JMB Papers in Press. First Published online Feb 1, 2018
DOI: 10.4014/jmb.1710.10037

Manuscript Number: JMB17-10037

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Article Type: Research article

Keywords: immobilization, encapsulation, protein–inorganic hybrid, reusability, Thermomyces lanuginosus, xylanase
Immobilization of xylanase using a protein-inorganic hybrid system

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Running Title: Protein-inorganic hybrid for xylanase immobilization

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Abstract

In this study, the immobilization of xylanase using a protein-inorganic hybrid nanoflower system, was assessed to improve the enzyme properties. The synthesis of hybrid xylanase nanoflowers was very effective at 4°C for 72 h, using 0.25 mg/mL protein, and efficient immobilization of xylanase was observed with a maximum encapsulation yield and relative activity of 78.5% and 148%, respectively. Immobilized xylanase showed high residual activity at broad pH and temperature ranges. Using birchwood xylan as a substrate, the \( V_{\text{max}} \) and \( K_m \) values of xylanase nanoflowers were 1.60 mg/mL and 455 \( \mu \text{mol/min/mg protein} \), compared with 1.42 mg/mL and 300 \( \mu \text{mol/min/mg protein} \), respectively, for the free enzyme. After 5 and 10 cycles of reuse, xylanase nanoflowers retained 87.5% and 75.8% residual activity, respectively. These results demonstrate that xylanase immobilization using a protein-inorganic hybrid nanoflower system is an effective approach for its potential biotechnological applications.

Keywords: immobilization; encapsulation; protein-inorganic hybrid; reusability; Thermomyces lanuginosus; xylanase
Introduction

Immobilization of enzymes can be a beneficial approach to improve their properties. Several methods of immobilization, including physical and covalent interactions, have been established, on solid supports and polymeric matrices [1-5]. However, lower enzyme efficiency after immobilization is a primary concern [4-8]. The encapsulation of enzymes, using protein-inorganic hybrid-based nanoflower systems, is a promising approach to improve their properties [9-11]. Protein-inorganic hybrids with nanoflower-like morphology has been conventionally synthesized as enzyme-Cu$_3$(PO$_4$)$_2$:3H$_2$O hybrids by combining copper sulfate (CuSO$_4$) with the enzymes in phosphate buffer saline (PBS) and incubating for 3 days at room temperature [10]. Nanoflower synthesis occurs in three steps, including nucleation, aggregation, and anisotropic growth [10]. The effective immobilization of enzymes such as $\alpha$-chymotrypsin, alcohol dehydrogenase, and lipase, using protein-inorganic hybrids, has also been reported, under similar incubation conditions and with other metals, including calcium, cobalt, and zinc [12-14]. The enzymes immobilized as nanoflowers had a higher catalytic activity and improved stability compared to their corresponding free forms because of their large surface area and effective immobilization [10, 15, 16]. Nanoflowers of various enzymes, including $\alpha$-chymotrypsin, catalase, chloroperoxidase, glucose oxidase, and laccase, have been successfully used in enzymatic biofuel cells; for degradation of phenolic compounds; and for digestion of proteins and human serum [10, 11, 13, 17, 18].

Xylanase ($\beta$-1,4-D-xylanohydrolase, EC 3.2.1.8) is an enzyme that hydrolyzes polysaccharides such as xylan [19]. By degrading xylan to fuels and chemicals, xylanase
has useful applications in the food, paper, and pulp industries, with a global market value of nearly $200 million [5, 20]. Owing to the high industrial importance of thermal xylanases from *Thermomyces lanuginosus* strains, immobilization on various supports, including aluminum hydroxide (gibbsite and amorphous), gold and magnetic nanoparticles, EUDRAGIT S-100, alginate, polyethylene glycol net cloth, and chitosan, has been investigated to improve their catalytic properties and stability [5, 7, 8, 20-22]. The lower relative activity observed after immobilization in these studies suggested that a suitable immobilization approach is still required. In addition, the higher reusability of immobilized xylanase makes its use more economical [5, 7, 21, 22]. Generally, the covalent immobilization of enzymes requires complex processes, such as the functional activation of supports, and results in lower efficiency compared with that of the free enzyme [5, 7]. The immobilization of both commercial and purified enzymes with nanoflowers caused significant variations in their relative activities after synthesis under different incubation conditions [23]. Thus, modification of the synthesis conditions is essential for the effective immobilization of enzymes. In this study, we examined the effects of xylanase immobilization on its enzymatic properties using enzyme-Cu₃(PO₄)₂·3H₂O hybrids synthesized under different conditions. The immobilized xylanase exhibited high relative activity at 4°C and reusability with improved catalytic properties. These results suggest that the immobilized xylanase using a protein-inorganic hybrid nanoflower system can be effectively used in industrial applications.

Materials and Methods
Materials

Birchwood xylan, CuSO$_4$, fluorescein isothiocyanate (FITC), and xylanase (from *Thermomyces lanuginosus*) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PBS and ultrapure water were purchased from Life Technologies (Carlsbad, CA, USA). All other reagents were of analytical grade.

Synthesis of Xylanase-Cu$_3$(PO$_4$)$_2$·3H$_2$O Hybrid Nanoflowers

Xylanase nanoflowers were synthesized in 5 mL of PBS (10 mM, pH 7.4), using 0.25 mg/mL of protein and different concentrations of CuSO$_4$ (0.8 and 2.0 mM) for up to 72 h at 4 or 25°C [23]. Furthermore, the effect of the protein concentration (0.05–1.00 mg/mL) on efficient xylanase immobilization was examined using the optimal CuSO$_4$ concentration and synthesis conditions. The protein concentration in the supernatant was determined by the Bradford method as described previously [2].

Enzyme Assay

Free and nanoflower-immobilized xylanase activity was measured using birchwood xylan (1%) as a substrate, by the modified Bailey’s method using dinitrosalicylic acid [19].

Characterization of Xylanase Nanoflowers

Free and nanoflower-immobilized xylanase activity was measured using standard assay conditions at different pH values in various buffers (50 mM): sodium-citrate (pH 3.0 and
3.5), sodium-acetate (4.0–5.5), and sodium-phosphate (pH 6.0–8.0). The effect of
temperature was investigated over the range of 30–80°C at the optimum pH value. Further,
to determine the kinetic parameters (K_m and V_max), the samples were incubated at 50°C
with different concentrations of substrate (1–20 mg/mL). The data were analyzed by non-
linear regression fitting of the Michaelis-Menten equation, using GraphPad Prism 5
(GraphPad Software, CA, USA) [24].

Stability and Reusability

The storage stability of free and immobilized xylanase was determined at 25°C. Samples were withdrawn at regular intervals over the incubation of 20 days to measure the residual activity. The reusability of the xylanase nanoflowers was measured at 50°C using xylan as a substrate under standard assay conditions. After each cycle, xylanase nanoflowers were separated by centrifugation for 10 min at 10,000 rpm and 4°C, and used for the next cycle after washing twice in the assay buffer [6]. The initial activity of the xylanase nanoflowers was considered 100%. The leaching of immobilized enzyme was evaluated by the measurement of activity in the supernatant of each cycle, as follows (Eq. 1):

\[
\text{Leaching (\%)} = \left( \frac{\text{total enzyme activity in supernatants}}{\text{initial immobilized enzyme activity}} \right) \times 100
\]

Instrumental Analysis
The morphology of the synthesized xylanase nanoflowers was analyzed by field emission scanning electron microscopy (FE-SEM; Jeol JSM-6060, Japan) [25-27]. Absorption spectra were monitored using a Varian Cary 100 Bio UV-Vis spectrophotometer (Palo Alto, CA, USA) [28-30]. The size distribution analysis of synthesized nanoflowers was analyzed by dynamic light scattering (DLS, Wyatt Technology, Santa Barbara, CA, USA) [31]. For the fluorescence labeling, 8 mg of FITC dissolved in 2.0 mg/mL of dimethyl sulfoxide was mixed with 1 mL of xylanase (1 mg) in 0.5 M of carbonate buffer (pH 9.5) and the resulting solution was incubated in the dark for 6 h at 300 rpm [32]. The unbound FITC was removed through dialysis against distilled water. Further, FITC-labeled xylanase was used for the synthesis of nanoflowers and microscopic analysis was performed using a confocal laser scanning microscope (CLSM; FV-1000 confocal microscope, Olympus, Japan) [2, 33, 34]. All experimental values are the mean ± standard deviation. Statistical analysis of variance (ANOVA, $\alpha = 0.05$) was performed using GraphPad Prism 5 software [35].

Results and Discussion

Synthesis of Xylanase Nanoflowers

The synthesis scheme for the xylanase nanoflower is presented in Fig. 1. To effectively immobilize xylanase in an enzyme-Cu$_3$(PO$_4$)$_2$$\cdot$3H$_2$O hybrid nanoflower system, nanoflower synthesis was evaluated using different concentrations of CuSO$_4$ (0.8 and 2 mM) for incubation periods up to 72 h at 4 and 25°C. The immobilized xylanase showed encapsulation yield (EY) and relative activity (RA) of 57.8–78.5% and 32.2–148%,
respectively (Table S1). The effective immobilization of xylanase was observed with EY and RA values of 76.9% and 148%, respectively, using 0.8 mM CuSO\textsubscript{4} for 72 h at 4°C. In contrast, synthesis using 2 mM CuSO\textsubscript{4} resulted in a significantly lower RA of 32.2. Further, the effect of protein concentration on xylanase immobilization was evaluated using optimized conditions [Table 1]. The EY and RA of synthesized enzyme-Cu\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} \cdot 3H\textsubscript{2}O hybrid nanoflowers varied widely with protein concentration. As the concentration increased from 0.05 to 1 mg/mL, a substantial decrease in the EY was observed, from 86.7% to 19.2%. On the contrary, the weight of bound enzyme in the synthesized hybrid nanoflowers increased from 7.6% to 13.9%, and the xylanase nanoflowers exhibited RAs of 94.7–148%. Initially, with an increase from 0.05 to 0.25 mg/mL, the RA increased from 115% to 148%. Thereafter, the RA decreased, to 94.7% at 1 mg/mL. The most efficient immobilization was observed at 0.25 mg/mL. The higher residual activity of xylanase nanoflowers at lower concentrations might be associated with the high surface area, the co-operative influence of the immobilized enzymes, or a favorable conformation [10, 23, 36].

**Instrumental Analysis of Xylanase Nanoflowers**

FE-SEM images of xylanase nanoflowers synthesized at 4°C for up to 72 h are presented in Fig. 2. These images confirmed the synthesis of xylanase-Cu\textsubscript{3}(PO\textsubscript{4})\textsubscript{2} \cdot 3H\textsubscript{2}O hybrids with flower-like morphology. The size of the hybrid nanoflowers increased with an increase in the incubation period. These results suggest that the synthesis of xylanase nanoflowers at 4°C followed a three-step mechanism (nucleation, aggregation, and anisotropic growth), similar to that demonstrated at 25°C [10]. Initially, xylanase interacted
with Cu$_3$(PO$_4$)$_2$ nanocrystals to initiate the nucleation. Then, a bulky aggregation of xylanase and nanocrystals was formed and finally, controlled anisotropic expansion resulted in a branched flower structure. Further, the morphological changes of the synthesized xylanase nanoflowers were evaluated at pH 6.5 and 8.5 (Fig. S1). The size of the synthesized nanoflowers was observed to be similar under both pH conditions, but the petal density decreased at pH 8.5. CLSM analysis confirmed the efficient immobilization of xylanase in protein-inorganic hybrid nanoflowers (Fig. 3a-b). Further, DLS analysis indicated that the synthesized xylanase nanoflowers had an average size of 8 µm (Fig. 3c). Whereas, nanoflowers synthesized at higher temperature (25°C) exhibited a larger size of about 15 µm (Fig. S2). High-resolution TEM images suggested the formation of a crystal lattice structure in the petals of xylanase nanoflowers (Fig. S3a-b). Moreover, the XRD patterns of synthesized xylanase nanoflowers exhibited sharp and strong peaks similar to those for the standard Cu$_3$(PO$_4$)$_2$·3H$_2$O (JCPDS 00-022-0548) (Fig. S3c-d) [15].

Characterization of Xylanase Nanoflowers

Free and nanoflower-immobilized xylanase showed specific activities of 270 and 400 U/mg protein, respectively. Previously, xylanase exhibited variable residual activity profiles at different pH and temperature values after immobilization through adsorption, covalent, and encapsulation methods on aluminum hydroxide and magnetic nanoparticles, and within polyethylene glycol net-cloth, respectively [5, 7, 8]. To examine the influence of pH and temperature on the activity of immobilized xylanase, the activity of synthesized nanoflowers was evaluated at different pH (3.0–8.0) and temperature (30–80°C) values (Fig.
Free and immobilized xylanase showed an optimum pH of 5.5. Here, the xylanase nanoflowers exhibited RA values of 39.8% and 92.1% at pH 3.0 and 8.0, respectively. Free enzyme under similar conditions showed lower RA values of 11.7% and 72.6%, respectively. The immobilized xylanase showed 3.4- and 1.3-fold increases in stability compared to the free enzyme at pH 3.0 and 8.0, respectively. Similarly, the optimum temperature for both free and nanoflower-immobilized xylanase was 65°C. Xylanase nanoflowers exhibited 2.0-fold higher RA than the free enzyme at 80°C. On the contrary, entrapping *T. lanuginosus* xylanase within polyethylene glycol net-cloth did not have a positive influence on its stability at similar pH and temperature ranges [8]. Similar profiles at different pH and temperature values were also observed for immobilized *T. lanuginosus* xylanase on nanoporous gold particles [20]. Overall, these outcomes suggest that xylanase immobilized using a protein-inorganic hybrid system is very effective, retaining higher stability and activity than the free enzyme.

**Kinetic Parameters**

The kinetic parameters (apparent $V_{\text{max}}$ and $K_{\text{m}}$ values) of xylanase are significantly influenced by immobilization [5, 7, 22]. The Michaelis-Menten model was used to determine the kinetic parameters of free and immobilized xylanase, using xylan as a substrate under standard assay conditions at 50°C (Table 2 and Fig S4). The $K_{\text{m}}$ and $V_{\text{max}}$ values of free xylanase were 1.42 mg/mL and 300 $\mu$mol/min/mg protein, respectively. After immobilization through encapsulation, the $K_{\text{m}}$ value slightly increased to 1.60 mg/mL, while the $V_{\text{max}}$ was significantly enhanced to 455 $\mu$mol/min/mg protein. A similar increase
in the $K_m$ value (from 4.16 to 5.27 mg/mL) was observed by immobilized *T. lanuginosus* xylanase on multifunctional hyperbranched polyglycerol-grafted magnetic nanoparticles [22], while significant increases in $K_m$ (up to 2.4-fold) were reported for immobilized xylanase on solid supports, including amorphous Al(OH)$_3$ and nanoporous gold and magnetic nanoparticles, compared to the free enzyme [5, 7, 20]. Generally, the increase in $K_m$ after immobilization is primarily associated with steric effects or mass transfer limitations, while enhancements in $V_{max}$ tend to result from conformation changes or co-operative effects of enzymes [7, 22, 23].

**Stability and Reusability**

The stability profiles of the free enzyme and xylanase nanoflowers at 25°C are presented in Fig. 5a. After 20 days of incubation, free and immobilized xylanase showed a residual activity of 4.7 and 76.6%, respectively. The reusability of immobilized enzyme is a crucial parameter to demonstrate its economic viability. To evaluate the reusability of nanoflower-immobilized xylanase, the residual activity was measured for up to 10 cycles of reuse under standard assay conditions at 50°C (Fig. 5b). Immobilized xylanase nanoflowers retained residual activities of 87.5% and 75.8% after 5 and 10 reaction cycles, respectively. The loss of activity during the successive cycles might be associated with enzyme inactivation or leaching of the enzyme [22]. We observed cumulative leaching of 8.9% of the residual activity in the supernatant after 10 cycles (Fig. 5b). A summary of the reusability of *T. lanuginosus* xylanase immobilized by different methods on various supports, including nanoparticles and polymeric matrices, is presented in Table 3. Among
these, immobilization of xylanase using a nanoflower hybrid system retained the highest residual activity after 10 cycles of reuse.

In summary, the immobilization of *T. lanuginosus* xylanase on various supports, including nanoparticles and polymeric matrices, has previously resulted in significantly lower efficiency than that of the free enzyme. However, this study has demonstrated that the immobilization of xylanase through encapsulation using a protein-inorganic hybrid system is an effective approach to retain significantly higher activity than that of the free enzyme. After immobilization, xylanase showed more stable pH and temperature profiles. The xylanase nanoflowers exhibited higher reusability than that observed in many previous reports, using adsorption and covalent immobilization approaches on different support materials such as amorphous Al(OH)₃, gibbsite, and gold, magnetic, and silica nanoparticles. This suggests that the immobilization of xylanase in a protein-inorganic hybrid nanoflower system is a useful method to enhance its use in potential industrial applications.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2012159 and NRF-2013R1A1A2007561). This research was supported by the KU Research Professor program of Konkuk University. This work was also supported by 2015 KU Brain Pool Fellowship of Konkuk University.

Conflict of Interest
The authors declare that they have no conflicting interests associated with this publication.

References


on functionalized magnetic nanoparticles and determination of its activity and stability. 
a polyethylene glycol net-cloth grafted on polypropylene nonwoven fabrics with 
exceptional operational stability and its application for hydrolysis of corncob 
in enzyme immobilization: Organic-inorganic hybrid nanoflowers with enhanced 
428-432.
complexes for reinforcing immobilized chloroperoxidase reusability. Chem. Commun. 53: 
3216-3219.
12. Lopez-Gallego F, Yate L. 2015. Selective biomineralization of Co$_3$(PO$_4$)$_2$-sponges 
triggered by His-tagged proteins: efficient heterogeneous biocatalysts for redox 
nanoflower based immobilized enzyme reactor with enhanced enzymatic activity. J. Mater. 


Figure Legends

Fig. 1. Schematic representation of xylanase immobilization in enzyme-
$\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ hybrid nanoflowers

Fig. 2. FE-SEM images of synthesized xylanase nanoflowers Nanoflowers were
synthesized by combining 0.25 mg/mL of protein and 0.8 mM CuSO$_4$ in 5 mL of PBS (10
mM, pH 7.4) at 4°C for (a) 6, (b) 12, (c) 24, and (d) 72 h.

Fig. 3. CLSM and DLS analysis of xylanase nanoflowers Nanoflowers were synthesized
using FITC-labeled xylanase (0.25 mg/mL) and 0.8 mM CuSO$_4$ at 4°C for 72 h, and
subjected to morphological analysis in (a) green channel and (b) bright field, along with (c)
size distribution measurements.

Fig. 4. Activity of free and immobilized xylanase at different pH and temperature
values (a) The effect of pH was measured using the standard assay with different buffers
(sodium-citrate, pH 3.0 and 3.5; sodium-acetate, 4.0–5.5; and sodium-phosphate, pH 6.0–
8.0; all at 50 mM). (b) The enzyme activity of free and immobilized xylanase was
monitored at various temperatures in sodium-acetate buffer (50 mM, pH 5.5) under
standard conditions. The maximum activities of free and immobilized xylanase are shown
as 100% residual activity. Each value represents the mean of triplicate measurements that
varied from the mean by no more than 10%. FE, free enzyme.
Fig. 5. **Stability and reusability of immobilized xylanase** (a) storage stability at 25°C and (b) reusability. The reusability of the xylanase nanoflowers was evaluated using xylan as a substrate under standard assay conditions at 50°C. The maximum activity is shown as 100% residual activity. Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.
Table 1 Encapsulation of Cu$_3$(PO$_4$)$_2$·3H$_2$O-xylanase hybrid nanoflowers

<table>
<thead>
<tr>
<th>Protein (mg/mL)</th>
<th>EY$^a$(%)</th>
<th>Weight (%)$^b$</th>
<th>RA$^c$(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>86.7 ± 3.8</td>
<td>7.6 ± 0.7</td>
<td>124 ± 12</td>
</tr>
<tr>
<td>0.10</td>
<td>81.2 ± 4.2</td>
<td>8.4 ± 0.9</td>
<td>132 ± 14</td>
</tr>
<tr>
<td>0.25</td>
<td>76.9 ± 4.6</td>
<td>11.5 ± 1.2</td>
<td>148 ± 13</td>
</tr>
<tr>
<td>0.50</td>
<td>34.9 ± 3.0</td>
<td>12.7 ± 1.2</td>
<td>125 ± 10</td>
</tr>
<tr>
<td>1.00</td>
<td>19.2 ± 1.6</td>
<td>13.9 ± 1.4</td>
<td>94.7 ± 7.8</td>
</tr>
</tbody>
</table>

Significant (p < 0.05) differences were observed by one-way ANOVA

$^a$EY, Encapsulation yield [(amount of enzyme immobilized/amount of initial enzyme) × 100].

$^b$Weight of enzyme (as a percentage of the total) in the hybrid nanoflowers

$^c$RA, Relative activity [(total specific activity of immobilized enzyme/total specific activity of free enzyme) × 100].
<table>
<thead>
<tr>
<th>Xylanase</th>
<th>$K_m$  (mg/mL)</th>
<th>$V_{max}$ (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>1.42 ± 0.24</td>
<td>300 ± 12.5</td>
</tr>
<tr>
<td>Nanoflower</td>
<td>1.60 ± 0.37</td>
<td>455 ± 27.5</td>
</tr>
</tbody>
</table>

Significant (p < 0.05) differences were observed by one-way ANOVA.
Table 3 Comparison of *Thermomyces lanuginosus* xylanase immobilization properties by different methods

<table>
<thead>
<tr>
<th>Support</th>
<th>Method</th>
<th>IE%</th>
<th>Reusability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorphous Al(OH)$_3$</td>
<td>Adsorption</td>
<td>64.0</td>
<td>24.0 (8)</td>
<td>[5]</td>
</tr>
<tr>
<td>Gibbsite</td>
<td>Adsorption</td>
<td>75.0</td>
<td>24.0 (8)</td>
<td>[5]</td>
</tr>
<tr>
<td>EUDRAGIT S-100 polymer</td>
<td>Adsorption</td>
<td>75.0</td>
<td>62.0 (6)</td>
<td>[17]</td>
</tr>
<tr>
<td>Magnetic nanoparticles supported hyper branched polyglycerol (MNP/HPG)</td>
<td>Adsorption</td>
<td>78.2</td>
<td>54.0 (10)</td>
<td>[18]</td>
</tr>
<tr>
<td>MNP/HPG-citric acid</td>
<td>Adsorption</td>
<td>89.7</td>
<td>66.0 (10)</td>
<td>[18]</td>
</tr>
<tr>
<td>Nanoporous gold particles</td>
<td>Adsorption</td>
<td>77.9</td>
<td>70.0 (10)</td>
<td>[16]</td>
</tr>
<tr>
<td>Chitosan polymer</td>
<td>Covalent</td>
<td>45.0</td>
<td>NA$^c$</td>
<td>[17]</td>
</tr>
<tr>
<td>Silica-encapsulated magnetic nanoparticles</td>
<td>Covalent</td>
<td>73.0</td>
<td>65.0 (9)</td>
<td>[7]</td>
</tr>
<tr>
<td>Alginate polymer</td>
<td>Encapsulation</td>
<td>70.0</td>
<td>NA</td>
<td>[17]</td>
</tr>
<tr>
<td>Protein-inorganic hybrid</td>
<td>Encapsulation</td>
<td>148</td>
<td>75.8 (10)</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$ IE, immobilization efficiency  
$^b$ RE, residual activity (number of cycles)  
$^c$ NA, not applicable
Fig. 1

CuSO₄ + Xylanase → PBS (1x) pH, 7.4 → Incubation 72 h, 4 °C → Nanoflower
Fig. 2
Fig. 3
Fig. 4

(a) Relative activity (%) of FE and Nanoflower at different pH values.

(b) Relative activity (%) of FE and Nanoflower at different temperatures.
Fig. 5