Prestained SDS-PAGE (ThermoFisher®, Mumbai, India) was used as the molecular weight marker. For protein visualization, gels were stained with Coomassie brilliant blue.

Section S-2: Synthetic Procedures and Methods

Preparation of NPs: Synthesis of Ni-incorporatedFe_3O_4 NPs and subsequent modification of the NPs with NTA was done by the following method. Core Fe_3O_4NPs were prepared using the method described elsewhere[1] with a few modifications for further Ni/NTA coating. Chemical co-precipitation of Fe^{2+} and Fe^{3+} ions in alkaline conditions was used for the synthesis of the magnetic NP. The obtained NP, after hydrothermal treatment, was thoroughly washed with nano-pure water to remove the excess un-reacted components; particularly the removal of excess ammonia was monitored by checking the pH of the supernatant. A neutral pH is preferred over the basic one. After this vigilant washing step with water, the NPs are washed with dry methanol several times to remove water. The ensuing methanol dispersed NP was estimated for concentration and kept under ambient conditions for further use. For the surface modification with Ni, a 100 mL (0.05 mg/mL) aliquot of methanolic solution of iron oxide NP was taken and kept under sonication at10500 J for 1 h. After 1 h filtered 50 mL (0.05 M) $NiCl_2$ solution prepared in 1:99 water/methanol was added in it and the sonication continued for another 30 min. Further, under sonication 10 mL of methanolic (0.5 M) KOH solution and 10 mL of 80% hydrazine were added in a dropwise manner. After the addition of KOH/hydrazine, the mixture was removed from sonication and kept under stirring at 1500 rpm for 1 h. Upon completion, the solution of the NP was washed with water to remove all unreacted chemicals and monitored for the pH to reach neutral. Properly washed NPs were re-dispersed in 100 mL dry methanol and kept under stirring at 1500 rpm, in this solution 10 mL (0.05 M) ethanolic solution of NTA was added, and the stirring was continued for a further 1 h. The final product was thoroughly washed with 1:1 water/ethanol then re-suspended in methanol until further use.



Scheme S1. Schematic representation of the synthesis procedure of NTA-Ni@Fe_3O_4 NPs.

Section S-3: NPs Characterizations

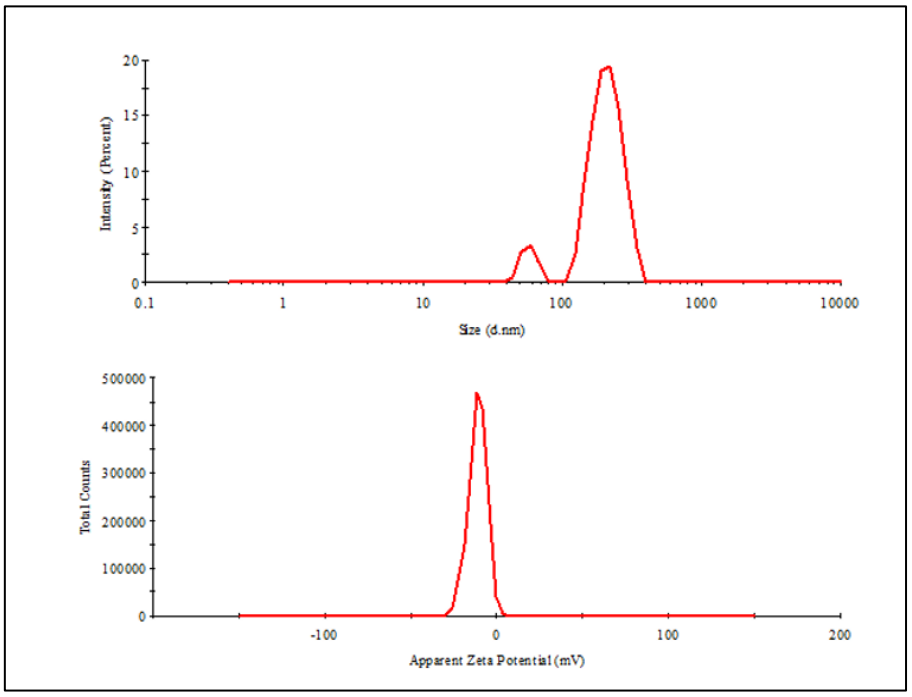


Figure S1. The upper panel shows the size distribution of NTA-Ni@Fe_3O_4 NPs measured by dynamic light scattering, representing a higher hydrodynamic radius due to the surface charge (presented in the lower panel) of the NP and the presence of NTA on the surface.

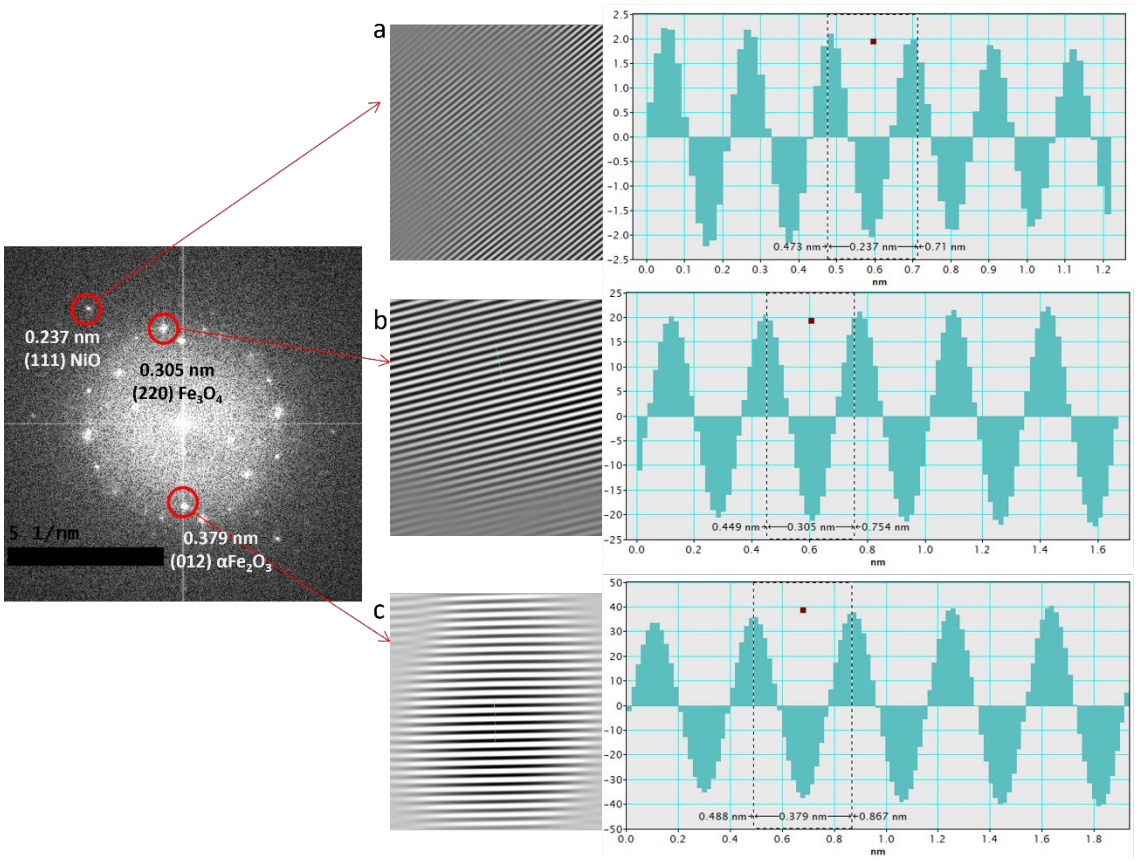


Figure S2. Localized FFT of the nanoparticle and corresponding lattice plane are shown here. a. Represents (111) plane of NiO, b. (220) of Fe_3O_4 and (012) of α- Fe_2O_3 represented in c.

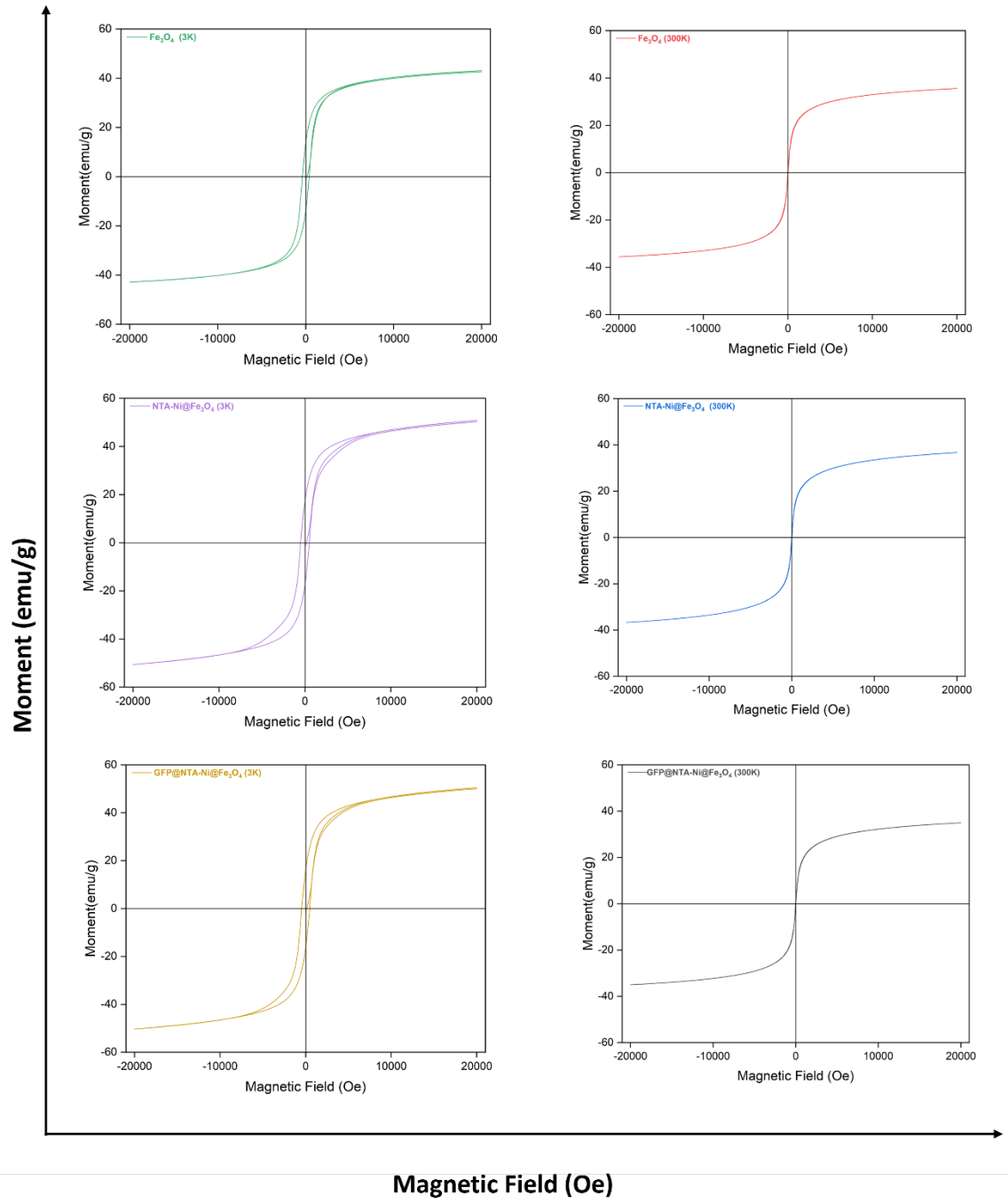


Figure S3. A representation of the magnetic moment vs. magnetic field curve is shown for each synthesized nanoparticle.

Table S1. Key magnetic parameters of the synthesized nanoparticles

	Saturation magnetization (emu/g)	Remnant magnetization (emu/g)	Coercivity (kOe)
Fe_3O_4@3K	38.5	13.3	0.41
Fe_3O_4 @300K	25.02	0.6	0.01
NTA-Ni@Fe_3O_4 NPs @3K	44.8	19.3	0.49
NTA-Ni@Fe_3O_4 NPs @300K	24.5	0.6	0.01
GFP@NTA-Ni@Fe_3O_4 NPs @3K	44.86	6.6	0.487
GFP@NTA-Ni@Fe_3O_4 NPs @300K	24.23	0.53	0.025

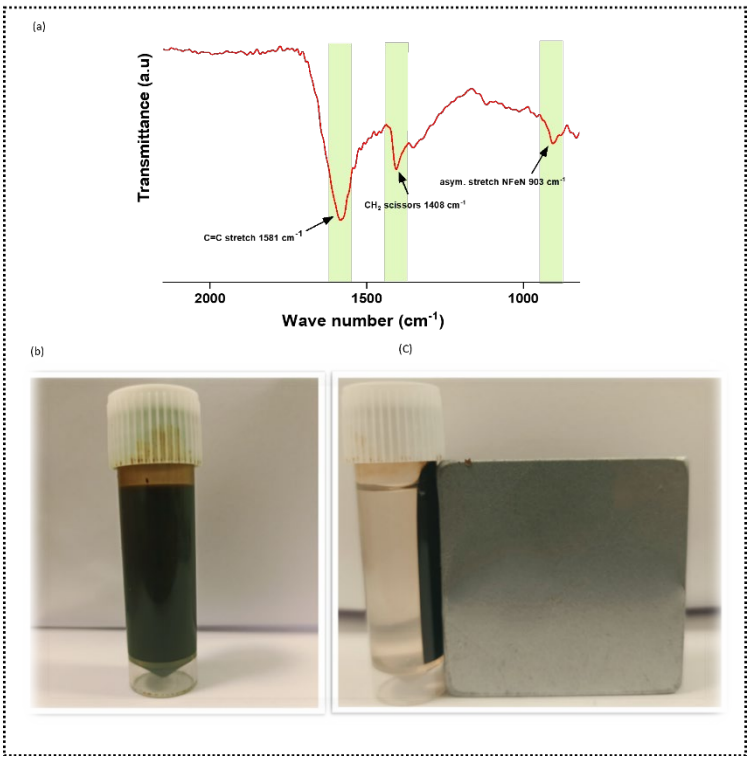


Figure S4. (a) FTIR spectrum of synthesized NTA-Ni@Fe_3O_4NPs manifests the signature of the presence of oxidized Ni and Fe along with NTA, (b) magnetic separation of NTA-Ni@Fe_3O_4 NPs, (c) A good dispersity and easy separation of the NPs was observed upon application of an external magnetic field.

Section S-4: PXRD patterns

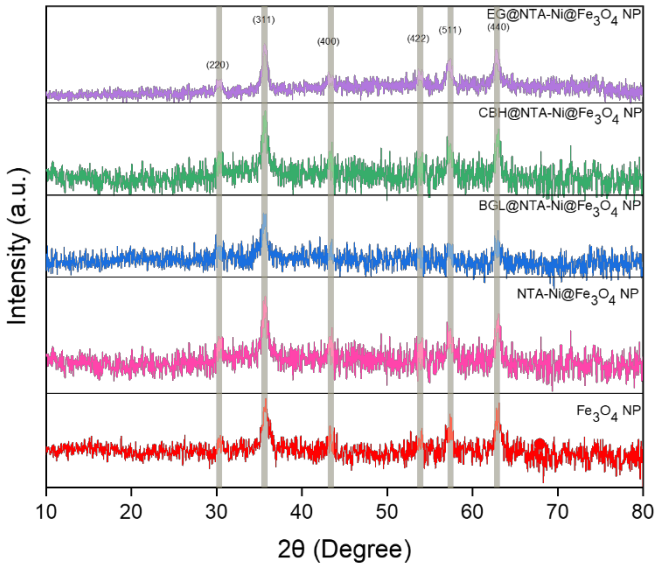


Figure S5. PXRD patterns of Fe_3O_4 NPs, NTA-Ni@Fe_3O_4 NPs, and EG, CBH, BGL immobilized NTA-Ni@Fe_3O_4 NPs.

Section S-5: TGA

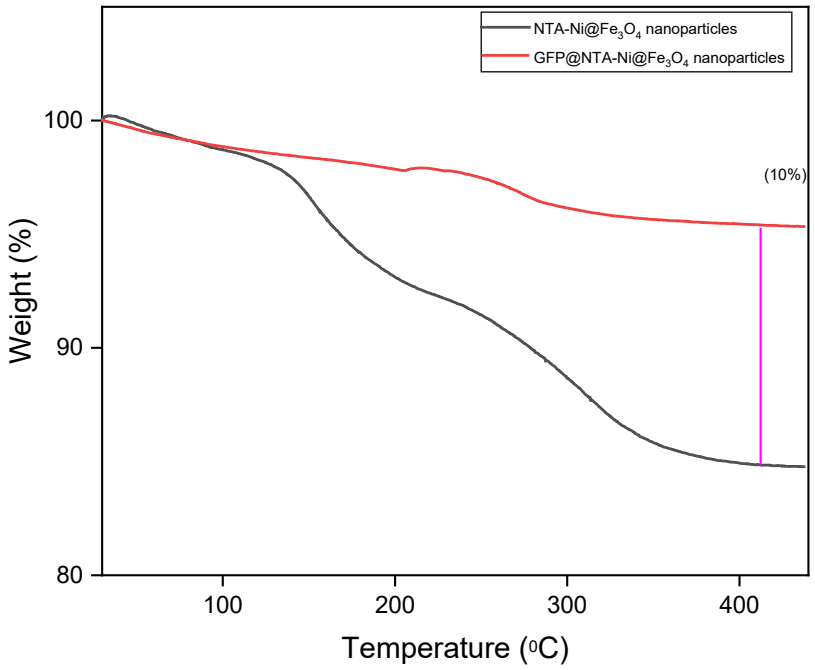


Figure S6. TGA analysis of NTA-Ni@Fe_3O_4 NPs and GFP@NTA-Ni@Fe_3O_4 NPs.

Section S-6: Fluorescence microscopy

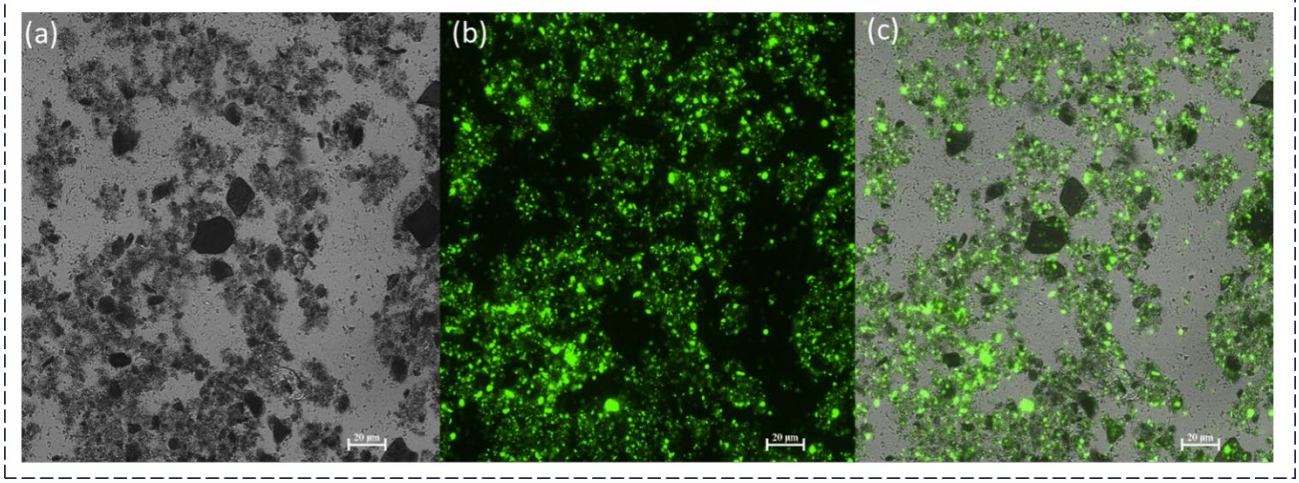


Figure S7. Epi-fluorescence microscopy images (a) NTA-Ni@Fe_3O_4NPs (b) GFP (c) overlay of (a) and (b).

Section S-7: SEM

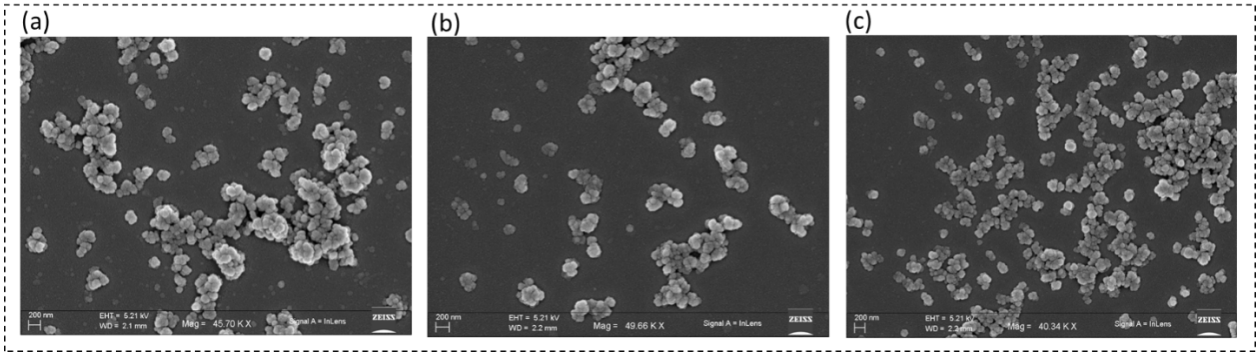


Figure S8. SEM images of NTA-Ni@Fe_3O_4NPs after (a) BGL, (b) CBH, (c) EG immobilization.

Section S-8: TEM

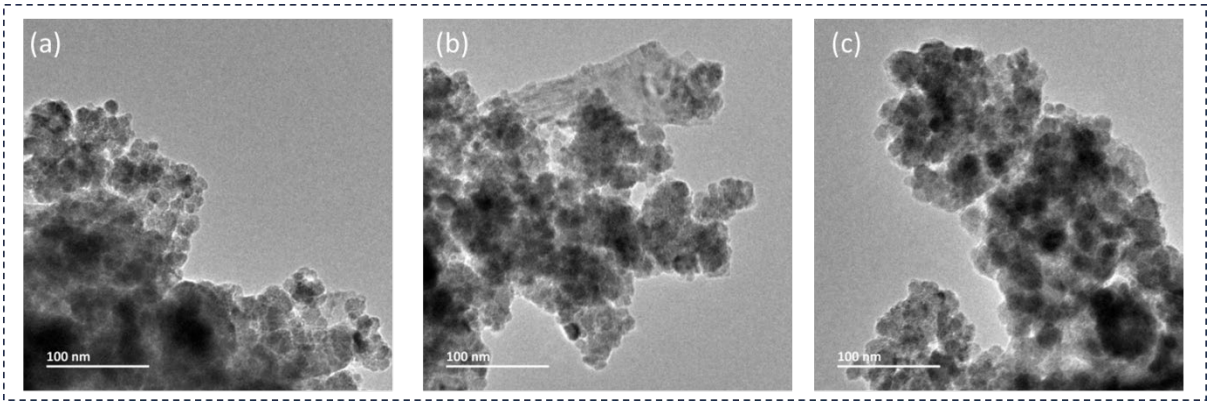


Figure S9. TEM images of NTA-Ni@Fe_3O_4 NPs after (a) BGL, (b) CBH, and (c) EG immobilization.

Section S-9: FTIR

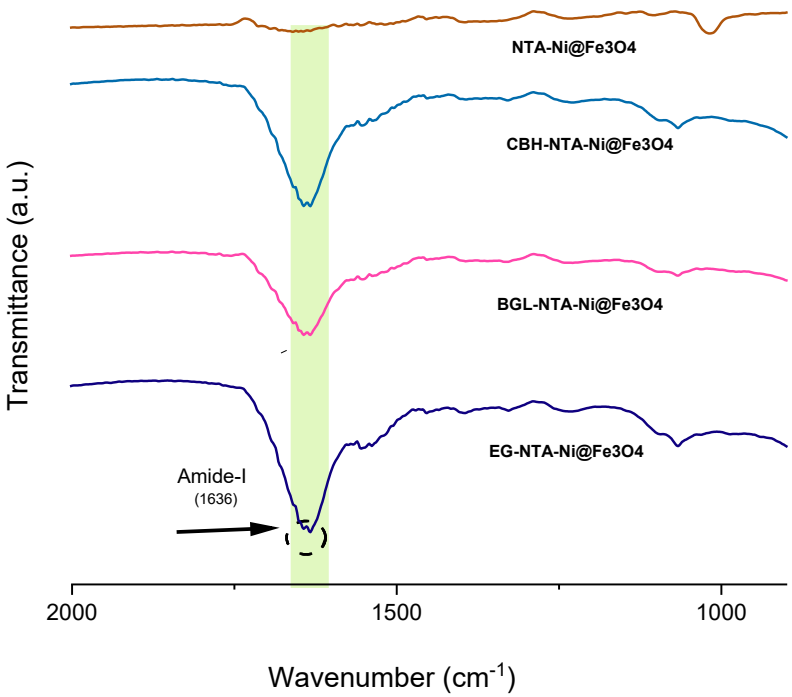


Figure S10. FTIR spectra of NTA-Ni@Fe_3O_4 NPs, CBH@ NTA-Ni@Fe_3O_4 NPs, BGL@ NTA-Ni@Fe_3O_4 NPs and EG@NTA-Ni@Fe_3O_4 NPs.

Section S-10: Enzyme expression, secretion and purification

Expression, Lysate preparation, and Purification of proteins:

The β-glucosidase (BGL), Cellobiohydrolase (CBH), and Endogluconase (EG) genes were amplified, and a C-terminal 6-His tag was incorporated. All genes were cloned into the pTU-1A expression vector and transformed into the Top10F′ *Escherichiacoli* bacterial strain. A Qiagen Miniprep kit was used to isolate the plasmid, and the gene sequence was confirmed at the IISER Kolkata sequencing facility. Later, the plasmid was transformed into the BL21 bacterial strain of *Escherichia coli* (DE3).

All clones' initial cultures were cultured in LB media containing ampicillin (100 μg mL^{-1}). overnight at 37 °C with constant shaking (200 rpm). The overnight grown saturated solution was used to inoculate a 400 mL secondary culture by diluting in a ratio of 1: 100. Protein synthesis was allowed for 6 hours at 37 °C after adding 0.5 mM IPTG to the culture to induce protein expression at an OD_{600} of 0.5-0.6. The cells were pelleted by centrifugation at 8000×g for 10 minutes at 4 °C and then kept at 20 °C until the protein was purified.

For protein purification, the cell pellet was thawed and resuspended in lysis buffer (10 mM potassium phosphate buffer, 10 mM imidazole, 500 mM NaCl, 1 mM PMSF, and 1.2 mg mL^{-1} lysozyme) at pH 7.4 and sonicated at 70 % amplitude, 5 cycles of 1 min each with a 1 min interval between two consecutive cycles. Following a protocol previously reported[2], the cell lysate was centrifuged at 13,400 rpm for 15 minutes at 4 °C, and the clear supernatant was used as cell lysate. Binding buffer washed 5 mg NPs, mixed with cell lysate at 4°C at 1000 rpm for 1 h in shaker. NTA-Ni@Fe_3O_4 NPs were separated from the solution of NPs and lysate through a magnet, and the supernatant was decanted. The NTA-Ni@Fe_3O_4 NPs were washed three times with a binding buffer. The desired His-tagged protein was eluted by adding elution buffer (10 mM phosphate buffer, 500 mM imidazole, 500 mM NaCl, pH 7.4) in the tube, and NTA-Ni@Fe3O4NPs were separated through the magnet. The eluted protein was buffer exchanged in their respective storage buffer, and the purity was checked by SDS-PAGE gel electrophoresis. Protein concentration was determined by measuring the absorbance at 280 nm and using the extinction coefficient (ε_{280} = 107 260 M^{-1} cm^{-1}) as per the modified Edelhoch and Gill/Von Hippel method available on the Expasy ProtParam website by Swiss Institute of Bioinformatics[3].

Secretion and Purification: ansB tag was used for secretion of enzyme into media. BGL, CBH, and EG encoding genes were amplified, and a C-terminal 6-His tag and N- N-terminal ansB tag were incorporated. All of the genes were cloned into the pTU-1A expression vector and transformed into the Top10F′ *Escherichia coli* bacterial strain. A Qiagen Miniprep kit was used to isolate the plasmid, and the gene sequence was confirmed at the IISER Kolkata sequencing facility. Later, the plasmid was transformed into the BL21 bacterial strain of *Escherichia coli* (DE3). All clones' initial cultures were cultured in LB media containing ampicillin (100 μg mL^{-1}). overnight at 37 °C with constant shaking (200 rpm). The overnight grown saturated solution was used to inoculate a 400 mL secondary culture by diluting in a ratio of 1 : 100. Protein synthesis was allowed for 6 hours at 37 °C after adding 0.5 mM IPTG to the culture to induce protein expression at an OD_{600} of 0.5–0.6. The cells were pelleted by

centrifugation at 8000g for 10 minutes at 4 °C, and supernatant media was used for enzyme purification. 40 mM imidazole and 500 mM NaCl were added to supernatant media, and pH was adjusted to 7.4 for proper binding of desired His-tagged protein to NTA-Ni@Fe_3O_4 NPs. 5mg of the washed NPs and supernatant media was mixed for 1 h, and NTA-Ni@Fe_3O_4 NPs were separated through a magnet. Separated NTA-Ni@Fe_3O_4NPs were washed three times with binding buffer (10 mM phosphate buffer, 40 mM imidazole, 500 mM NaCl, pH 7.4). The desired His-tagged protein was eluted by adding elution buffer (10 mM phosphate buffer, 500 mM imidazole, 500 mM NaCl, pH 7.4) in the tube, and NTA-Ni@Fe_3O_4 NPs were separated through the magnet. The eluted protein was buffer exchanged in their respective storage buffer, and the purity was checked by SDS-PAGE gel electrophoresis.

Section S-11: Protein immobilization & Determination of the loading capacity

Synthesis of GFP-loaded NTA-Ni@Fe_3O_4 NPs: 5 mg of the oven-dried NTA-Ni@Fe3O4NPs was taken in a reaction tube. 250 µL of freshly purified GFP (4 mg/mL) was poured into the reaction tube. The reaction mixture was then incubated at 4°C for 2 h. After incubation, the NTA-Ni@Fe_3O_4NPs were separated from the mixture by a magnet. 20 µL of the supernatant solution was diluted five times, and UV-vis absorbance was measured at 480 nm.

GFP Adsorption Isotherm NTA-Ni@Fe_3O_4 NPs: For binding experiments, the hexa-histidine tagged GFP were incubated with 1mg Ni-NTA@Fe_3O_4 nanoparticles at varying masses of GFP in binding buffer at 4 ºC under shaking conditions (1000 rpm) for 2 hours. After incubation, the NTA-Ni@Fe_3O_4 NPs were separated from the mixture by a magnet. 20 µL of the supernatant solution was diluted five times, and UV-vis absorbance was measured at 480 nm. The adsorbed amount of protein with NPs were calculated by using the following equation:

$$q_t = (C_0 – C_t) \times V \quad \ldots\ldots(1)$$

where q_t (µg) is the total adsorbed GFP in the NTA-Ni@Fe_3O_4 NPs, C_0 is the initial concentration of GFP solution, C_t is the final concentration of GFP solution, and V is the volume of the loading buffer (mL). We cloned GFP with a His tag and a thrombin cleavage site to allow for the removal of the His tag after purification. Both His-tagged GFP and tag-free GFP were used as controls. We immobilized both variants of GFP on NTA-Ni@Fe_3O_4 nanoparticles, washed them, and measured UV absorbance to quantify binding efficiency. The results showed that only about 10 % of the tag-free GFP was immobilized on NTA-Ni@Fe_3O_4 nanoparticles, compared to the His-tagged GFP. This confirms the specificity of the NTA-Ni@Fe_3O_4 system for binding histidine-tagged proteins.

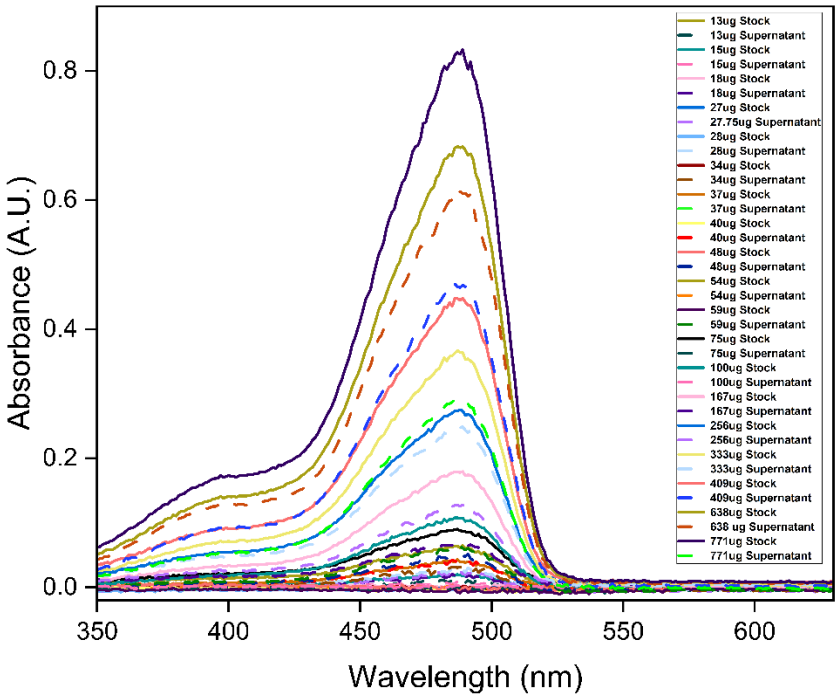


Figure S11 UV-vis absorption spectra of the stock solutions and supernatants after the loading of GFP with increasing stock quantity at NTA-Ni@Fe_3O_4 nanoparticles.

The adsorption of His_6-GFP on the nanoparticles fitted with Langmuir isotherm equation;

$$q = \frac{q_{max}KC}{1+KC} \text{.....(2)}$$

The q_{max} obtained from the fitting is 124.36 µg of protein per mg of nanoparticles and the Langmuir constant (K) is found to be 2.78 g/L.

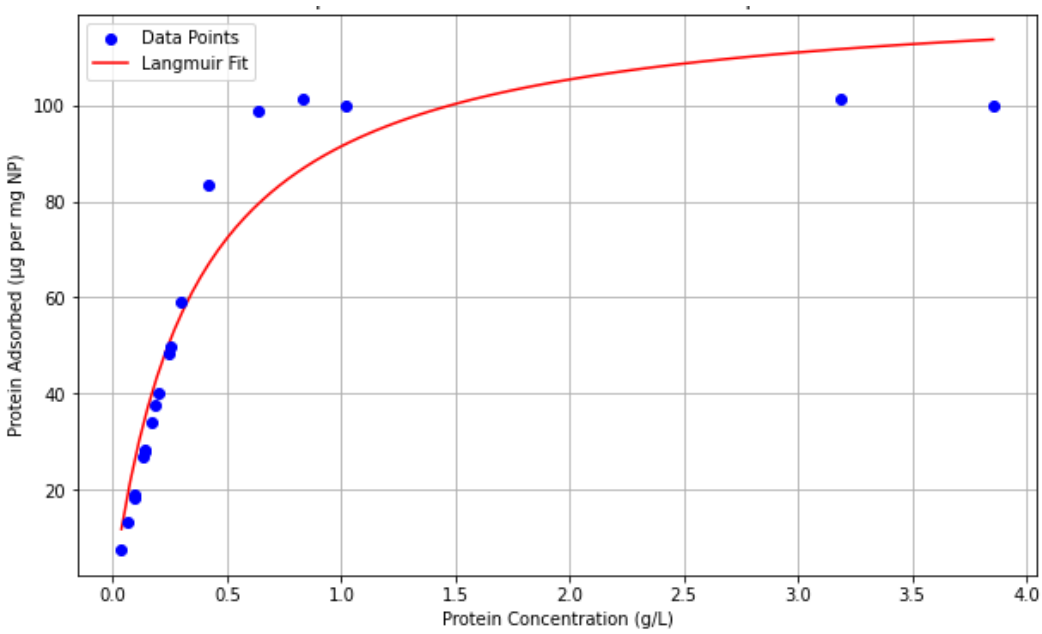


Figure S12. His-GFP adsorption isotherm fitting data points (blue) and Langmuir fit (red line).

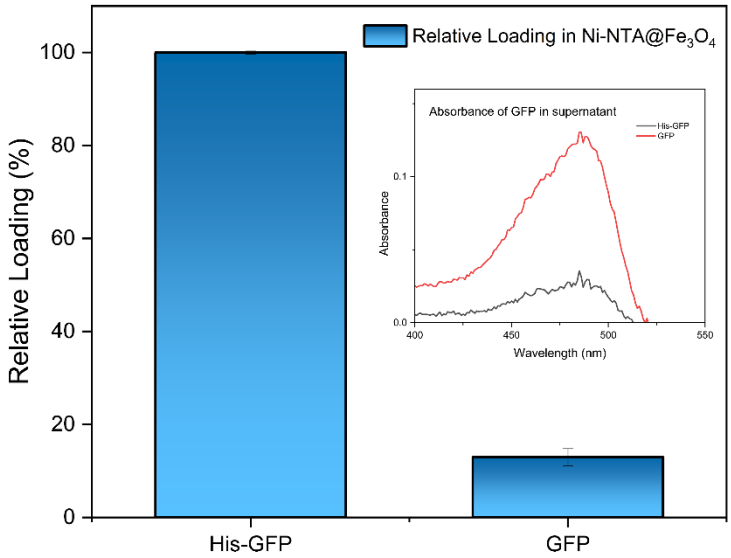


Figure S13. Maximum immobilization of His-GFP and non-His tagged GFP at NTA-Ni@Fe_3O_4 nanoparticles. The data are presented as mean ± SD; n = 3; The error bars represent standard deviations from three replicates measurements. Inset is the UV absorbance of His-GFP and non-His tagged GFP supernatant of samples.

Recovery Rate (RR) calculation: Assessing the GFP protein's recovery rate (RR) from the loaded NTA-Ni@Fe_3O_4 NPs and commercial Ni-NTA resins. To ascertain this rate, we employed a high-concentration solution of pure GFP for loading into the NTA-Ni@Fe_3O_4 NPs and commercial Ni-NTA resins and utilized an elution buffer to release the enzyme from the NTA-Ni@Fe_3O_4 NPs and commercial Ni-NTA resins. Comparing the concentration of the recovered GFP to the initially loaded GFP was done using a UV-VIS spectrophotometer at 480 nm. Quantification of the immobilized enzyme was achieved using the following equation:

$$q_t = (C_0 – C_t) \times V \quad \ldots\ldots(1)$$

where q_t (µg) is the total adsorbed GFP in the NTA-Ni@Fe_3O_4 NPs and commercial Ni-NTA resins, C_0 is the initial concentration of GFP solution, C_t is the final concentration of GFP solution, and V is the volume of the loading buffer (mL). The recovery rate was calculated by eluting the GFP in an elution buffer by following the equation.

$$RR\ (\%) = etq_t \times 100 \ldots\ (2)$$

where RR is the recovery rate percentage, *et* (µg) is the total eluted enzyme with elution buffer from the NTA-Ni@Fe_3O_4 NPs and commercial Ni-NTA resins.

Synthesis of enzyme-loaded NTA-Ni@Fe_3O_4 NPs: 5 mg of the oven-dried NTA-Ni@Fe_3O_4 NPs were taken in a reaction tube. 250 µL of freshly purified BGL, CBH, and EG, (all 4 mg/mL) was poured into the reaction tube. The reaction mixture was then incubated at 4 °C for 2 h. After incubation, the NTA-Ni@Fe_3O_4 NPs were separated from the mixture by a magnet.

20 µL of the supernatant solution was diluted five times, and UV-vis absorbance was measured at 280 nm.

The following procedure is maintained throughout all the experiments to determine the adsorbed enzyme. After incubating the NTA-Ni@Fe_3O_4 NPs with a known concentration of enzyme solution, the final concentration of the solution was measured by the UV-spectroscopy of the supernatant. Now, the adsorbed amount of enzyme at equilibrium is calculated by the following equation

$$E_t = (C_0 - C_t) \times V, \qquad L_p(\%) = \frac{(C_0 - C_t) \times v}{m} \times 100$$

where E_t is the total immobilized enzyme in the NTA-Ni@Fe_3O_4 NPs (mg/mg), L_p (%) is the loading percentage of the enzyme, $C_0$is the initial concentration of enzyme solution, C_t is the final concentration of enzyme solution, V is the volume of the treated solution (ml), m is the mass of the NTA-Ni@Fe_3O_4 NPs (mg).

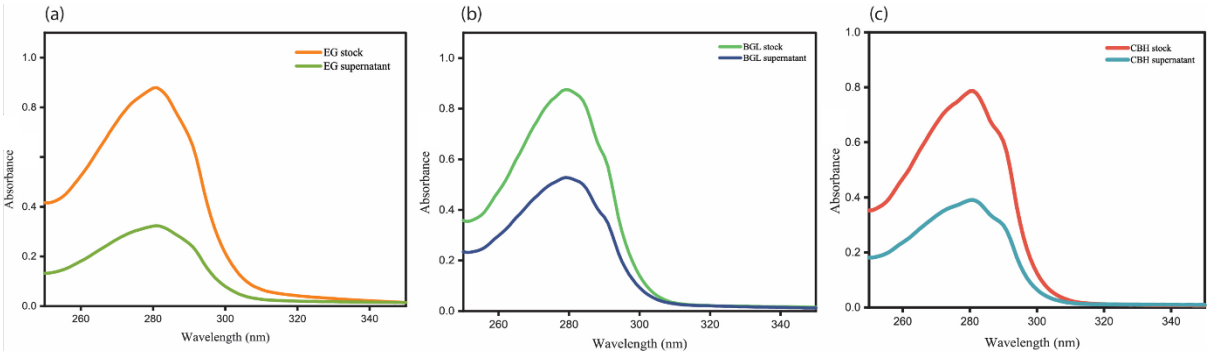


Figure S14. UV-vis absorption spectra of the stock enzyme solutions (a: EG, b: BGL, c: CBH)and supernatant after surface immobilization of respective enzyme stock in NTA-Ni@Fe_3O_4.

Section S-12: Enzyme Activity Assay

BGL@NTA-Ni@Fe_3O_4 NPs recyclability assay: The BGL@NTA-Ni@Fe_3O_4 NPs recycled activity was measured on the chromogenic substrate, *p*NPGlc. For recycling assays, a 100 µL reaction mixture containing 1 µL of BGL@ NTA-Ni@Fe_3O_4 NPs (equivalent to 0.01 µg of free BGL) and 40 mM of *p*NPGlc (final concentration) was incubated at 70 °C for 5 min with constant shaking at 800 rpm. To precipitate the BGL@ NTA-Ni@Fe_3O_4 NPs, the reaction contents were centrifuged at 10000 rpm for 10 min, and the supernatant was decanted. The *p*NP in the supernatant was quantified by measuring absorbance at 405 nm by UV-visible spectrophotometry. The BGL@ NTA-Ni@Fe_3O_4 NPs were reused for another product-forming reaction up to five cycles to compare the catalytic efficiency between the first and the later cycles.

CBH@NTA-Ni@Fe_3O_4 NPs recyclability Assay: The effect of recycling CBH@NTA-Ni@Fe_3O_4 NPs was determined by measuring enzyme activity on CMC. A 150 µL reaction mixture containing 10 µL of CBH@NTA-Ni@Fe_3O_4 NPs (equivalent to 0.01 µg of free CBH) and 1 % of CMC (10 mg/mL) was incubated at 55 °C for 15 min with constant shaking in a Thermomixer (Eppendorf, Hamburg, USA) at 800 rpm. To precipitate the CBH@NTA-Ni@Fe_3O_4 NPs, the reaction contents were centrifuged at 10000 rpm for 10 min.150 µL DNS reagent was added to the supernatant and incubated at 95 °C for 15 min. Absorbance was measured at 540 nm, and the reducing sugars were quantified. The CBH@NTA-Ni@Fe_3O_4 NPs were reused for five cycles, and activity was determined to compare the catalytic efficiency between the first and the later cycles.

EG@NTA-Ni@Fe_3O_4 NPs recyclability assay: The EG@NTA-Ni@Fe_3O_4 was measured on CMC. A 150 µL reaction mixture containing 1 µL of EG@ NTA-Ni@Fe_3O_4 (equivalent to 0.01 µg of free EG) and 1 % CMC (10 mg/mL) was incubated at 55 °C for 15 min with constant shaking at 800 rpm in a thermomixer (Eppendorf, Hamburg, USA). To precipitate the EG@ NTA-Ni@Fe_3O_4, the reaction contents were centrifuged at 10000 rpm for 10 min, and the supernatant was decanted. Then 150 µL DNS reagent was added to the supernatant and incubated at 95°C for 15 min. to measure the reducing sugar generated, as described previously. The EG@ NTA-Ni@Fe_3O_4 was reused for another reaction, and the measurements were repeated up to five cycles to compare the catalytic efficiency between the first and the later cycles.

One-Pot reaction assay: The glucose oxidase–peroxidase (GOD-POD) assay was used to measure the amount of glucose generated during the one-pot reaction. In one pot reaction, the substrate CMC was used. 150 µL reaction mixture containing McIlvaine buffer (pH 6.0), 10 µL of EG@NTA-Ni@Fe_3O_4 (equivalent to 0.5 µg of free EG), 10 µL of CBH@NTA-Ni@Fe_3O_4 (equivalent to 0.5 µg of free CBH), 10 µL of BGL@NTA-Ni@Fe_3O_4 (equivalent to 0.5 µg of free BGL) and 1 % CMC (10 mg/mL) was incubated at 55 °C for 30 min with constant shaking at 800 rpm on a Thermomixer. To precipitate the enzymes@TpAzo-foam, the reaction contents were centrifuged at 10000 rpm for 10 min, and the supernatant was decanted. The amount of glucose generated was measured by the glucose oxidase–peroxidase assay (Glucose Oxidase kit, Sigma-Aldrich, St Louis, USA) following the manufacturer's protocol.

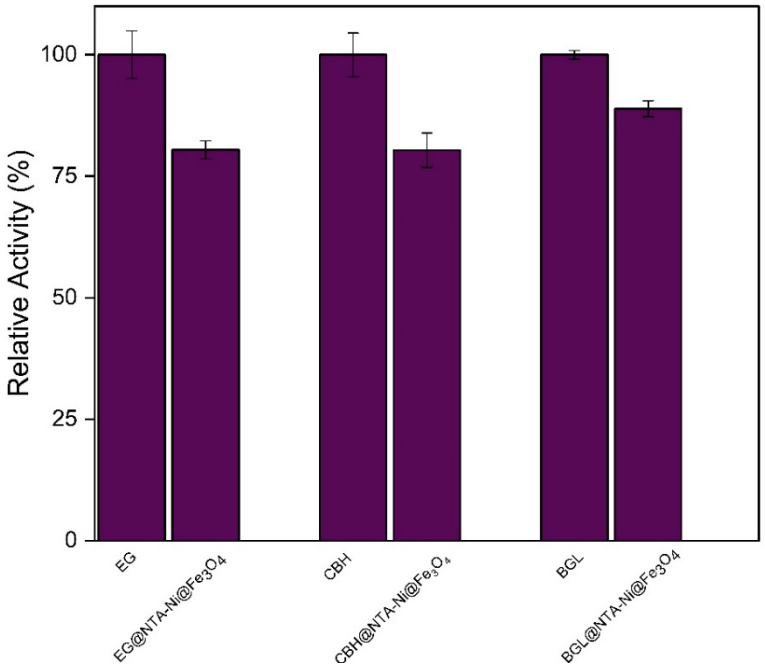


Figure S15: Relative activity comparison of free EG, EG@NTA-Ni@Fe_3O_4 NPs, free CBH, CBH@NTA-Ni@Fe_3O_4 NPs, free BGL and BGL@NTA-Ni@Fe_3O_4 NPs. The data are presented as mean ± SD; n = 3; The error bars represent standard deviations from three replicate measurements.

Table S2. Comparative studies described in the literature on the different supports of magnetic nanoparticles

Carrier	Enzyme	Recovered Activity (%)	Recyclable	Reference
GO-Fe_3O_4 nanocomposites	Catalase	98	yes	Yang et al. (2016)
Fe_3O_4 nanoparticles with chitosan, and glutaraldehyde crosslinkers	Laccase	80	yes	Lin et al. (2017)
Graphene oxide (GOMNP) superparamagnetic nanofilms functionalized with polyethene glycol bisamine (PEGA).	Xylanase	40	yes	Mehnati-Najafabadi et al. (2018)
Cobalt ferrite ($CoFe_2O_4$) composites	β-Glucosidase, Xylanase, Phytase	78-100	yes	Coutinho et al. (2020)
Magnetic graphene oxide (GO-MNP)	Cellulase, Xylanase	76	yes	Paz-Cedeno et al. (2021)

Section S-13: References

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