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Enzyme-magnetic nanoparticle conjugates as rigid biocatalyst for the elimination of toxic aromatic hydrocarbon

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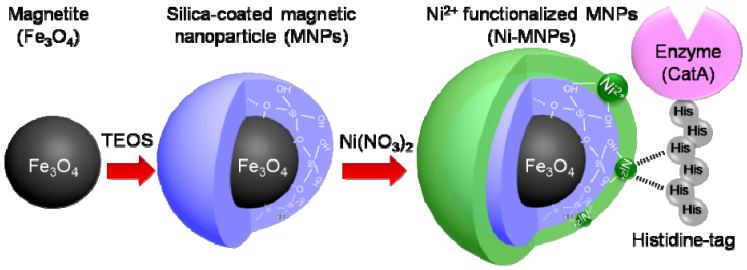
Preparation of Ni-MNPs



Synthesis of magnetite (Fe_3O_4): Magnetites were prepared by coprecipitation with Fe^{2+} and Fe^{3+}. To prepare the Fe_3O_4 magnetites a fresh mixture of 2 M $FeCl_2 \cdot 4H_2O$ and 1 M $FeCl_3 \cdot 6H_2O$ was added to 0.7 M ammonia solution with vigorous stirring. The obtained Fe_3O_4 magnetites were separated by magnet and the supernatant was decanted. The collected Fe_3O_4 magnetites were washed 4 times with ethanol and then air-dried.

Silica-coated magnetic nanoparticles (MNPs): Before the silica-coating process, the 100 mg Fe_3O_4 magnetites were dispersed in 100 mL cyclohexane under sonication for 3h in the presence of 3.3 mL oleic acid. The 44 g Igepal CO-520 dissolved solution in 900 mM cyclohexane was added to Fe_3O_4 magnetites solution and then further stirred for 15 min. Then, 8mL of aqueous ammonia solution was added drop wisely into the freshly prepared solution with stirring for 15 min. After stirring, a solution of 98 % tetraethyl orthosilicate (TEOS) was added dropwise. The mixed solution was further stirred for 20 h. Methanol was added to the solution to form dark precipitates, which were collected through centrifugation after removal of supernatants. The dark precipitate was washed repeatedly with ethanol and then vacuum dried.

Ni^{2+} functionalized MNPs (Ni-MNPs): $Ni(NO_3)_2 \cdot 6H_2O$ and MNPs were reacted in deionized water through shacking at room temperature for 1h. The final product was collected and washed with deionized water three times and then air-dried. The particle size and morphology of the Ni-MNPs were determined by high-resolution transmission electronic microscopy (HR-TEM) using a JEM-2000EX TEM (JEOL, Japan) and dynamic light scattering spectrophotometer (DLS) using a DLS-8000 (Otsuka Electronics, Japan) (Fig. S1)

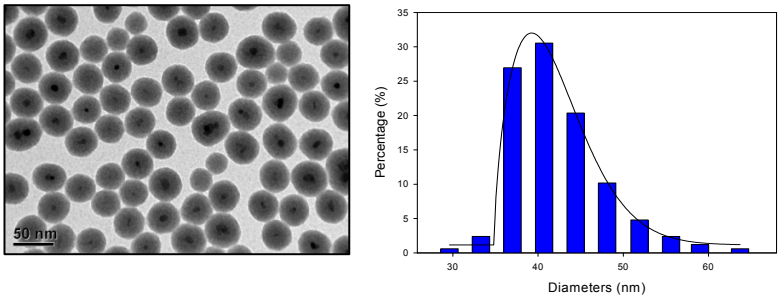


Fig. S1 TEM image and diameter distribution of Ni-MNPs.

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Synthesis of histidine-tagged CatA enzyme and conjugation with Ni-MNPs (CatA@Ni-MNPs)

The *catA* coding region was amplified from the chromosomal DNA of *C. glutamicum* ATCC 13032 by polymerase chain reaction (PCR) using the primer pair. The PCR product was digested with *Nde*I/*Not*I and ligated into the *Nde*I/*Not*I-restricted plasmid, pET-21a (Fig. S2). The resulting plasmid pSY-Cat was transferred to *E. coli* BL21 (DE3) by $CaCl_2$ transformation. The synthesis of the six histidine-tagged CatA fusion protein was induced in recombinant *E. coli* BL21 (pSY-Cat) by the addition of 0.5 mM IPTG after the culture had reached an optical density at 600 nm of 0.6. The cells were grown for 3 h. Purification of the fusion protein was carried out by affinity chromatography (Ab Frontier Inc., Korea). 1 mg of Ni-MNPs were added to purified CatA solution in 10 mM phosphate buffered saline (PBS, pH 7.4) and incubate with rotation (30 rpm) for 1 h at room temperature. CatA conjugated Ni-MNPs (CatA@Ni-MNPs) were separated from the solution using a magnet for 1 min, and washed with PBS briefly. Proteins were analyzed by SDS-PAGE and their concentrations estimated by standard bicinchoninic acid protein assay using Pierce BCA Protein Assay kit (Thermo Scientific Inc., USA) (Fig. S3).

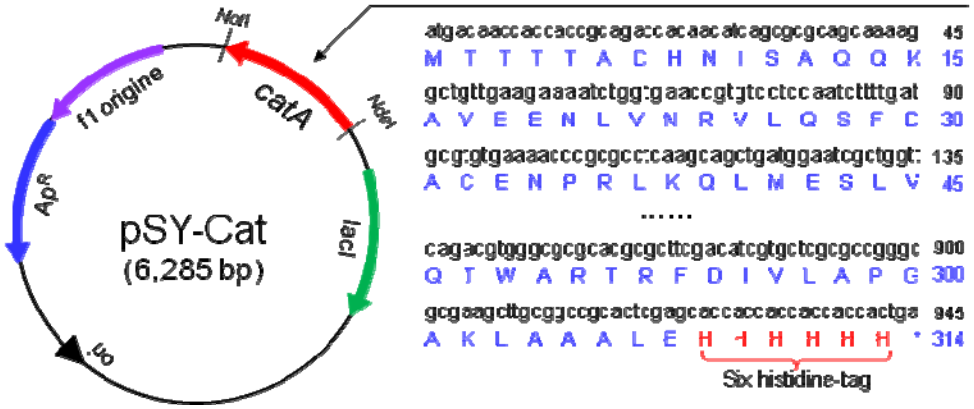

Fig. S2 Plasmid construction for six histidine-tagged catechol 1,2-dioxygenase (CatA-His_6) synthesis.

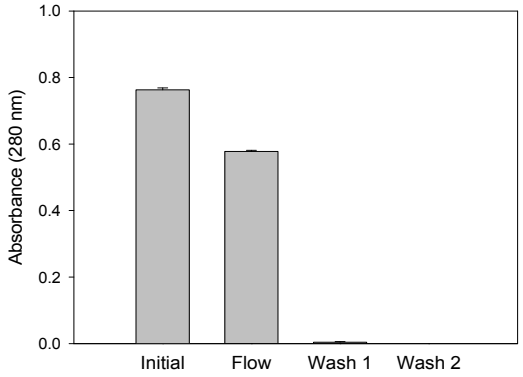

Fig. S3 Analysis of protein concentration of Ni-MNPs conjugated CatA enzyme by UV/VIS spectrophotometer.

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Reusability of CatA@Ni-MNPs by repetitive magnetic separations from the catechol solution

The catalytic activity of catechol degradation from recycled CatA@Ni-MNPs was observed. For this, we used the recovered CatA@Ni-MNPs in repetitive magnetic separations from the catechol solutions. For each cycle, a solution of 100 µM catechol was exposured to CatA@Ni-MNPs and after 60 min the concentration of catechol was quantified. As shown in Fig. S3, catalytic activity was over 72 ± 4.02 % after recycling it up to six times.

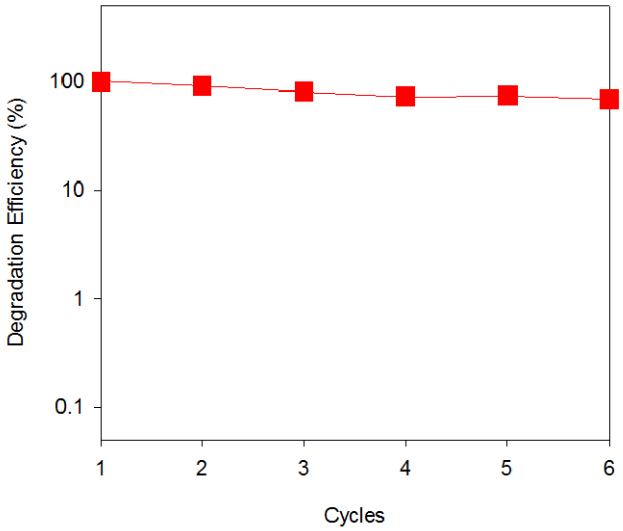

Fig. S3 Reusability efficiency of CatA@Ni-MNPs

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Real time RT-PCR analysis for evaluation of catechol toxicity

To evaluate toxicity of catechol and its product during our process, transcriptional analysis of these genes was performed using real time-RT PCR. PCR primers were designed for the four stress responsive genes, *grpE, katG, fabA,* and *recA,* as well as for the *rrnH* gene, used as endogenous control by using the Primer Express software version 3.0 (Applied Biosystems, USA) (Table S1). mRNA expression levels of target genes were quantified by real-time reverse transcription (RT)-PCR using One step SYBR® PrimeScript kit (TaKaRa Bio, Japan) according to the manufacturer's protocol. The total RNA from *E. coli* DH 5α strain cultured under Luria-Bertani medium containing catechol solutions for 2 h was extracted using RNeasy Mini kit (Qiagen, Germany). Real time RT-PCR was performed by a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA). Data analysis was performed using StepOne™ software version 2.1 (Applied Biosystems, USA). The gene expression levels of target genes were normalized against the expression level of the *rrnH* gene, which encodes for the 16S rRNA. The induction ratio was calculated using the function $2^{-\Delta\Delta Ct}$ according to the handbook of StepOne™ software. In this study, cultures grown in pure distilled water (DW) were used as controls for normalization when tests were done with the catechol solution. All the results represent the data from at least three independent experiments and include a mean value.

Table S1. DNA oligonucleotides used in this study

Name	Sequence (5′-3′)	Note
grpE-F	5′- cgt aag ttt ggc gtt gaa gtg a -3′	heat shock regulon (protein damage)
grpE-R	5′- tgg cct gat gca cat tcg -3′	
katG-F	5′- gag cac gtc aga cga tat cca taa -3′	catalase (oxidative stress)
katG-R	5′- gtg acc gcc ctg atg gaa -3′	
fabA-F	5′- ctg gtc gcg gtg aac tgt t -3′	fatty-acid synthesis (membrane
fabA-R	5′- acg gtc cat cat cag cat gtt -3′	damage)
recA-F	5′- agc act ggg cca gat tga ga -3′	SOS regulon (DNA damage)
recA-R	5′- cat cca tgg aac ggt ctt cac -3′	
rrnH-F	5′- tcg tgt tgt gaa atg ttg ggt ta -3′	16S rRNA[a]
rrnH-R	5′- gga ccg ctg gca aca aaa -3′	(internal standard)

[a] The *rrnH* gene encoding 16S rRNA was selected and tested as an internal standard to normalize the data obtained from the target genes.

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Determination of catalytic activity of CatA@Ni-MNPs

To determine catechol degradation by CatA, high-performance liquid chromatography (HPLC) was carried out using a reverse-phase XTerra™ RP_{18} column and Waters 2487 Dual λ absorbance detector (Waters Co., USA). An acetonitrile-water (30:70, v/v) mobile phase containing 1 % acetic acid was pumped at 1 mL min^{-1} by a high pressure pump (515 HPLC pump) (Waters Co., USA). The chromatograms were plotted at 275 nm. The catalytic parameters (Michaelis-Menten constant, K_m) of the free and Ni-MNPs supported enzyme were calculated by measuring the initial linear rates of the reaction after addition of different concentrations of substrate, catechol, ranging from 10 to 300 μM at 25 °C. Three independent measurements were carried out for each substrate concentration. The experimental data were fitted to the Michaelis-Menten curve by using the Sigma Plot software version 10.0.

Instrumental analyses

X-ray photoelectron spectroscopy (XPS) was used to determine the surface functionalization with Ni^{2+} ion (Sigma Probe equipped with monochromatic Al source, 15 kV and 100 W) (Thermo Scientific, UK). The magnetization of functionalized magnetic nanoparticles at room temperature up to 10 kOe was measured using a vibrating sample magnetometer (VSM 4179) (Oxford Instruments, UK).

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Prediction of stereostructure of CatA@Ni-MNPs conjugates by *in silico* analysis

The stereostructure of CatA@Ni-MNPs conjugates was predicted by *in silico* analysis using bioinformatic tools such as PSI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and protein modeling programs provided by ExPASy Proteomics Server (http://us.expasy.org/tools). From the analysis, we guess that active site of CatA enzyme is located in the core site of whole enzyme structure (Fig. S5). In this case, six histidine residues located in the C-terminal region of CatA enzyme might be attributed to exposoure of the active site to its surroundings even though the enzyme immobilized onto Ni-MNPs surface.

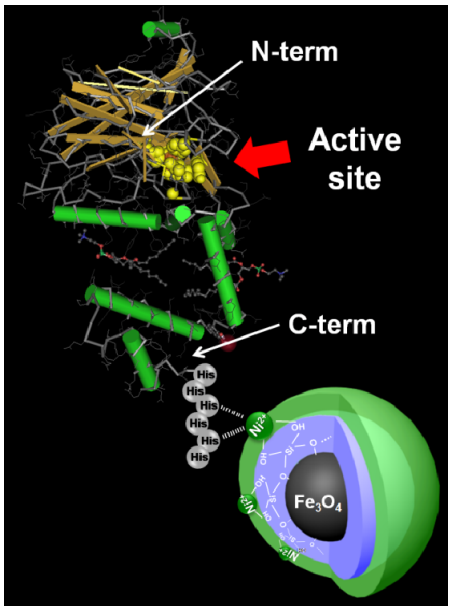

Fig. S5 Prediction of stereostructure of CatA@Ni-MNPs