

Immobilization of Xylanase onto Starch Nanoparticles: A Reusable and Robust Nanobiocatalyst for Juice Clarification

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Nanoparticles (NPs) have attracted interest recently as a promising support for the immobilization of enzymes. Xylanase (Xyl) has been utilized in the food industry for several purposes. To improve its stability and reusability, starch NPs (SNPs) are synthesized and assessed for Xyl immobilization for the first time. The characterization results reveal that SNPs are successfully synthesized and Xyl@SNPs are effective as the immobilization carrier. The Xyl is immobilized on SNPs under optimized conditions leading to 91% immobilization efficiency. A shift in optimum pH (from 6.0 to 8.0) and temperature (from 70 to 60 °C) of Xyl is observed after immobilization. The Xyl@SNPs possess improved pH stability and thermal stability as well as operational stability. The Xyl@SNPs are easily reutilized and the residual activity is still higher than 62% after seven times. More importantly, the Xyl@SNPs have a lower K_m value and enhance affinity for the substrate compared to the free Xyl. Additionally, the clarity of orange juice is increased by 76.0% by using Xyl@SNPs after 2 h incubation at 60 °C, pH 8.0. These positive results show promising support for the Xyl immobilization of SNPs, confirming that this immobilized enzyme-based strategy is an effective technique for rapid clarification of targeted juices.

material remediation, and textile bio-processing.^[2] Among them, xylanase (1,4- β -xylan xylanohydrolase, EC 3.2.1.8, Xyl), which hydrolyzes xylan, is a biotechnologically important commercial enzyme.^[3] Xyl enzyme has been utilized in the food industry for various purposes^[1] such as improving the sensory properties of wheat bread,^[4] clarifying fruit juices,^[5] converting agricultural residues into useful products,^[6] increasing oil extracted from plants,^[7] optimizing the ethanol production process,^[8] and improving animal feed digestibility.^[9] Even though Xyl enzyme is used in a wide range of industrial applications, the high production cost due to drawbacks including short life, poor operational stability, high sensitivity to environmental conditions and not being reusable limits the use of Xyl enzyme.^[3] These annoying problems can be overcome by the immobilization of enzymes on solid supports that allow them to be easily separated from the reaction medium after catalytic

cycling. Moreover, the immobilization strategy allows the repeated use of enzymes as well as maintaining pH, thermal and storage stability of the enzymes.^[10] In addition, enzyme immobilization offers better operation control, flexibility of reactor design, and ease separation of enzyme from the reaction product; thereby minimizing the chances of product contamination.^[11–16]

The selection of an appropriate support is an important key to the immobilization process. Otherwise, the activity of the enzyme may be significantly inhibited or even completely denatured. In general, a support is considered ideal when it has low cost, high physical and chemical stability, good biocompatibility, and many points for enzyme binding.^[17] To date, Xyl has been immobilized on various kinds of supports such as organic,^[18] inorganic,^[19] and hybrid materials.^[3] Among them, nanoparticles (NPs) as immobilization support have received great attention due to their specific surface area, facile modification, simple separation, mass transfer resistance, and effective enzyme loading.^[20,21] Owing to their excellent properties, NPs have been used widely in the study of immobilized enzymes in recent years. For instance, trichlorotriazine-functionalized polyethylene glycol grafted magnetic NPs,^[2] 1,3,5-triazine-functionalized silica-encapsulated magnetic NPs,^[19] SiO₂ NPs,^[22] and carbon-coated chitosan NPs^[23] were employed as support for Xyl immobilization.

1. Introduction

Xylan is a linear homopolymer containing D-xylose monomers linked via β -1,4-glycosyl bonds and is considered the second most abundant renewable biomass in nature after cellulose.^[1] Nowadays, xylan-degrading enzymes have managed to attract the attention of researchers as one of the most important commercial enzymes because of their widespread applications in several industrial processes such as juice purification, power generation, raw

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Starch containing amylose and amylopectin is one of the natural biopolymers, which is biocompatible, biodegradable, and bio-adhesive.^[24] Due to its biodegradability, abundance, and low cost, starch is widely used as a support in drug and enzyme carrier systems.^[25] Most importantly, starch can form hydrogen bonds between the enzyme due to its hydroxyl units, increasing the immobilization capacity and also providing structural stability.^[26] For example, the lipase enzyme was immobilized on the magnetic NPs by dialdehyde starch from Yang et al.^[27] Similarly, starch-polyester biosupport was used for lipase immobilization.^[28] In our previous works, poly(2-hydroxyethyl methacrylate) (pHEMA)-starch composites,^[29] poly(methyl methacrylate) (PMMA)-starch composites,^[30] and starch functionalized biodegradable p(MAA-co-methacrylic acid)^[26] were utilized for immobilization of L-asparaginase. On the other hand, starch nanoparticles (SNPs) have been shown to have advantageous physicochemical and biological properties compared to natural starch such as larger reaction surface, absorption capacity, and biological penetration rate; therefore, they are more suitable to be used as carriers to facilitate the transport of bioactive compounds.^[31]

In recent years, the increase in healthy nutrition has also increased the demand for fruit juice consumption. This situation has increased the importance of fruit juice in the beverage industry at the commercial level. The most challenging process in commercially produced fruit juices is the removal of turbidity caused by hemicellulose, pectin, and starch in fresh fruit juices.^[32] In this context, enzymatic processing or clarification of fruit juices increases juice yield, shelf life, and storage stability, and preserves the natural nutritional quality of fruit juice. Enzymatic processes are gaining importance in juice clarification due to the low toxicity, mild reaction conditions, green chemistry, and substrate specificities of the enzymes.^[33] Xyl enzyme, which has recently gained more importance in the food industry, attracts the attention of researchers in the juice clarification process.

To the best of our knowledge, it is worth noting that this study is the first approach to investigate the use of SNPs as support for Xyl immobilization. Besides, we anticipate that effectiveness of Xyl@SNPs in fresh juice clarification process will lead to its application in the industrial field. Motivated by the superior features and many applications of SNPs, herein, the bare SNP of hydrodynamic diameter range between 200 and 300 nm were synthesized by a simple nanoprecipitation method from native potato starch (*Solanum tuberosum*). Subsequently, using the adsorption technique, Xyl from *Aspergillus oryzae* was immobilized into SNPs. It is well known that adsorption is the easiest immobilization method without changing the natural structure of the enzyme.^[34] The functional groups, crystal structure, surface morphology, elemental composition, and hydrodynamic diameters of the synthesized SNPs were analyzed. The aforementioned features of the Xyl@SNPs were evaluated further. The effect of temperature and pH was extensively investigated to achieve outstanding results. Kinetic parameters changed by immobilization were determined and compared with commercial free Xyl. In addition, the reusability of Xyl@SNPs was examined by performing successive catalytic cycles under optimized conditions. Eventually, Xyl@SNPs were used by measuring the transmittance for orange juice clarification.

2. Experimental Section

2.1. Materials

All the reagents were of analytical grade and were used without any further purification. Xyl from *A. oryzae* (≥ 2500 units g^{-1} , recombinant), soluble starch from potato, absolute ethanol, and xylose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Beechwood xylan used as substrate was obtained from (Carl Roth, Karlsruhe, Germany). 3,5-Dinitrosalicylic acid (DNS) was supplied by Alfa Aesar (Haverhill, MA, USA). Urea and sodium hydroxide (NaOH, $\geq 98\%$) were purchased from Merck (Darmstadt, Germany). Enzyme and substrate solutions were prepared freshly just before use. Type 1 ultrapure water (Millipore Direct-Q 3 water purification system, Merck KGaA, Darmstadt, Germany) was used to prepare all buffers and aqueous solutions.

2.2. Analytical Instruments

The functional groups of the samples were analyzed by FTIR spectroscopy (PerkinElmer) in the range of $650\text{--}4000\text{ cm}^{-1}$ at a resolution of 4 cm^{-1} . The crystallographic spectra were recorded on XRD (Rigaku RadB-DMAX II) with Cu $K\alpha$ radiation in the 2θ range of $10^\circ\text{--}80^\circ$. The SEM micrographs of SNPs and SNPs@Xyl were collected using an EVO 40-LEO computer-controlled SEM device (LEO Ltd., Cambridge, UK). The elemental composition was determined via an EDX detector (model 125 eV; Bruker AXS, Berlin, Germany). The hydrodynamic diameters of the synthesized SNPs along with the polydispersity index (PDI) were obtained using a particle size analyzer (Litesizer 500, Anton Paar, Graz, Austria). The absorbance measurements were performed on an Eon Microplate Reader controlled by Gen5 2.0 Data Analysis Software (BioTek Instruments, Inc., Winooski, VT, USA).

2.3. Preparation of SNPs and Xyl@SNPs

The SNPs were prepared by the addition of starch solution into excess absolute ethanol under controlled conditions according to Chin et al.^[24] with minor modifications. Briefly, NaOH/urea (0.8:1.0 wt%) solution mixture was used as a solvent system to dissolve potato starch. Afterward, 1% wt of potato starch solution was prepared by using the prepared solvent medium at room conditions. One milliliter of starch solutions was added dropwise into 15 mL of absolute ethanol that was stirred continuously using a magnetic stirrer. The resulting mixtures were then centrifuged at 14 000 rpm for 15 min and the supernatants were rinsed three times with absolute ethanol to remove NaOH and urea. The washed SNPs were dried in a vacuum oven (50°C) overnight. The preparation method of Xyl@SNPs was performed as follows: 50 U mL^{-1} of Xyl solution was placed in the tube with 25 mg of SNPs. Then the solution was stirred at 250 rpm at 4°C overnight. Finally, the regenerated SNPs and SNPs@Xyl were collected under similar centrifugation conditions and freeze-dried overnight. The lyophilized SNPs and Xyl@SNPs were stored at 4°C for characterization processes and further use. **Figure 1** shows a schematic representation of the preparation of SNPs and immobilization of Xyl.

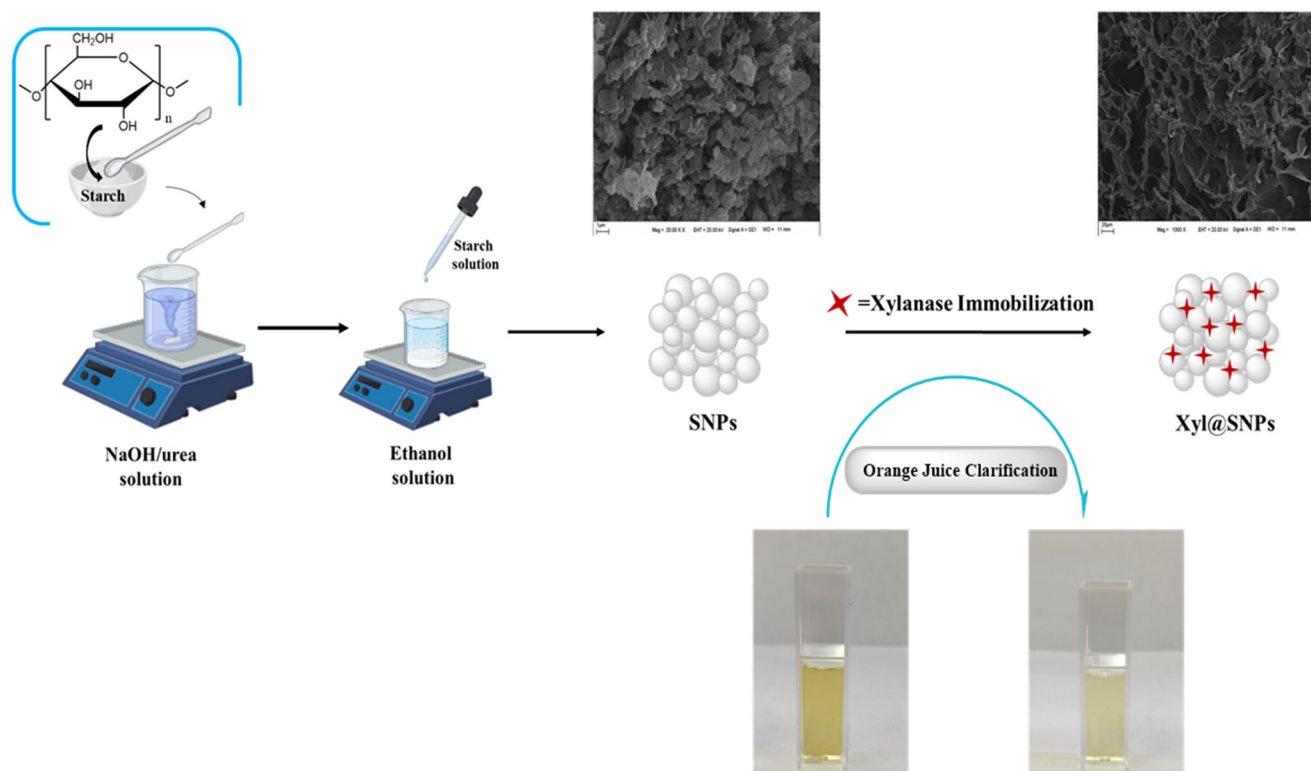


Figure 1. Schematic representation of the preparation of SNPs and immobilization of Xyl. SNP, starch nanoparticle; Xyl, xylanase.

2.4. Catalytic Assay of Free Xyl and Xyl@SNPs

Xyl activity was determined by measuring the amount of reducing sugars (xylose equivalents) produced from beechwood xylan hydrolysis using the DNS method.^[2] In brief, a xylan solution (1% w/v) was prepared by dissolving xylan in 50 mM acetate buffer, pH 6.0. An appropriate amount of free Xyl (0.4 U) and Xyl@SNPs (2 mg) was incubated with 1 mL of xylan solution for 10 min at 50 °C. Afterward, the Xyl@SNPs were recovered using a centrifuge process, and 1.5 mL DNS was added into the test tubes containing both free Xyl and Xyl@SNPs to terminate the process. The test tubes containing the reaction solution were heated in boiling water. After boiling for 5 min, it was cooled at 25 °C and the absorbance measurements at 540 nm of released reducing sugars were performed on an Eon Microplate Reader controlled by Gen5 2.0 Data Analysis Software (BioTek Instruments, Inc., Winooski, VT, USA). The amount of released reducing sugars was determined from the xylose standard curve. One unit (IU) of Xyl activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of reducing sugar per minute equivalent of xylose under optimal assay conditions.^[35] All measurements were performed in triplicate and results were presented as mean ± standard deviation.

2.5. Effect of pH and Temperature

The effect of pH and temperature on the activity of free Xyl and Xyl@SNPs was evaluated at varying pH and temperature values,

according to the standard assay condition. The effect of pH was estimated by incubating free Xyl and Xyl@SNPs with 1% of xylan solution at varying pH values ranging from 4.0 to 9.0. For the effect of temperature, the free Xyl and Xyl@SNPs were incubated for 10 min at a temperature range of 30–80 °C, with 10 °C intervals. The highest activity was considered as 100% for each enzyme sample and the other results were presented as relative activity. In addition, the pH and temperature values, where the highest relative activity was observed, were recorded as optimum.

Additionally, the activation energy (E_a) of both free Xyl and Xyl@SNPs was calculated from the slope of the Arrhenius plot according to the following Equation (1). The slope was determined by plotting the graph of the log of relative activity versus $1000/T$ [K].^[36]

$$\text{Slope} = -\frac{E_a}{R} \quad (1)$$

The temperature coefficient value (Q_{10}) for optimal temperatures of free Xyl and Xyl@SNPs was calculated via the following Equation (2).^[37]

$$Q_{10} = \text{antilog} (E_a 10/RT) \quad (2)$$

where E_a is the activation energy (kJ mol^{-1}), R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), and T is the absolute temperature (K).

2.6. Reusability of Xyl@SNPs

Reusability (operational stability) can make the enzyme economically viable and is one of the key parameters for cost-effective use in large-scale applications. The activity of Xyl@SNPs was measured in optimal assay conditions (60 °C and pH 8.0) according to the mentioned method for determination of activity. Following each reaction step, the Xyl@SNPs were removed by centrifugation and washed with Tris-HCl buffer (50 mM, pH 8.0). After washing, it was used for the next test of substrate hydrolysis. The above steps were repeated seven times. Using the obtained data, the residual activity was measured using the initial activity of 100%.

2.7. Kinetic Profiling of Free Xyl and Xyl@SNPs

The kinetic constant (K_m) and maximum reaction rate (V_{max}) of free Xyl and Xyl@SNPs were evaluated by using different xylan concentrations (1–10 mg mL⁻¹). Linear regressions were fitted to Lineweaver–Burk plots and K_m and V_{max} values were calculated according to slope and intercept, respectively. Additionally, the turnover number (k_{cat}), catalytic efficiency (k_{cat}/K_m), and specificity constants (V_{max}/K_m) were estimated according to Wehaidy et al.^[38]

2.8. Preparation and Clarification of Orange Juice

The clarification process was carried out as reported by Haile et al.^[39] Oranges (*Citrus sinensis*) were purchased from the local market. The fresh orange juices were prepared by a blender and fruit pulps were separated from the juices by centrifuging at 5000 rpm for 10 min. The juice yield was calculated via Equation (3). After that, 2 mL of juice pulp was treated with 15 mg of Xyl@SNP at 60 °C for 2 h versus the untreated juice sample as a control. The samples were heated for 5 min in a boiling water bath to inactivate them at the end of the incubation period. Afterward, the samples were centrifuged at 5000 rpm for 5 min after treatments. By measuring the transmittance (T) at a wavelength of 660 nm through a UV–Vis spectrophotometer (Shimadzu 1601, Kyoto, Japan), the clarity of the juice was evaluated.

$$\text{Juice yield (\%)} = \frac{\text{weight of juice}}{\text{weight of fruit}} \times 100 \quad (3)$$

2.9. Statistical Analysis

Experiments were performed in triplicate and data were expressed as mean $\pm$ standard deviation. Statistical analysis was performed by GraphPad Prism software version 8.0 (GraphPad Software, USA). Statistical significance was considered significant when the p -value was less than 0.05.

3. Results and Discussion

3.1. FTIR Characterization

The FTIR spectra of the native starch, SNPs, and Xyl@SNPs are shown in **Figure 2**. FTIR spectra of native starch and SNP were

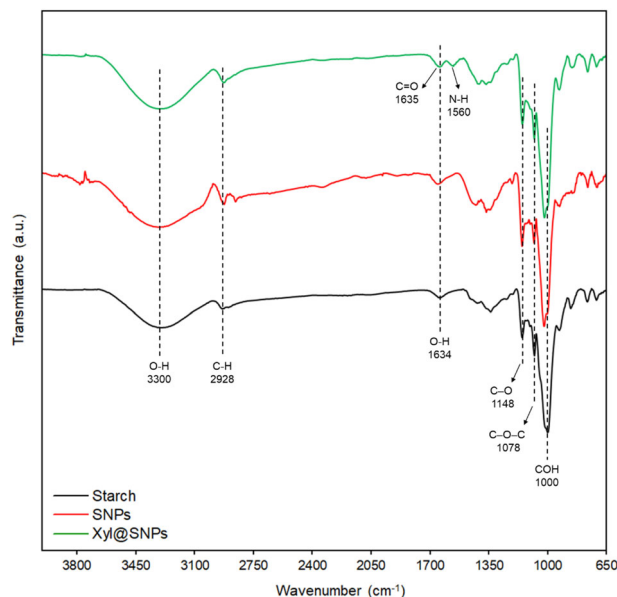


Figure 2. FTIR spectra of starch, SNPs, and Xyl@SNPs. FTIR, Fourier transform infrared spectrometry; SNP, starch nanoparticle; Xyl, xylanase.

remarkably similar. The spectra of the native starch and SNPs exhibited a characteristic peak around 3300 cm⁻¹ that may be assigned to inter- and intramolecular hydrogen-bonded hydroxyl groups.^[40] A small peak at 2928 cm⁻¹ was also observed due to the C–H stretching vibrations.^[40,41] The different wavenumber and their identification were attributed to 1634 cm⁻¹ (OH bending of water), 1148 cm⁻¹ (C–O stretching), 1078 cm⁻¹ (C–O–C symmetrical stretching), and 1000 cm⁻¹ (COH bending).^[42] However, as noted by Andrade et al.,^[43] some bands in the SNP spectrum showed different intensities compared to native starch, especially in the fingerprint region (under 800 cm⁻¹ and between 800 and 1500 cm⁻¹) where the characteristic peaks of starch were observed. Similarly, the characteristic bands of the FTIR spectrum of Xyl@SNPs were identical to those of the SNPs. Additionally, compared with SNPs, absorption bands at 1635 and 1560 cm⁻¹ can be seen in the spectrum of Xyl@SNPs, which were related to C=O stretching (amide I) and N–H bending (amide II) of the enzyme, respectively.^[44,45] All these spectral results indicated that the SNPs were successfully synthesized and Xyl was immobilized into SNPs.

3.2. XRD Investigation

To check the crystallinity of SNPs and Xyl@SNPs, XRD analysis was performed. **Figure 3** shows the XRD diffraction patterns of the SNPs and Xyl@SNPs. The SNPs displayed a V-type structure with 2 θ sharp characteristic diffraction peaks at 13.8° (111) and 20.0° (211) with low intensity and broad amorphous diffraction peaks. Similar results were obtained from previous studies.^[46,47] For Xyl@SNPs, the same two major characteristic peaks were also observed, but, the changes in peak intensities occurred, which is probably due to the immobilization process. Altogether, this implies that there was no phase change in SNPs after Xyl immobilization.

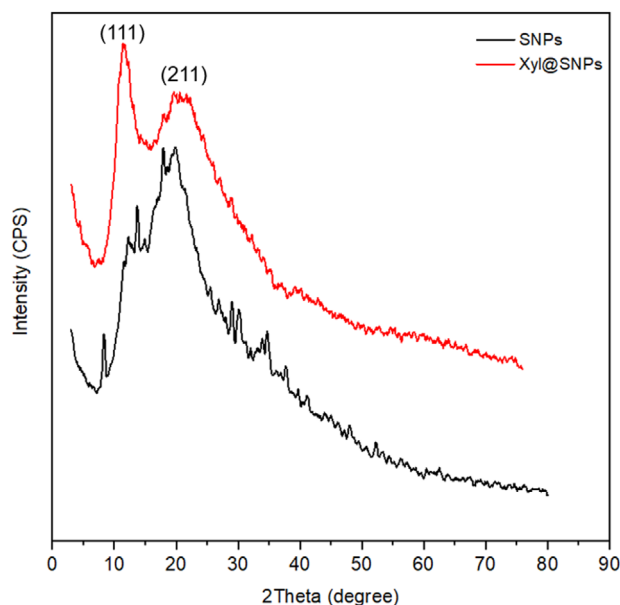


Figure 3. XRD patterns of SNPs and Xyl@SNPs. SNP, starch nanoparticle; XRD, X-ray diffraction; Xyl, xylanase.

3.3. SEM-EDX Characterization

The SEM micrographs of the SNPs and Xyl@SNPs are shown in **Figure 4**. As shown in Figure 4A, the SNPs were irregular in shape and size. Chin et al. reported^[24] that a mixture of spherical SNPs and elongated fiber-like particles were formed when the volume ratio of starch solution to absolute ethanol was changed to 1:15. Based on the previous characterization methods, SNP particles were successfully prepared, which was consistent with the conclusions of Chin et al.^[24] and Ahmad et al.^[48] In addition, the micrographs of the Xyl@SNPs demonstrated an increase in particle size and the formation of porous cavities on the surface of SNPs (Figure 4B), which can be attributed to the successful immobilizing of the Xyl enzyme.

EDX measurement was also carried out for the elemental analysis of the SNPs and Xyl@SNPs as shown in **Figure 5**. EDX measurements were performed at three different regions. Since starch consists of glucose units, the presence of carbon (C), hydrogen (H), and oxygen (O) elements is expected. As expected, peaks of C and O were observed. However, H could not be distinguished because there are no core electrons in H, only valence electrons.^[49] The presence of the sodium (Na) peak was caused by the NaOH used in the synthesis process. The EDX analysis also confirmed the successful synthesis of Xyl@SNPs, as the peaks showed the presence of nitrogen (N) and sulfur (S) in SNPs. The N and S peaks indicated that the Xyl enzyme was successfully immobilized into SNPs.

3.4. DLS Analysis

The hydrodynamic diameters of the synthesized SNPs and Xyl@SNPs were measured by DLS (**Figure 6**). The hydrodynamic diameters of the SNPs and Xyl@SNPs were found to be 200 ± 9.8 nm and 286 ± 5.2 nm, respectively. The increase in hydrody-

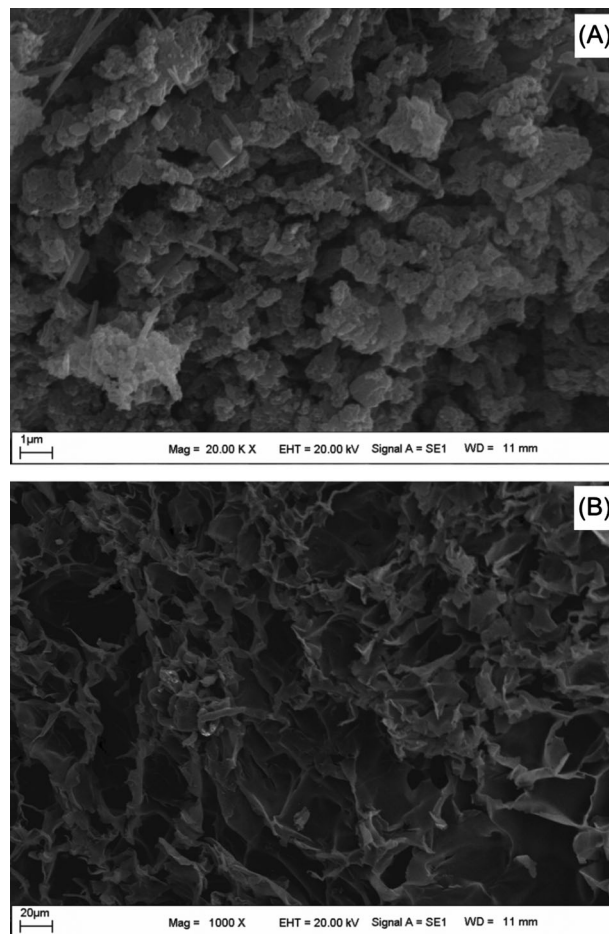


Figure 4. Micrographs obtained by SEM of SNPs A) and Xyl@SNPs B). SEM, scanning electron microscopy; SNP, starch nanoparticle; Xyl, xylanase.

namic diameter of Xyl@SNPs compared to SNPs was due to the presence of immobilized Xyl. This is consistent with our previous report.^[50] The polydispersity of the synthesized SNPs was also noted. The PDI values were determined to be 0.284 ± 0.004 for SNPs and 0.241 ± 0.011 for Xyl@SNPs. As a result, the low PDI values obtained for both formulations indicated that the suspension of SNPs produces narrow size distribution and consequently homogeneous distribution.^[51]

3.5. Effect of pH and Temperature

In this study, two key variables that affect enzyme activity (pH and temperature) were examined. **Figure 7A** shows the activity profiles of the free Xyl and Xyl@SNPs, the pH of which ranged from 4 to 9. As illustrated in Figure 7A, the behavior of free Xyl and Xyl@SNPs varied in buffers with different pH levels. The free Xyl has an optimum pH of 6.0 whereas that of the Xyl@SNPs shifted 2.0 units to a more alkali pH value (8.0). Similarly, Rezayat et al.^[52] and Kharazmi et al.^[2] reported the optimum pH value for free and immobilized Xyl as 6.5 and 7.5, respectively. The binding of the support material and enzyme, which caused the enzyme to change in conformation, was the primary cause of the altered

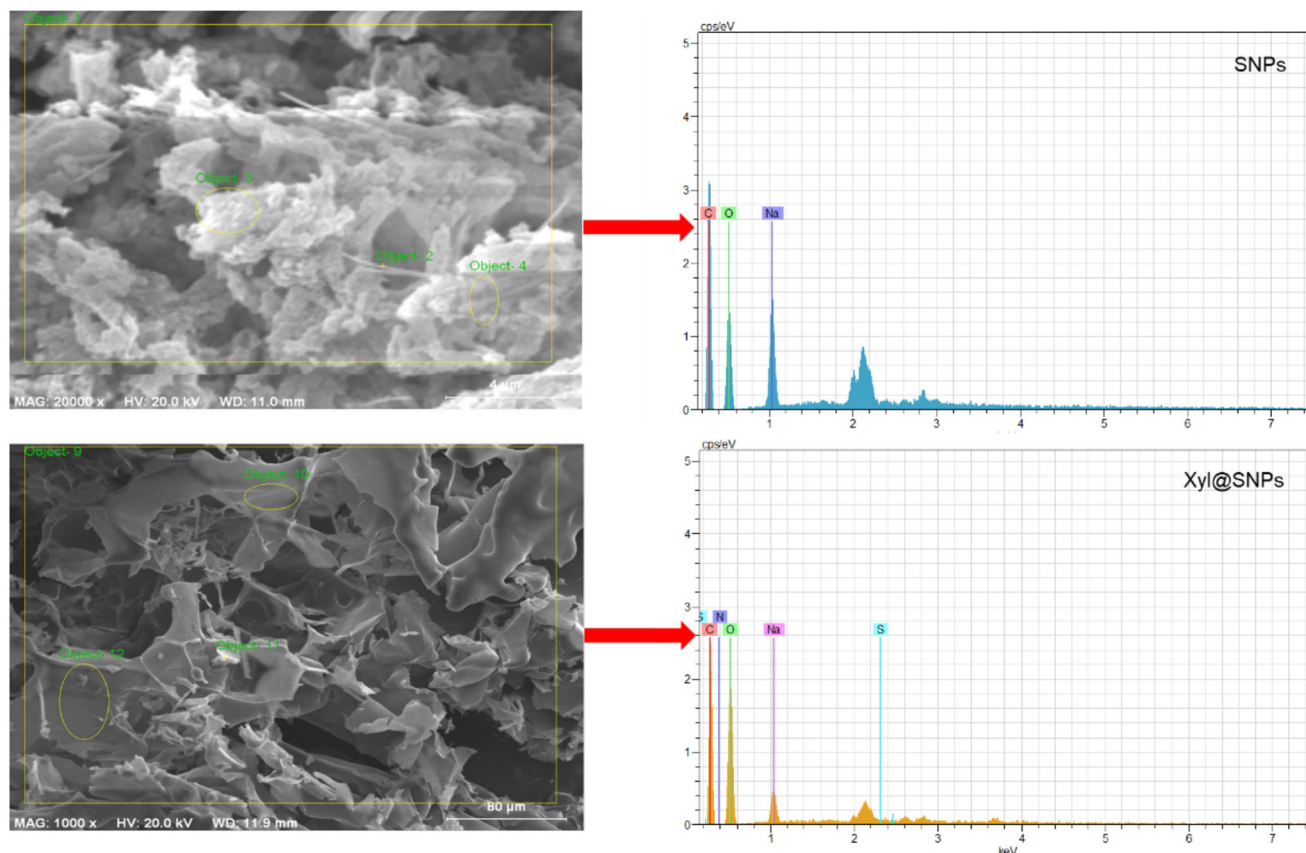


Figure 5. SEM-EDX analysis of SNPs and Xyl@SNPs. EDX, energy dispersive X-ray; SEM, scanning electron microscopy; SNP, starch nanoparticle; Xyl, xylanase.

properties of Xyl after immobilization. Comparatively, at lower pH values, free Xyl was more tolerant to pH changes, whereas the Xyl@SNPs were more stable than the free Xyl in the pH range of 7.0–9.0. Analysis results showed that immobilization changed

the pH stability of the enzyme. Similarly, these observations were consistent with previous studies.^[33,53]

The effect of temperature on the activity of commercial enzymes such as Xyl is of vital importance in bioprocesses. As shown in Figure 7B, the effect of temperature on the reactions was monitored between 30 and 80 °C. For free Xyl and Xyl@SNPs, the optimal temperatures were 70 and 60 °C, respectively. Both free Xyl and Xyl@SNPs showed a sharp decline in relative activity at temperatures above 70 °C due to thermal denaturation. Amazingly, they still had more than 50% activity at 80 °C. However, the interaction between SNPs and Xyl does not allow the enzyme molecules to be freer. Although this resulted in decreased enzymatic activity, Xyl@SNPs showed high relative activity over a wide temperature range of 30–80 °C, suggesting that their immobilization effectively stabilized the enzyme. The temperature tolerance for free and immobilized Xyl in the literature was similar to these results in a broad sense.^[1,54,55] For instance, Amaro-Reyes et al.^[56] reported that as a result of the immobilization of Xyl to the magnetic chitosan carrier support, the temperature at which free and immobilized Xyl had maximum activity was 70 °C. In another study, Kumar et al.^[57] reported that the optimum temperature of free Xyl and immobilized Xyl to nanoflowers remained unchanged at 65 °C.

The E_a values of both the free Xyl and Xyl@SNPs were estimated using the Arrhenius plot (Figure 8A, B). The E_a of Xyl@SNPs (8.43 kJ mol⁻¹) was higher than the free Xyl

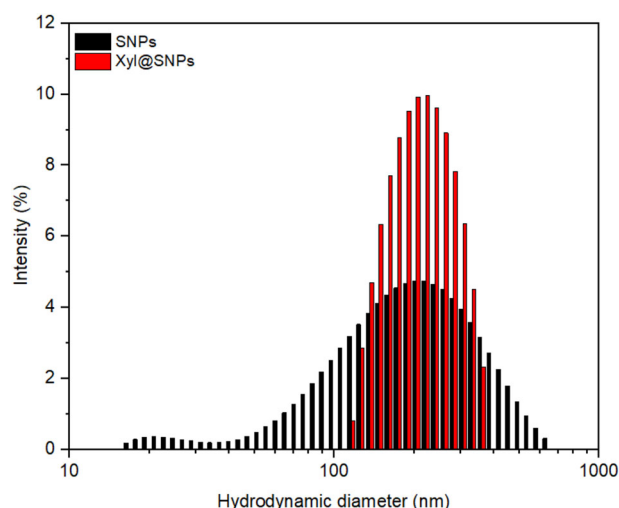


Figure 6. DLS examination for the hydrodynamic diameter distribution of SNPs and Xyl@SNPs. DLS, dynamic light scattering; SNP, starch nanoparticle; Xyl, xylanase.

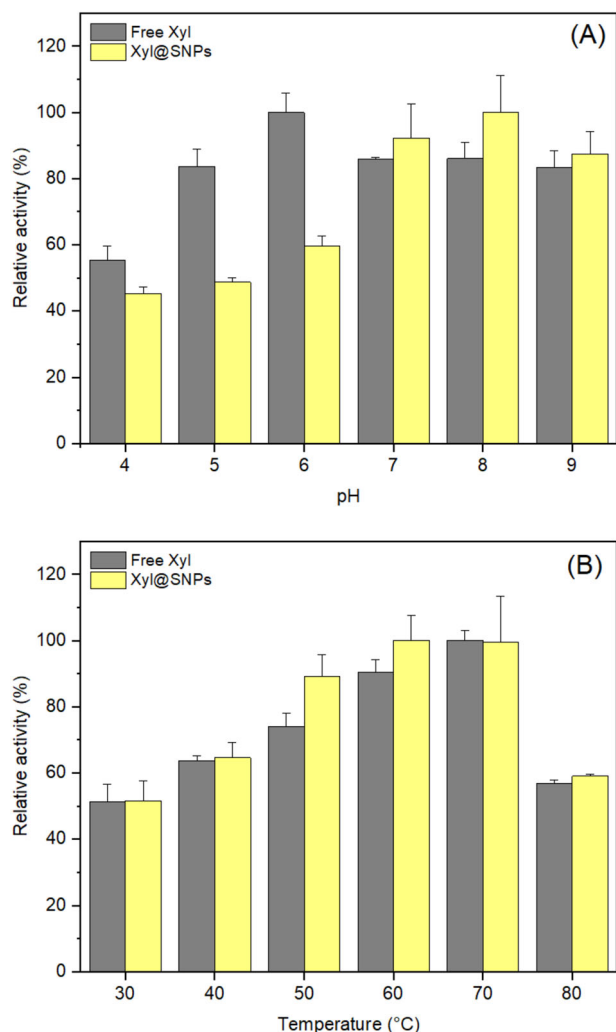


Figure 7. The effect of pH A) and temperature B) on the activity of free Xyl and Xyl@SNPs. SNP, starch nanoparticle; Xyl, xylanase.

(6.32 kJ mol⁻¹). E_a values for enzyme-catalyzed reactions are typically lower than those for non-enzyme-catalyzed reactions. The immobilization process, however, may or may not cause the E_a value of an enzymatic reaction to change, and any change is typically unpredictable.^[36] Several studies reported a similar increase in E_a value for various immobilized enzymes.^[58,59]

As another important kinetic parameter, Q_{10} (temperature coefficient) was calculated using E_a . The Q_{10} values for the free Xyl and Xyl@SNPs at their optimal temperatures were found to be 1.05 and 1.07, respectively. Generally, the Q_{10} for enzymes ranges from 1 to 2.^[60] The deviation from the Q_{10} value shows that reactions are not temperature dependent (other assay factors participate in controlling the reaction rate than temperature). This result is consistent with that reported by Glekas et al.^[37]

3.6. Reusability of Xyl@SNPs

Another limitation in the industrial application of free enzymes is reusability as free enzymes cannot be recovered after their use.^[61]

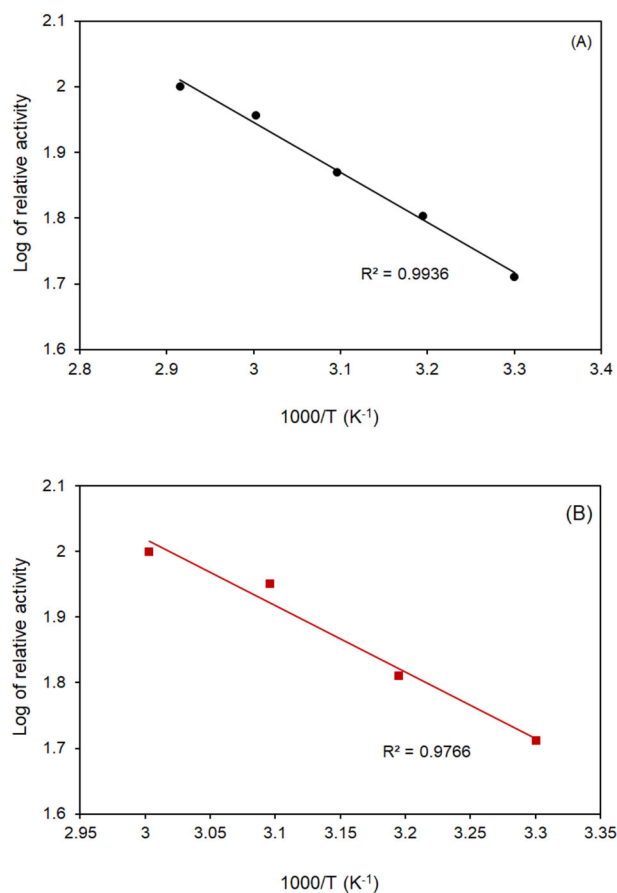


Figure 8. Arrhenius plots to calculate E_a for the free Xyl A) and Xyl@SNPs B). SNP, starch nanoparticle; Xyl, xylanase.

In the present study, the reusability of Xyl@SNPs was evaluated by repeated use for seven cycles. As shown in **Figure 9**, high relative activity was even observed after seven recycles. Xyl@SNPs remained at 83.2% and 69.1% of their initial activity, respectively after three and five reuses. However, recycling performance decreased significantly as the number of recycling increased. After the seventh use, Xyl@SNPs retained 62.8% of its initial activity. This loss in activity may be related to weakened bonds formed between the SNPs and the Xyl, which simply causes the enzyme to leak. Additionally, the loss of a few particles and denaturation of the Xyl from continued use diminishes residual activity.^[62]

3.7. Kinetic Profiling of Free Xyl and Xyl@SNPs

The K_m and V_{max} of free Xyl and Xyl@SNPs calculated from their corresponding Lineweaver–Burk plot (**Figure 10**) are shown in **Table 1**. In addition, the kinetic parameters such as the turnover number (k_{cat}), catalytic efficiency (k_{cat}/K_m), and specificity constants (V_{max}/K_m) of the free Xyl and Xyl@SNPs were presented in Table 1. K_m and V_{max} of the free Xyl were 13.73 ± 2.79 mg mL⁻¹ and 0.54 ± 0.06 μ mol min⁻¹ mL⁻¹, respectively, whereas K_m and V_{max} of the Xyl@SNPs were 8.33 ± 1.64 mg mL⁻¹ and 0.33 ± 0.04 μ mol min⁻¹ mL⁻¹, respectively. The k_{cat} of the free Xyl and Xyl@SNPs was 0.023 ± 0.002 and 0.014 ± 0.001 s⁻¹, respectively.

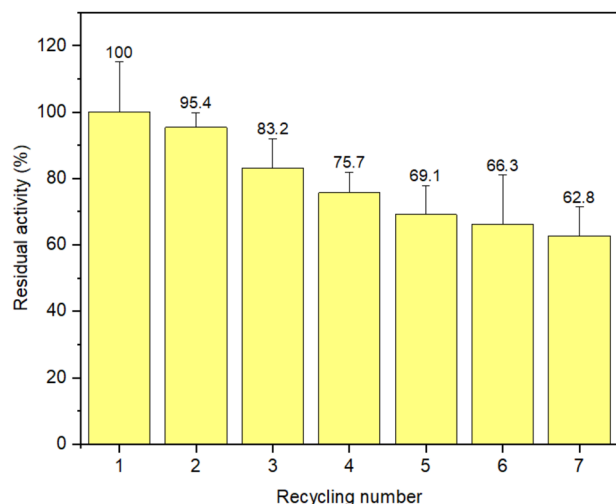


Figure 9. The reuse stability of Xyl@SNPs at different catalytic cycles. In each reaction, Xyl@SNPs were incubated with the fresh substrate at 60 °C for 10 min. SNP, starch nanoparticle; Xyl, xylanase.

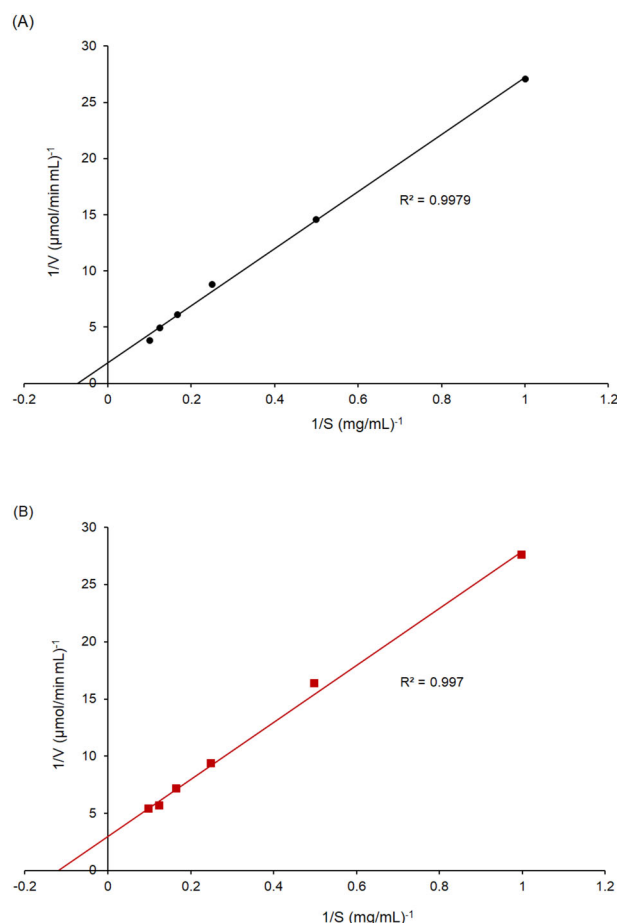


Figure 10. Comparative Lineweaver–Burk plots of free Xyl A) and Xyl@SNPs B). SNP, starch nanoparticle; Xyl, xylanase.

Table 1. Kinetic constants of free Xyl and Xyl@SNPs.

Parameter	Enzyme	
	Free Xy	Xyl@SNPs
K_m [mg mL ⁻¹]	13.73 ± 2.79 ^a	8.33 ± 1.64 ^a
V_{max} [μmol min ⁻¹ mL ⁻¹]	0.54 ± 0.06 ^a	0.33 ± 0.04 ^a
k_{cat} [s ⁻¹]	0.023 ± 0.002 ^a	0.014 ± 0.001 ^b
k_{cat}/K_m [mL mg ⁻¹ s ⁻¹]	0.0017 ± 0.0001 ^a	0.0019 ± 0.0001 ^a
V_{max}/K_m	0.040 ± 0.003 ^a	0.040 ± 0.004 ^a

Different letters, on the same line, indicate statistically significant difference ($p < 0.05$). SNP, starch nanoparticle; Xyl, xylanase.

After Xyl immobilization, Xyl@SNPs showed decreased kinetic parameters compared to the free Xyl: 1.64-fold in K_m , 1.63-fold in V_{max} , and 1.64-fold in k_{cat} . Among them, only the change in k_{cat} value is statistically significant ($p < 0.05$). K_m is a measure of the affinity of an enzyme to substrate and a low K_m value represents high affinity. A decrease in the K_m was seen from the Xyl@SNPs compared with the free Xyl, indicating the increased affinity between enzyme and substrate. k_{cat} represents the maximum number of reactions catalyzed per minute. On the other hand, V_{max} and k_{cat} were decreased because the substrate could not bind to the active sites of Xyl@SNPs due to increased diffusion limitation.^[63] In contrast, Xyl@SNPs compared to the free Xyl did not change in catalytic efficiency (k_{cat}/K_m), and specificity constants (V_{max}/K_m). Overall, the results of the study clearly show that the immobilization process increased substrate affinity and did not result in a significant change in the catalytic activity of the enzyme. These findings were in line with the majority of studies on the immobilization of enzymes.^[56,63,64]

3.8. Preparation and Clarification of Orange Juice

The presence of components such as hemicellulose, starch, pectin, and cellulosic causes the turbidity of natural juices. Therefore, the degradation of these components by appropriate enzymes positively affected the clarification, quality characteristics, and time storage of the juices. Hydrolysis of xylan, one of the most abundant components of fruit cell wall polysaccharides, is beneficial in the clarification of fruit juices.^[19] In this investigation, the potential of the Xyl@SNPs was evaluated in clarification of the juice obtained from orange pulps with 88.77% yield. As shown in **Figure 11**, 70.8% of transmittance was observed after 1 h treatment of orange juice with the Xyl@SNPs, while control exhibited 55.9%. In continuous processing, the results demonstrated that the Xyl@SNPs had 76% of transmittance after 2 h treatment. An increase in transmittance (%) indicates degradation of xylan, thus making the juices clear and palatable.^[65] The clarification data in this study appear comparable to previous studies. For instance, according to Alagöz et al.,^[33] the clarity of orange juice treated with Xyl/Si-NH₃ was increased from 58.9% to 75.4%. In another work, the Xyl-MNPs illustrated 77.4(±1.3)% of turbidity for orange juices, after 2 h incubation at 50 °C.^[19] Similarly, an increase in orange juice clarity (24%–29%) was noted when using the Xyl immobilized on glutaraldehyde-activated aluminum oxide pellets, and this

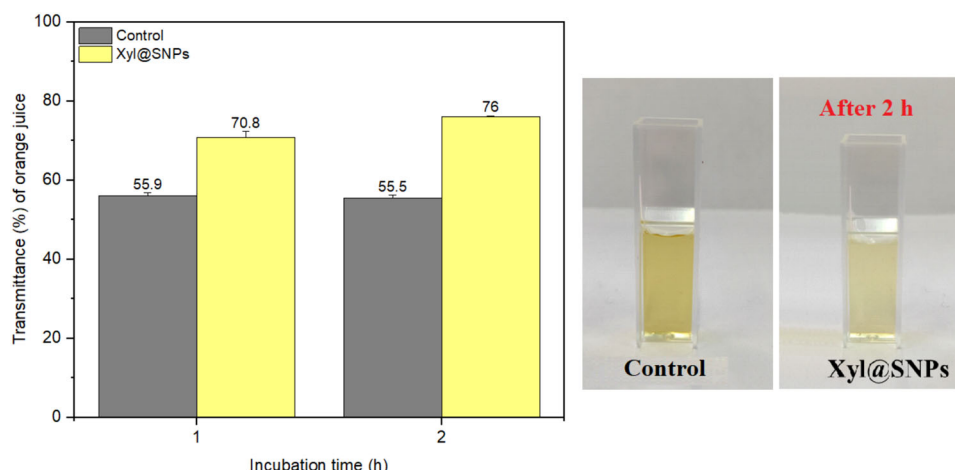


Figure 11. The effect of Xyl@SNPs on orange juice clarification. SNP, starch nanoparticle; Xyl, xylanase.

was slightly greater when immobilized compared to free Xyl.^[66] These results demonstrated that immobilization of Xyl on SNPs not only improves its stability and reusability but also provides cost-effective benefits in the juice industries. These advantages are due to the superior properties of SNPs such as low toxicity, significant biocompatibility, and good reusability.

4. Conclusion

Recent years further witnessed the application of support tools to attain more stable and well-recovered biocatalysts. Herein, we validated a novel, robust, and suitable biocatalyst based on SNPs for Xyl immobilization. This study selected SNPs as the immobilization support because of their large surface area and high adsorption capacity. The SNPs and Xyl@SNPs were successfully prepared through a simple nanoprecipitation method. The SNPs and Xyl@SNPs were elucidated by FTIR, XRD, SEM, EDX, and DLS. The FTIR and EDX analysis confirmed that the immobilization of the Xyl was achieved. The use of SNPs as a support was promising for Xyl with an immobilization efficiency of 91%. The effect of pH and temperature on the enzyme activity was examined. Optimum pH values for free Xyl and Xyl@SNPs were found to be 6.0 and 8.0, respectively, while optimum temperature values were 70 and 60 °C, respectively. Besides, the Xyl@SNPs displayed better tolerance to acidic/alkaline pH values and high temperatures compared with free Xyl, and excellent operational stability. The Xyl@SNPs can be reused for up to seven cycles. After optimization of different conditions, the K_m of the Xyl@SNPs was $8.33 \pm 1.64 \text{ mg mL}^{-1}$, which was 1.64 times lower than the free Xyl form. This affinity was supported by the resulting interactions between the SNPs and the Xyl enzyme. To summarize, this study not only presents a new carrier material for Xyl immobilization to the literature but also provides a basis and reference with scientific and promising results. Meanwhile, the proposed immobilized enzyme is expected to find wide application in juice clarification. In future work, the authors will investigate the possibility of using the Xyl@SNPs in some industrial processes. In conclusion, the prepared SNPs can be used as a suitable new carrier for the stabilization of the Xyl enzyme by immobilization and juice clarification.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

enzyme immobilization, juice clarification, starch nanoparticles, xylanase, improved stability

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- [1] J. P. dos Santos, E. R. da Zavareze, A. R. G. Dias, N. L. Vanier, *Int. J. Biol. Macromol.* **2018**, *118*, 1676.
- [2] S. Kharazmi, A. Taheri-Kafrani, A. Soozanipour, M. Nasrollahzadeh, R. S. Varma, *Int. J. Biol. Macromol.* **2020**, *163*, 402.
- [3] P. Jampala, M. Preethi, S. Ramanujam, B. S. Harish, K. B. Uppuluri, V. Anbazhagan, *Int. J. Biol. Macromol.* **2017**, *95*, 843.
- [4] J. Both, B. Biduski, M. Gómez, T. E. Bertolin, M. T. Friedrich, L. C. Gutkoski, *J. Food Sci. Technol.* **2021**, *58*, 3902.
- [5] J. Kaushal, M. Khatri, G. Singh, S. K. Arya, *Int. J. Biol. Macromol.* **2021**, *193*, 1350.
- [6] C. Azeri, A. U. Tamer, M. Oskay, *Afr. J. Biotechnol.* **2012**, *9*, 63.
- [7] A. Marasabessy, M. R. Moies, J. P. M. Sanders, R. A. Weusthuis, *Appl. Microbiol. Biotechnol.* **2011**, *90*, 2027.
- [8] M. A. Al-Ahdal, E. M. Ali, O. A. Baothman, A. I. Al-Sieni, H. A. Al-Talhi, *Biofuels*, <https://doi.org/10.1080/17597269.2023.2201731>.

- [9] A. L. Petry, N. F. Huntley, M. R. Bedford, J. F. Patience, *J. Anim. Sci.* **2021**, 99, 7.
- [10] F. Kazenwadel, H. Wagner, B. E. Rapp, M. Franzreb, *Anal. Methods* **2015**, 7, 10291.
- [11] F. Amin, H. N. Bhatti, M. Bilal, M. Asgher, *Int. J. Biol. Macromol.* **2017**, 95, 974.
- [12] F. Amin, H. N. Bhatti, M. Bilal, M. Asgher, *Appl. Biochem. Biotechnol.* **2017**, 183, 426.
- [13] F. Amin, A. Mohsin, H. N. Bhatti, M. Bilal, *Biochim. Biophys. Acta Proteins Proteom.* **2020**, 1868, 140379.
- [14] F. Amin, T. Arooj, Z.-H. Nazli, H. N. Bhatti, M. Bilal, *Biomass Convers. Biorefin.* **2023**, 13, 11141.
- [15] F. Amin, S. A. Asad, M. Bilal, in *Nanomaterials for Biocatalysis*, Elsevier, Cambridge, MA 02139, United States **2022**, pp. 595–621.
- [16] F. Amin, H. N. Bhatti, M. Bilal, M. Asgher, *Int. J. Food Eng.* **2017**, 13, 20160256.
- [17] S. A. M. Abdel-Hameed, S. A. Ahmed, F. A. Mostafa, O. N. Almasarawi, W. A. Abdel Wahab, *Heliyon* **2022**, 8, e09960.
- [18] A. Pal, F. Khanum, *Process Biochem.* **2011**, 46, 1315.
- [19] H. Shahrestani, A. Taheri-Kafrani, A. Soozanipour, O. Tavakoli, *Biochem. Eng. J.* **2016**, 109, 51.
- [20] R. Ahmad, M. Sardar, *Biochem. Anal. Biochem.* **2015**, 4, 2.
- [21] K. Thakur, C. Attri, A. Seth, *3 Biotech* **2021**, 11, 427.
- [22] R. Pagolu, R. Singh, R. Shanmugam, S. Kondaveeti, S. K. S. Patel, V. C. Kalia, J. K. Lee, *Bioresour. Technol.* **2021**, 331, 125063.
- [23] M. Q. Liu, W. K. Huo, X. Xu, D. F. Jin, *J. Mol. Catal. B Enzym.* **2015**, 120, 119.
- [24] S. F. Chin, S. C. Pang, S. H. Tay, *Carbohydr. Polym.* **2011**, 86, 1817.
- [25] P. V. F. Lemos, H. R. Marcelino, L. G. Cardoso, C. O. de Souza, J. I. Druzian, *Int. J. Biol. Macromol.* **2021**, 184, 218.
- [26] A. Ulu, S. Koytepe, B. Ates, *Carbohydr. Polym.* **2016**, 153, 559.
- [27] X. Yang, Y. Chen, S. Yao, J. Qian, H. Guo, X. Cai, *Carbohydr. Polym.* **2019**, 218, 324.
- [28] B. Matheus Simões, M. Victória Eiras Grossmann, A. Machado Baron, M. Martins Andrade, M. da Conceição de Castro, T. Larissa da Silva Farias, D. Aparecida de Almeida, P. Salomão Garcia, *Prep. Biochem. Biotechnol.* **2020**, 51, 580.
- [29] A. Ulu, S. Koytepe, B. Ates, *Polym. Bull.* **2016**, 73, 1891.
- [30] A. Ulu, S. Koytepe, B. Ates, *J. Appl. Polym. Sci.* **2016**, 133, 43421.
- [31] P. H. Campelo, A. S. Sant'Ana, M. T. Pedrosa Silva Clerici, *Curr. Opin. Food Sci.* **2020**, 33, 136.
- [32] L. Dal Magro, P. F. Hertz, R. Fernandez-Lafuente, M. P. Klein, R. C. Rodrigues, *RSC Adv.* **2016**, 6, 27242.
- [33] D. Alagöz, N. E. Varan, A. Toprak, D. Yildirim, S. S. Tukul, R. Fernandez-Lafuente, *Process Biochem.* **2022**, 113, 270.
- [34] X. Liu, X. Li, Y. Bai, X. Zhou, L. Chen, C. Qiu, C. Lu, Z. Jin, J. Long, Z. Xie, *Ind. Eng. Chem. Res.* **2022**, 61, 18646.
- [35] V. Mehnati-Najafabadi, A. Taheri-Kafrani, A. K. Bordbar, A. Eidi, *J. Iran. Chem. Soc.* **2019**, 16, 21.
- [36] S. A. Ali Noma, Ö. Acet, A. Ulu, B. Önal, M. Odabaşı, B. Ateş, *Polym. Test.* **2020**, 93, 106980.
- [37] P. D. Glekas, S. Kalantzi, A. Dalios, D. G. Hatzinikolaou, D. Mamma, *Biomolecules* **2022**, 12, 790.
- [38] H. R. Wehaidy, M. A. Abdel-Naby, W. G. Shousha, M. I. Y. Elmallah, M. M. Shawky, *Int. J. Biol. Macromol.* **2018**, 111, 296.
- [39] S. Haile, C. Masi, M. Tafesse, *BMC Microbiol.* **2022**, 22, 145.
- [40] C. Liu, M. Li, N. Ji, J. Liu, L. Xiong, Q. Sun, *J. Agric. Food Chem.* **2017**, 65, 8363.
- [41] G. Hafizosmanoğlu, A. Ulu, S. Köytepe, B. Ateş, *Starch–Stärke*, <https://doi.org/10.1002/star.202100152>.
- [42] X. H. Vu, T. T. T. Duong, T. T. H. Pham, D. K. Trinh, X. H. Nguyen, V. S. Dang, *Adv. Nat. Sci. Nanosci. Nanotechnol.* **2018**, 9, 025019.
- [43] I. H. P. Andrade, C. G. Otoni, T. S. Amorim, G. P. Camilloto, R. S. Cruz, *Colloids Surf. A Physicochem. Eng. Asp.* **2020**, 586, 124277.
- [44] Y. Tu, H. Li, T. Tu, Q. Zhang, *CCS Chem.* **2022**, 4, 872.
- [45] A. Ulu, M. Karaman, F. Yapıcı, M. Naz, S. Sayın, E. İ. Saygılı, B. Ateş, *Catal. Lett.* **2020**, 150, 1679.
- [46] K. M. Mostafa, H. A. El-Meged, M. S. Morsy, A. El-ebsiy, A. A. Elsanabary, *J. Polym. Environ.* **2019**, 27, 996.
- [47] K. M. Mostafa, A. A. EL-Sanabary, *J. Polym. Res.* **2020**, 27, 92.
- [48] M. Ahmad, A. Gani, I. Hassan, Q. Huang, H. Shabbir, *Sci. Rep.* **2020**, 10, 3533.
- [49] E. Sabella, A. Aprile, A. Genga, T. Siciliano, E. Nutricati, F. Nicoli, M. Vergine, C. Negro, L. De Bellis, A. Luvisi, *Sci. Rep.* **2019**, 9, 9602.
- [50] A. Ulu, S. A. A. Noma, S. Koytepe, B. Ates, *Artif. Cells Nanomed. Biotechnol.* **2018**, 46, 1035.
- [51] R. Vivek, R. Thangam, V. Nipunbabu, T. Ponraj, S. Kannan, *Int. J. Biol. Macromol.* **2014**, 65, 289.
- [52] A. Rezayat, A. Kakanejadifard, A. Taheri-Kafrani, F. Trotta, *J. Mol. Liq.* **2023**, 387, 122660.
- [53] S. A. A. Noma, *MANAS J. Eng.* **2022**, 10, 129.
- [54] P. R. Heinen, M. G. Pereira, C. G. V. Rechia, P. Z. Almeida, L. M. O. Monteiro, T. M. Pasin, J. M. Messias, M. Cereia, M. K. Kadowaki, J. A. Jorge, M. L. T. M. Polizeli, *Process Biochem.* **2017**, 53, 145.
- [55] S. Madakbaş, Ö. Daniş, S. Demir, M. V. Kahraman, *Starch–Stärke* **2013**, 65, 146.
- [56] A. Amaro-Reyes, A. Díaz-Hernández, J. Gracida, B. E. García-Almendárez, M. Escamilla-García, T. Arredondo-Ochoa, C. Regalado, *Catalysts* **2019**, 9, 966.
- [57] A. Kumar, S. K. S. Patel, B. Mardan, R. Pagolu, R. Lestari, S. H. Jeong, T. Kim, J. R. Haw, S. Y. Kim, I. W. Kim, J. K. Lee, *J. Microbiol. Biotechnol.* **2018**, 28, 638.
- [58] A. Karim, Z. Bibi, H. U. Rehman, A. Aman, S. A. U. Qader, M. H. Rashid, *Colloids Surf. B Biointerfaces* **2021**, 200, 111583.
- [59] N. Z. Adham, H. M. Ahmed, N. Naim, *J. Microbiol. Biotechnol.* **2010**, 20, 332.
- [60] A. K. Singh, H. S. Chhatpar, *Appl. Biochem. Biotechnol.* **2011**, 164, 77.
- [61] Z. Cheng, Z. Sun, F. Wei, J. Yu, J. Zhao, J. Chen, J. Wang, Y. Zhang, *Environ. Funct. Mater.*, <https://doi.org/10.1016/j.efmat.2023.02.001>.
- [62] S. A. Ahmed, S. A. A. Saleh, S. A. M. Abdel-Hameed, A. M. Fayad, *Heliyon* **2019**, 5, e01674.
- [63] D. Qi, M. Gao, X. Li, J. Lin, *ACS Omega* **2020**, 5, 20062.
- [64] L. Cai, Y. Chu, X. Liu, Y. Qiu, Z. Ge, G. Zhang, *Microb. Cell Fact.* **2021**, 20, 37.
- [65] R. K. Suryawanshi, U. K. Jana, B. P. Prajapati, N. Kango, *Food Chem.* **2019**, 289, 95.
- [66] L. Kumar, S. Nagar, A. Mittal, N. Garg, V. K. Gupta, *J. Food Sci. Technol.* **2014**, 51, 1737.