Zeolite-based nanocomposite as a smart pH-sensitive nanovehicle for release of xylanase as poultry feed supplement

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Xylanase improves poultry nutrition by degrading xylan in the cell walls of feed grains and release the entrapped nutrients. However, the application of xylanase as a feed supplement is restricted to its low stability in the environment and gastrointestinal (GI) tract of poultry. To overcome these obstacles, Zeozyme NPs as a smart pH-responsive nanosystem was designed based on xylanase immobilization on zeolitic nanoporous as the major cornerstone that was modified with L-lysine. The immobilized xylanase was followed by encapsulating with a cross-linked CMC-based polymer. Zeozyme NPs was structurally characterized using TEM, SEM, AFM, DLS, TGA and nitrogen adsorption/desorption isotherms at liquid nitrogen temperature. The stability of Zeozyme NPs was evaluated at different temperatures, pH, and in the presence of proteases. Additionally, the release pattern of xylanase was investigated at a digestion model mimicking the GI tract. Xylanase was released selectively at the duodenum and ileum (pH 6–7.1) and remarkably preserved at pH ≤ 6 including proventriculus, gizzard, and crop (pH 1.6–5). The results confirmed that the zeolite equipped with the CMC matrix could enhance the xylanase thermal and pH stability and preserve its activity in the presence of proteases. Moreover, Zeozyme NPs exhibited a smart pH-dependent release of xylanase in an in vitro simulated GI tract.

Enzyme supplements are used widely in poultry diets in an attempt to improve poultry performance, feed consumption, and also minimized environmental pollutions due to the reduced output of excreta¹. Different enzyme supplements have been developed with respect to their target substrates in feed ingredients. Exogenous xylanase is an example of an enzyme supplement to improve nutritional factors by efficiently decomposing the non-starch polysaccharides (NSP)², specifically xylans found in the cell walls of cereals. Consequently, a high proportion of entrapped energy in the NSP feed matrices would be liberated³. Furthermore, xylanases help relieves certain gastrointestinal problems such as decreased villus height, raised levels of pathogenic bacteria in the intestine, unnecessary mucin excretion⁴, and chronic inflammation⁵. They also improve poultry immunity⁶ and also minimize the harmful effects of Salmonella Typhimurium infection⁷ or Clostridium perfringens⁸.

However, xylanase would be inactivated by various external and internal factors that limited its efficient exploitation in the feed industry⁹. An important one of the external factors is faced during the industrial feed processes (e.g. pelleting) via the heat treatments, high pressures, and chemical treatments (such as pH, surfactant, and solvents)¹⁰ or during the shelf-life via the environmental parameters like sunlight, moisture, and temperature¹¹. Moreover, physiological barriers are an example of the internal factors of the inactivation of xylanase¹².

Whilst the poultry's crop has a slightly acidic medium, the proventriculus and gizzard media are acidic, and the proximal end of the intestine (duodenum) is slightly acidic that toward the distal part included jejunum, ileum, and colon it becomes neutral to slightly alkaline. Since the optimum pH for most of the exogenous enzymes is between 4–6¹³, it is most likely that the exogenous enzymes would be active in crop or duodenum. Unfortunately, the limited retention time of the feed in the crop restricts the enzyme function. On the other hand, before any function, the exogenous enzymes may be degraded under the harsh acidic conditions of some

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parts of GI such as crop, proventriculus, and gizzard or by the endogenous proteolytic enzymes such as pepsin and trypsin. Therefore, a convenient poultry feed xylanase must tolerate the physiological barriers imposed by pH, digesta retention time, and internal enzymes within the gastrointestinal (GI) tract. A smart nanosystem is equipped with a micro-environmentally sensitive group that enables it to release the loaded cargo at a desired time and location when exposed to the given external stimuli, such as temperature, pH, light, redox properties, enzyme activity, electric & magnetic fields, and ultrasound. These structural changes are basically important characteristics of the smart nanosystem, as they provide and facilitate the exploitation of the inherent characteristics of the GI microenvironment. Among them, the nanosystems with the pendant groups that undergo specific physicochemical changes in response to pH changes are deemed “pH-sensitive”. The smart pH-sensitive delivery nanosystems appear to be highly appealing candidates due to the intrinsic differences of the target tissues in terms of the relative acidity.

Carboxymethyl cellulose (CMC), is an anionic linear polymer, water-soluble, and pH-sensitive. CMC is used in the food industry as an emulsion stabilizer, thickener, moisture binder to create desirable textural of food. CMC has been used in drug delivery widely. Therefore, the CMC-based polymer was used as a bio-compatible cottage for pH-sensitive release of xylanase in different parts of GI. There are many reports about stabilizing xylanases as feed supplements against external sources. However, to the best of our knowledge, there is no report in the stabilization of xylanase against the internal factors through the GI.

To address these challenges, enzyme immobilization has been exploited to improve the operational stability of enzymes. An efficient enzyme immobilizing in feed industries requires an appropriate support matrix with definite properties such as inertness, biocompatibility, biodegradability, cost-effectiveness, physical strength, stability, renewability, and ability to preserve enzyme activity. Zeolites are a kind of natural and biocompatible nanoparticles with hydroxyl-rich surface functional groups such as ion adsorption and cation exchange capacity they are ideal for different applications such as enzyme immobilization. Due to their useful and unique properties, using zeolites in different sectors of poultry industries provides significant opportunities for obtaining an improvement in the performance and quality of production as well as the mitigation of environmental pollutions and waste control processes produced by the poultry and swine industries.

Herein, to mitigate the mentioned issues, a smart nanosystem enabled to efficient release and protecting enzyme activity was designed for providing enough enzyme concentrations for the desirable function at the duodenum, jejunum, and ileum parts of GI. Generally, to design an efficient support–enzyme interaction, the tailored pendant groups of the organic linker can be grafted onto the surface of nanocarriers. It has been reported that amino acids are a suitable candidate as the linker molecules for enzyme immobilization. Therefore, Lys was selected as the pendant organic linker, which as adopted for poultry feed supplementation. Then, xylanase was immobilized onto the surface of Lys modified zeolite (xylanase@Lys-zeolite) to stabilize xylanase against external deactivation sources. Furthermore, to overcome the internal deactivation of xylanase in GI and to improve its pH-controlled release, the xylanase@Lys-zeolite was encapsulated by CMC and chemically cross-linked using glutaraldehyde (denoted as Zeozyme NPs). As a feed supplement, the structural and functional analysis has revealed that Zeozyme NPs as a smart pH-responsive nanosystem could be preserved enzyme stability and activity in the acidic environment of the stomach.

Results
Synthesis and characterization of Zeozyme NPs. The use of exogenous xylanase as a feed supplement would improve poultry performance. However, exogenous xylanase was subjected against two defined deactivation factors: environmental changes (external) and physiological conditions (internal). Consequently, we aimed to introduce the biocompatible Zeozyme NPs programmed for the smart pH-responsive release of xylanase due to the pH changes in GI microenvironment, as a potential feed supplement. The preparation of Zeozyme NPs has been demonstrated in Fig. 1. As illustrated, the nano-encapsulated xylanase called Zeozyme NPs was prepared by immobilizing xylanase onto the nanopores of zeolite (xylanase@Lys-zeolite) and then wrapped by CMC-based polymer. Although the zeolite surface possesses multifunctional groups, these hydroxyl groups interact with each other and form deactivated surface. Hence, the surface of zeolite clinoptilolite particles was activated by refluxing in HCl overnight to break these rings and make reachable hydroxyl groups on the surface. The XRD studies showed that zeolite was not changed after this mild acidic treatment (Fig. 2). In the next step, the zeolite surface was modified by lysine (Lys). The amino acid modified zeolite not only makes a biocompatible surface for xylanase immobilization but also provides more functional groups for the effective binding of xylanase to the surface. Therefore, to achieve a suitable organic linker for improving the physical binding of the enzyme with the rigid surface of zeolite, Lys (an abundant amino acid in poultry feed supplement) was investigated at different weight ratios. The TGA analysis showed that the grafting of Lys was performed more successfully compared to methionine. Therefore, the Lys pendant moieties were applied as a biocompatible and eco-friendly linker for modifying the zeolite surface. Afterwards, xylanase was immobilized onto the surface of Lys-zeolite. The supernatant was examined to determine the enzyme content of the sample. The immobiliza-
tion efficiency (IE) of Lys-zeolite was calculated by the following equation (Eq. 1), while bovine serum albumin (BSA) was used as standard.

\[ \text{IE} = \frac{E_i - E_f}{E_i} \times 100 \]  

where \( E_i \) is the amount of the initial xylanase used in the immobilization and \( E_f \) is the amount of free xylanase detected in the supernatant after centrifugation of the aqueous colloidal solution. The IE for zeolite was 40% while for the Lys modified zeolite (Lys-zeolite), IE was 90%.

Then the xylanase@Lys-zeolite was encapsulated using a glutaraldehyde cross-linked CMC-polymeric shell (Zeozyme NPs) which is capable of preserving the xylanase activity and stability at low pH of the poultry digestion system. One of the common chemical reactions that involves the formation of polymer-based compounds is Schiff base reaction. This reaction comprises of an interaction between two functional groups of amine and aldehyde groups. Some examples of Schiff base reaction involves applying the cross-linking agents. At acidic pH, the cross-linking agent glutaraldehyde can also react with the hydroxyl groups present on the CMC polymer.

Figure 1. Schematic illustration on the preparation of Zeozyyme NPs from zeolite, Lys, xylanase, and CMC polymer as the starting materials.

Figure 2. XRD analysis of clinoptilolite zeolite before and after acidic treatment.
chains (Fig. 1). The glutaraldehyde contains aldehyde groups at the terminal end of the molecule, which react with the hydroxyl groups on the adjacent CMC chains. To roll out the leaching of xylanase during the CMC-encapsulation, the amount of xylanase in the supernatant of this step measured by UV–Vis spectroscopy was zero. The Zeozyme NPs structure was characterized by different techniques such as SEM, TEM, AFM, DLS, BET and BJH, and TGA analyses. The nanoporous network has revealed an ordered array of hexagonal honeycomb faces of pores as visualized using SEM (Fig. 3c). The SEM image of Zeozyme NPs would be compared with their starting materials as zeolite (Fig. 3a), xylanase@Lys-zeolite (Fig. 3b). TEM images of zeolite, xylanase@Lys-zeolite, and Zeozyme NPs were illustrated in Fig. 3d–f. The TEM image showed that Zeozyme NPs are spherical with average diameters below 200 nm (Fig. 3f).

Figure 3. SEM images of zeolite (a), xylanase@Lys-zeolite (b), and Zeozyme NPs (c). TEM images of zeolite (d), xylanase@Lys-zeolite (e), and Zeozyme NPs (f).

The AFM imaging of Zeozyme NPs and the related intermediate materials have been demonstrated in Fig. 4. The AFM images (Fig. 4) have revealed that its particles were easily agglomerated, which makes different size ranges of particles (Fig. 4a). However, after immobilization and encapsulation, the uniform morphology with high dispersity was occupied for the xylanase@Lys-zeolite (Fig. 4b) and Zeozyme NPs (Fig. 4c).
The particle size of zeolite, xylanase@Lys-zeolite, and Zeozyme NPs was determined using DLS and compared in Fig. 4d, e and f, respectively. As seen, the average diameters of diluted zeolite were slightly larger than that of Zeozyme NPs (800 and 120 nm, respectively). Comparison between DLS data of the starting material and final product revealed that the microscale size of zeolite (800 nm, PDI 0.38) was reduced to the nanoscale and homogeneous nanoparticles of Zeozyme NPs (120 nm, PDI 0.23). Reducing the size from microscale to nanoscale and decreasing the polydispersity is mainly due to encapsulation by CMC. In fact, in this method, immobilized xylanase on zeolite (xylanase@Lys-zeolite) was exposed to CMS that was cross-linked by glutaraldehyde. In such a mixture, CMC polymer nano-network often forms to entrap the xylanase@Lys-zeolite.

The zeta potentials measurement results showed that the charges of zeolite, Lys-zeolite, xylanase@Lys-zeolite, and Zeozyme NPs were −31, −22, −54, and −25 mV, respectively. This observation suggested that the hydroxyl ions on the surface caused the negative zeta potential of zeolite and upon the surface modifying with positively charged L-lysine amino acid, the zeta potential became a little positive compared to the native zeolite surface. This confirmed that the zeolite surface was modified by L-lysine amino acid. The zeta potential of used xylanase in this research was −30 mV. The xylanase was immobilized on Lys modified zeolite by covalent binding of amine and carboxylic groups. In this regard, the zeta potential of xylanase@Lys-zeolite reached −54 mV. The pI of CMC depends on the pKa values of the two functional groups including \( \text{NH}_2\text{RCOO}^- \) and \( \text{NH}_3^+\text{RCOO}^- \). This pI is 5.14 and the zeta-potential is negative above the pI due to the larger amount of \( \text{COO}^- \) groups and the zeta-potential is positive below pI due to larger amount of \( \text{NH}_3^+ \) groups. Consequently, the zeta-potential of CMC is positive because of the used acetate buffer (pH 5) for immobilization. As the xylanase@Lys-zeolite (−54 mV) interacted with CMC the zeta potential of the final product turns −25 mV. Hence, the zeta potential of Zeozyme NPs was more positive compared to the xylanase@Lys-zeolite, which indicates the loading of enzyme on the surface through the electrostatic interaction.

The \( \text{N}_2 \) adsorption–desorption isotherm experiment has shown the pore volume and surface area in the zeolite. The specific surface area \( S_{\text{BET}} \) of the samples was calculated based on the multiple-point Brunauer–Emmett–Teller (BET) method. As the results of BET analysis summarized in Table 1, a comparison of the data shows that the amount of surface area and pore size of zeolite after xylanase immobilization has decreased. This decrease indicated that the L-lysine, which modified the zeolite, was located both inside and outside surface of the porous network of zeolite. Also, the decreased surface area and pore volume of zeolite after xylanase immobilization confirmed that xylanase was immobilized on the modified zeolite. The nitrogen adsorption–desorption isotherm at 77 K of the zeolite and Zeozyme (Fig. 5) has revealed that the isotherms were of hybrid type I and type IV(a) according to the IUPAC classification with a H3/H4 hysteresis cycle. Although the 2D layered materials were mostly or fully microporous, some of them showed a mesoporosity as revealed by the hybrid type I and type IV isotherms and the type H3/H4 hysteresis loops.

Table 1. The results obtained from BET and BJH of Zeolite and zeozyme NPs.

| Material          | \( S_{\text{BET}} \) (m² g⁻¹) | Total pore volume (cm³ g⁻¹) | \( r_p \) (nm) |
|-------------------|-------------------------------|-----------------------------|----------------|
| Zeolite           | 103.77                        | 0.18                        | 1.93           |
| xylanase@Lys-zeolite | 73.965                      | 0.1534                      | 1.21           |
| Zeozyme NPs       | 28.292                        | 0.1418                      | 3.2            |

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TGA analysis of bare zeolite, Lys-zeolite, xylanase@Lys-zeolite, and Zeozyme NPs are shown in Fig. 5. In all of the samples, the observed weight loss within about 100 °C is related to the elimination of the adsorbed water.

Figure 5. (a) The nitrogen sorption isotherm and BJH analysis (inset) of zeolite (dotted line), Lys-zeolite (black solid line), xylanase@Lys-zeolite (dash-dotted line), and Zeozyme NPs (blue solid line). (b) TGA analysis of zeolite, Lys-zeolite, xylanase@Lys-zeolite, and Zeozyme NPs.
molecules. The TGA analysis of Zeozyme NPs showed a second peak at about 200 °C, corresponding to the loss of the Lys as the organic linker group. The third peak at 600 °C was related to the loss of xylanase immobilized on the surface of the zeolite. The amount of loaded Lys and glutaraldehyde cross-linked CMC in Zeozyme NPs were determined ~ 10%, and ~ 30%, respectively, as measured using TGA. These results were further confirmed using elemental analysis. Also, the amount of immobilized xylanase onto the zeolite surface measured by thermal analysis was ~ 100 mg xylanase per gram of Zeozyme NPs.

**In vitro evaluating study of zeozyme NPs.**  
**Activity assay.** The function of xylanase was based on degrading of the linear polysaccharide xylan into xylose by catalysis the hydrolysis of the glycosidic linkage (β-1,4) of xylosides (Eq. 2). The catalytic activity of xylanase entrapped in xylanase@Lys-zeolite (52 U mL\(^{-1}\)) and Zeozyme NPs (54 U mL\(^{-1}\)) were measured and compared with free xylanase (55 U mL\(^{-1}\)). Any significant difference in the specific activity between free and entrapped xylanase was not observed. It means that immobilization and encapsulation did not affect the catalytic activity. A comparison of the enzymatic activity before and after encapsulation showed litter changes. This is because encapsulation with a polymer makes better dispersity and size reduction. These two parameters are very significant in catalytic activity\(^{36}\).

**Thermal and pH stability.** The stability of immobilized xylanase in the form of xylanase@Lys-zeolite and Zeozyme NPs were evaluated against temperature and pH verification. The thermal stability of free xylanase, xylanase@Lys-zeolite, and Zeozyme NPs from 60 to 90 °C for 45 min has been demonstrated in Fig. 6a. The immobilized and encapsulated enzyme was more stable against the thermal treatment than the free enzyme (Fig. 6a). Furthermore, the pH stability of free xylanase, xylanase@Lys-zeolite, and Zeozyme NPs in the pH and retention time simulated GI tract was studied. The samples were incubated in various pH from 1.1 to 7.1 followed by the xylanase activity assay. In acidic pH, free xylanase was deactivated but xylanase@Lys-zeolite and Zeozyme NPs retained hydrolysis activity (Fig. 6b). A Comparison of the pH-stability between xylanase@Lys-
zeolite and Zeozyme showed that xylanase@Lys-zeolite preserved about 60% activity in acidic pH compared to its activity in neutral pH, but Zeozyme NPs completely maintained its activity in all of the pH ranges.

This thermal stability of xylanase@Lys-zeolite and Zeozyme NPs would be explained by zeolite role in the immobilization. Zeolite as an electric insulator possesses a low dielectric constant, but it is able to transport heat away from the electric components. For example, the immobilization of lipase or glucose oxidase on zeolite enhanced the enzymes' thermos-stability. Zeolite with a high surface area shows high stability against variation in the temperature, pH and organic solvents. Hence, with regard to these properties zeolite would shield xylanase against thermal and pH changes. In addition, xylanase unfolding was restricted by proper linkage via Lys on the porous surface of zeolite. As a whole, Zeozyme NPs up to 90% and xylanase@Lys-zeolite about 60% can preserve hydrolytic activity of xylanase comparing to free xylanase.

Pepsin and trypsin resistance. The free xylanase, xylanase@Lys-zeolite, and Zeozyme NPs varied in the protease resistance over a range of protease/enzyme mass ratios (Fig. 6c). After the treatment with pepsin, the enzyme activity in Zeozyme NPs remained almost unchanged. A slight decrease in Zeozyme NPs activity was observed from the protease: enzyme ratios of 1:100–1:5. In the ratio of 1:1 the Zeozyme NPs activity reduced to 90% of the initial value (Fig. 6c). The pepsin resistance of free xylanase decreased with the increased pepsin/enzyme ratios, retaining 45% of its activity after the pepsin treatment at a ratio of 1:1 (Fig. 6d). These results indicated that Zeozyme NPs is highly resistant to trypsin and pepsin degradation, while the free xylanase is highly sensitive to pepsin and moderately resistant to trypsin. Enzyme immobilization moderately protected enzyme from denaturation in the acidic pH and proteases in gastrointestinal conditions such as pepsinase immobilized on hydroxyapatite NPs. Thus, zeolite may play a suitable role in the stabilizing the xylanase against pepsin. But, CMC coating plays a superior role in stabilizing enzyme against acidic environment and pyrolytic enzymes of the digestive system.

In vitro pH-controlled release of xylanase. To evaluate the efficacy of Zeozyme NPs as a tailored pH-controlled released system for xylanase delivery in GI tract, the xylanase release from xylanase@Lys-zeolite and Zeozyme NPs were investigated in vitro. This evaluation also confirmed the necessity of the presence of CMC polymer to enhance the enzymes' thermos-stability. The passage time of feed and the pH value of different parts of the digestive tract of poultry were also illustrated in Fig. 7b by color changes. The passage time of feed in the anterior of the digestive tract of poultry is relatively short and the xylanase could not have enough time to hydrolysis the feed completely. Therefore, xylanase showed the best activity in neutral pH. The free exogenous feed enzyme must be released in the posterior digestive tract where enough time and suitable pH for degrading feed effectively is available. As seen in Fig. 7c, about 50% of xylanase was rapidly released from xylanase@Lys-zeolite in pH 1.1–2.5 during the first hour. In contrast, Zeozyme NPs released less than 4% of xylanase within the first hour and the majority of xylanase (about 95%) was released within 4 h. Figure 7d showed the enzyme activity of released xylanase from xylanase@Lys-zeolite and Zeozyme NPs in vitro under different simulated conditions of GI. The enzyme activity data (Fig. 7d) was in correlation with the amounts of released enzyme in Fig. 7c. This data indicates that the glutaraldehyde cross-linked CMC polymeric coating hinders xylanase release in the acidic pH condition of the stomach. Hence, CMC as a smart response moiety would ensure the safe delivery of high xylanase concentration at the target site (duodenum, jejunum, and ileum), minimizing xylanase release in the stomach, and subsequently promote the poultry growth. Thus, Zeozyme NPs may be a promising platform for xylanase delivery that is triggered by pH.

Discussion
The results have revealed that the Zeozyme NPs containing zeolite, Lys, and CMC-based nanocage increased xylanase stability against the external deactivating factors arisen from environmental changes, industrial feed processes (e.g. pelleting), or the shelf-life conditions. Moreover, to validate the merits of Zeozyme NPs, its stability was assessed under different deactivation factors using mimicked physiological conditions of GI tract. The Zeozyme showed a smart pH-dependent release of xylanase in different sections of the simulated poultry GI tract. This fantastic stability of xylanase would be clarified the intelligent application of a combined stabilization method (included immobilization and encapsulation) of the enzyme for preparing of Zeozyme NPs. Xylanase immobilization onto the surface of zeolite nanonetwork via Lys as an applicable organic linker has made it robust against the harsh external deactivation factors such as varying ranges of pH and temperature. The distinctive physicochemical properties of zeolites and Lys modified zeolites including hydroxyl rich surface, thermo-stability, and biocompatibility make it a potential candidate for enzyme immobilization to improve its function and stability.

Furthermore, CMC polymer as a hydrophilic matrix and pH-sensitive system has offered the physiological stability for xylanase enzyme against internal deactivation such as pyrolytic internal enzymes of the digestive tract and provided a tailored controlled xylanase release, especially in the neutral and alkaline pH. Since the digestions of non-starch polysaccharides take place through the last sections of the poultry GI tract (duodenum, jejunum, and ileum) which is alkaline, the polymeric shield retained the xylanase in the harsh acidic parts of the digestive tract. In addition, the polymeric nanocage system preserved xylanase against the pyrolytic digestion...
enzyme secreted in the GI tract. Considering the useful components of Zeozyme NPs including zeolite, Lys, and CMC as biocompatible and poultry feed supplementations the Zeozyme NPs would efficiently be applied as a supplementation in feed and promote the poultry industry. The development of biodegradable and smart delivery nanosystems for improving the efficacy of enzyme-based supplementations in the animal feed industry is worth to be investigated in vivo in future studies.

**Experimental section**

**Materials and apparatus.** A specific natural zeolite, called clinoptilolite, has been purchased from Afrand Toska Company (Iran). It has been approved by EU (70/524/EEC) and FDA (21 CFR 582–2727) to apply in animal feed. L-lysine (Lys), carboxymethyl cellulose sodium (CMC), potassium phosphate buffer, and acetate buffer were purchased from Merck. DNS (3,5-di-nitrosalicylic acid), sodium potassium tartrate, xylan, and xylose were obtained from Sigma-Aldrich. Deionized water was used in all solutions preparation. The particle size distribu-

| pH | Crop | Proventriculus | Gizzard | Duodenum | Jejunum | Ileum |
|----|------|----------------|---------|-----------|----------|-------|
| 5  | Green| 1.6            | 2.5     | 6         | 6.2      | 7     |
| Time (min) | 12 | 17 | 20 | 87 | 60 |
Preparation of zeolyme NPs. Firstly, acidic activation of zeolite clinoptilolite surface was done by refluxing 2.0 g of zeolite with hydrochloric acid (1%) overnight. Then it was washed and centrifuged (6000 rpm, 5 min) until the neutral pH. After that, the surface of zeolite was modified by Lys. Briefly, the zeolite (100 mg) and Lys (50 mM) were dispersed by use of sonication in acetate buffer (2 mL, pH 5.0) and the colloidal solution was stirred for 5 h at room temperature. Next, it was centrifuged at 6000 rpm for 5 min, and the solid phase was separated and washed as Lys modified zeolite (Lys-zeolite).

Afterwards, a previously reported thermostable xylanase was immobilized by mixing 100 mg of Lys-zeolite with 2.5 mL of xylanase solution (5 mg mL⁻¹) and allowed to stir overnight at 4 °C in phosphate buffer (20 mM, pH 7.5). Then the mixture was centrifuged (6000 rpm, 5 min) to collect the residual solid. The xylanase@Lys-zeolite was washed twice with the phosphate buffer to eliminate any non-immobilized xylanase. The supernatant was gathered to determine the enzyme content of the sample. The enzyme concentration at first was identified by monitoring the protein specific adsorption peak at 280 nm Nano-drop UV. Then, by subtracting the enzyme concentration before immobilization and remained in supernatant after immobilization the amount of immobilized xylanase would be obtained.

Finally, a clear solution of 75 mg of CMC in 5 mL of deionized water was added to a mixture of 100 mg of xylanase@Lys-zeolite in 2.5 mL of deionized water and then it was left for 20 min at room temperature. After that, glutaraldehyde 4% was poured into the mixture dropwise, and stirred for 2 h at room temperature. In the next step to obtain CMC-based polymer encapsulated xylanase@Lys-zeolite (Zeozyme NPs), the solid residue was separated by centrifugation and it was then washed thoroughly with water and phosphate buffer.

Loading efficiency. Xylanase concentration entrapped in zeolitic-based nanocarriers was quantified by the Bradford method. Briefly, into a 96-well titer plate, the resulting supernatant (may contain xylanase) (5 μL) and then 245 μL of Bradford reagent were added. The blended sample and Bradford reagent were incubated at 22 °C for 10 min. Subsequently, the absorbance of solution was recorded at 595 nm, and the concentration of xylanase was assessed via a bovine serum albumin-based Bradford standard curve.

Additionally, the stability of xylanase after loading was performed. The xylanase@Lys-zeolite was re-suspended and stirred in a phosphate buffer pH 7 for 2 h. Then the sample was centrifuged (6000 rpm, 5 min). The supernatant was collected and the xylanase concentration was evaluated using the Bradford method to assay the amount of xylanase leaves out from zeolite surface.

Enzymatic activity assay. Xylanase activity was measured based on Bailey, et al. Briefly, birchwood xylan solution (900 μL, 1%) as the substrate was added to the xylanase sample and incubated at 50 °C for 30 min. 1.5 mL DNS reagent was added and boiled for 5 min in a water bath to stop the reaction. Afterward, the absorbance intensity at 540 nm was recorded. The calibration standard graph for different concentrations of xylose (0–1 mg) was obtained. One unit of xylanase activity was equal to the amount of enzyme that liberated 1.0 μmol of xylose product per minute under the specified conditions of the assay method as described by Bailey et al.

Temperature and pH stability of xylanase. The thermal stability of free xylanase, xylanase@Lys-zeolite, and Zeozyme NPs was assayed after incubating samples separately in a water bath for 60 min at different temperatures (from 30 to 90 °C in phosphate buffer, 10 mM, and pH 7.0). The Effects of pH changes on the enzymatic activity of xylanase were measured and the results were compared for all of the enzyme samples (free xylanase, xylanase@Lys-zeolite, and Zeozyme NPs). The phosphate/acetate buffer was used at 10 mM for different pH values. The xylanase@Lys-zeolite and Zeozyme NPs samples were incubated at the specific temperature or pH, then the samples were centrifuged (at 6000 rpm, 5 min) and the pellet re-suspended in phosphate buffer, (10 mM, and pH 7.0). For free xylanase sample, the pH of buffer was just adjusted to phosphate buffer (pH 7.0). Then all the samples were assayed for evaluating the efficiency of immobilization or encapsulation.

Pepsin and trypsin resistance. The proteases resistance of enzyme samples (free xylanase, xylanase@Lys-zeolite, and Zeozyme NPs) was evaluated by incubating amount of 40 μg of each sample with various proteases (1:1, 1:5, 1:10, 1:20, and 1:40) at 41 °C for 2 h, and then their xylanase activities were assayed. The examined proteases were pepsin in a glycine–HCl buffer (0.25 M, pH 2.0) or trypsin in a Tris–HCl buffer (0.25 M, pH 7.0).

pH-responsive release assay. The assessment of pH-controlled release of xylanase from xylanase@Lys-zeolite and Zeozyme NPs was performed using the corresponded stimulated standard buffers. The buffer...
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Author contributions
F.D. synthesis and evaluation of nanobiomaterials, and writing the manuscript. F.J. helping in doing encapsulation synthesis. M.A.F. editing and revising manuscript. M. M. providing xylanase, L. M. and G. H.S. project administration, design synthesis and evaluation of nanobiomaterials, writing-review and editing, and Supervision.

Competing interests
The authors declare no competing interests.

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