

Layered polymeric carbon nitride as a green support for cellulase immobilization: Improved stability, activity, and reusability

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Abstract

Polymeric carbon nitride (PCN) attracted considerable attention in recent years due to its semiconducting properties. In addition to its semiconducting properties, it is renowned as a support material due to its layered structure and donor groups such as terminal amines and triazine units. In this study, PCN was employed for the first time as a novel support material for the immobilization of cellulase, a key enzyme used in many industrial applications. Cellulase from *Aspergillus sp.* was immobilized onto PCN in three methods. In the first way, cellulase was adsorbed on PCN (PCN@cellulase). In the second way, PCN@cellulase was crosslinked with glutaraldehyde (PCN@cellulase/Glu). Finally, the primary amino group of PCN was modified with glutaraldehyde and the cellulase was immobilized on this support by covalent attachment (PCN/Glu@cellulase). Each cellulase preparation was individually assessed for its optimal pH, temperature conditions, heat stability, and enzyme kinetics. The optimal pH was 5.5 for all cellulase preparations, while the optimal temperature was 45°C for free cellulase and 55°C for all immobilized cellulase preparations. Thermal stability of PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase increased by 2.1-, 2.7-, and 3.7-fold, respectively, compared to the free cellulase. PCN/Glu@cellulase showed 1.4-fold higher catalytic efficiency than the free cellulase and retained 80% of its initial activity after 10 reuses. These results indicate that the use of metal-free, nitrogen-rich PCN, synthesized from abundant and low-cost melamine, aligns with the principles of green chemistry and offers a sustainable alternative to traditional immobilization supports.

Introduction

Cellulases (EC 3.2.1.4) are a group of hydrolytic enzymes that catalyze the breakdown of cellulose, the most abundant renewable biopolymer on Earth, into glucose and other soluble sugars (33). These enzymes have attracted considerable attention due to their wide range of industrial applications, including biofuel production, textile processing, animal feed formulation, food and beverage production, and waste management (30, 26). Cellulase systems are typically composed of three main types of enzymes: endoglucanases, exoglucanases (cellobiohydrolases), and β -glucosidases, which act synergistically to convert cellulose into fermentable sugars (43, 19). Despite their broad utility, the industrial application of free cellulase enzymes is often limited by several factors, such as low stability, high cost, and the inability to reuse the enzymes in continuous processes (44, 31, 37). To overcome these limitations, enzyme immobilization has emerged as a promising strategy. Immobilization refers to the confinement of enzyme molecules to or within solid supports, enabling their repeated use, enhanced operational stability, and improved resistance to changes in environmental conditions such as pH and temperature (25, 28). Various techniques, including adsorption, covalent binding, entrapment, and cross-linking, have been employed for the immobilization of cellulase enzymes (34, 11, 5, 36).

Choosing a suitable immobilization technique and support material is essential for preserving the enzyme's activity and stability (39). Natural and synthetic polymers, inorganic carriers, and hybrid materials have all been explored for this purpose (8). Among all other materials, carbon nitrides have

gained significant interest in recent years because of their 2-D layered structure, ease of manufacture, excellent thermal and chemical stability, and semiconductor properties (16, 42, 38). When carbon and nitrogen-containing molecular compounds such as melamine, urea, cyanamide, dicyandiamide etc. are pyrolyzed over 525°C, a carbon nitride material can be produced as a yellow-orange solid. This structure is very commonly called "graphitic carbon nitride", "graphitic CN" or "g-C₃N₄" in the scientific literature (22). In fact, the structure obtained in this way is a polymeric carbon nitride (PCN) (14, 15). In addition to being a semiconductor, PCN has been used to stabilize and support numerous metal nanoparticles due to the nitrogen donors (amine and tri-s-triazine units) in its structure. On the other hand, these donor groups in the structure, especially the amine groups, allow other molecules to chemically bind to PCN. In recent years, PCN has emerged as the perfect metal-free scaffold for enzyme immobilization because of its hydrophobic qualities, surface-active sites, high biocompatibility, and economical manufacturing (35, 6, 24). However, there is no work in the literature about the immobilization of cellulase enzymes onto the PCN support.

This study presents a comparative approach for the immobilization of cellulase enzymes onto PCN. While PCN has been widely studied for photocatalytic applications, its potential as a biocompatible, metal-free scaffold for enzyme immobilization remains relatively underexplored, especially in the context of cellulase. In this study, cellulase from *Aspergillus sp.* was immobilized onto using three different techniques. All immobilized cellulase preparations were investigated using FTIR, SEM, SEM-EDX and TEM analysis. Both free and immobilized cellulase preparations were analyzed for their optimal pH, temperature conditions, resistance to thermal stress, and kinetic behavior. This side-by-side comparison of immobilization methods on the same support material provides valuable insights into how different strategies affect enzyme binding, activity, and stability.

Materials and methods

Materials

Cellulase from *Aspergillus sp.* (≥ 1000 units/g), melamine, glutaraldehyde, cellulose microcrystalline (CMC), and 3,5-dinitrosalicylic acid were bought from Sigma-Aldrich Chemie GmbH (Germany). All other chemicals were analytical grade and used without further purification.

Methods

Synthesis of PCN

PCN was synthesized using the literature method (13), with a few modifications. Briefly, 10 g of melamine was ground in a mortar and then taken into a crucible and heated to 550°C in a muffle furnace at a heating rate of 12°C per minute and kept at this temperature for 4 h. The solid cooled to room temperature was coded as PCN and used in the relevant processes.

Activation of PCN

1 g of PCN support was treated with 25 mL of glutaraldehyde activation solution (2.5% (w/w) glutaraldehyde in 50 mM sodium phosphate, pH 7.0) at room temperature for 2 hours (3). The mixture was stirred continuously at 100 rpm during the activation reaction and then, the activated PCN supports were collected by filtration under vacuum and washed with distilled water to remove unreacted glutaraldehyde molecules. The glutaraldehyde-activated PCN supports were stored at 5°C until use.

Immobilization of cellulase

Two grams of PCN support was treated with 8 mL of cellulase solution (1 mg/mL in 50 mM pH 5.5 citrate buffer). The mixture was gently shaken for 4 h at 5°C. The resulting immobilized cellulase samples were vacuum-filtered and rinsed with the immobilization buffer to eliminate any unbound enzyme. Filtrates were gathered for subsequent protein analysis. The immobilized enzymes were kept at 5°C until further use.

One gram of cellulase-adsorbed PCN support was treated with 4 mL of 1.25% (w/w) glutaraldehyde solution prepared in phosphate buffer (50 mM, pH 7.0). After 2 h of the crosslinking reaction, the immobilized cellulases were filtered under vacuum and stored at 5°C until use.

Cellulase was immobilized onto glutaraldehyde-activated PCN according to Alagöz et al (3). One gram of the activated support was suspended in 4.0 mL of 25 mM sodium phosphate solution at pH 7.0 containing 1 mg/mL cellulase. The immobilization suspension was continually shaken at 100 rpm and at 5°C for 2 h. The immobilized cellulases were filtered under vacuum and filtrates were collected for protein analysis. The immobilized cellulases were stored at 5°C until use.

Protein concentrations in the collected filtrates were measured using the Lowry method (17). The immobilization yield (IY%) and recovered activity (RA%) were determined following the approach described by Boudrant et al (7).

Instrumental analysis

FT-IR spectra were collected over the 400–4000 cm^{-1} range. SEM images were recorded at different magnifications (10000x-100000x). TEM analysis was operated at an acceleration voltage of 200kV. The detailed information about the instrumental analysis of the samples was given in supplementary information file.

Cellulase assay

The cellulase activity was measured according to Miller with slight modification (20). The detailed information about the cellulase assay was given in supplementary information file.

Effect of pH and temperature on the activity

In order to determine the effect of pH, a series of citrate-phosphate buffer were prepared in a pH range of 4.0-6.5 and free and immobilized cellulase activities were separately measured in these pH solutions at 40°C using 0.5% crystalline micro cellulose (CMC) as substrate. To determine the optimal temperature, the free and immobilized cellulase activities was assayed at different temperatures in a range of 35–65°C at pH 5.5.

Kinetic parameters of free and immobilized cellulase preparations

The free or immobilized cellulase activity was determined for different CMC concentrations ranging from 0.1 to 2.0% under the optimum pH and temperature of each sample. The apparent Michaelis constant (K_M) and maximum velocity (V_{max}) of each sample were estimated from Lineweaver-Burk plots. To determine the catalytic efficiency ratio (CER), the V_{max}/K_M of each immobilized cellulase was divided by the V_{max}/K_M of the corresponding free cellulase.

Thermal stress

The thermal stress of the cellulase samples was tested at 45°C and 55°C in 50 mM sodium acetate at pH 5.5. The residual activity of each sample was determined for the specified time intervals under optimal conditions. The thermal stress parameters of such as inactivation constant (k_d), half-life ($t_{1/2}$) and stabilization factor (SF) were calculated according to Alagöz et al (3).

Reuse of the different cellulase biocatalysts

The reuse experiments were performed in batch assays by incubating 0.1 g immobilized cellulases with 5 mL of 0.5% cellulose solution at 55°C. After 10 min reaction, the suspension was centrifuged (1 min, 5,000 x g) and the residual activity of each immobilized cellulase preparations was measured. After each cycle, the biocatalysts were washed with 50 mM sodium acetate buffer at pH 5.5, filtrered and added to a new hydrolysis cycle with new substrate solution. The activity after each cycle was expressed in relation to the activity after the first cycle.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was conducted using analysis of variance (ANOVA) to determine significant differences among experimental groups. Group means were compared using the Least Significant Difference (LSD) method in SigmaPlot software (Version 12), with a significance level set at $P < 0.05$.

Results and discussion

In this study, the use of PCN support for the immobilization of *A. niger* cellulase and comparison of immobilization methods on the activity and stability of immobilized cellulase were aimed. The pH of the immobilization medium plays a crucial role in determining the efficiency of enzyme binding and its subsequent catalytic performance. Table 1 summarizes the immobilization of cellulase on PCN supports via adsorption at different pH values ranging from 4.5 to 7.0. The highest IY% was obtained at an immobilization pH of 5.0. Increasing the pH resulted in a slight decrease in IY% values. However, RA values decreased significantly from 80.8% to 59.0% as the immobilization pH increased from 5.0 to 7.0. At higher pH values, the enzyme's tertiary structure may undergo conformational changes that adversely affect the integrity of the active site, thus reducing catalytic efficiency. As shown in Table 1, the amount of bound protein increased as the initial protein concentration increased from 0.25 to 2 mg/mL. However, the highest RA value of 98.7% was obtained at an initial protein concentration of 0.5 mg/mL. Further increasing the protein concentration to 2 mg/mL resulted in a decrease in RA value to 48.9%. At higher protein concentrations, excessive enzyme molecules may adsorb onto the surface of the PCN support. This can lead to molecular crowding, where enzymes are packed closely. Such crowding can hinder proper enzyme orientation, restrict substrate accessibility to the active sites, and increase steric hindrance, all of which reduce the overall catalytic activity (18).

Table 1
Effect of immobilization pH and initial added protein on IY (%) and RA (%) values for the adsorption of cellulase on PCN support.

Immobilization pH	Immobilization temperature	Added protein amount	Bound protein amount	IY (%)	RA (%)
4.5	5°C	1 mg	0.89 mg	89.0	76.4
5.0			0.92 mg	91.8	80.8
5.5			0.91 mg	90.8	77.4
6.0			0.90 mg	89.9	74.1
6.5			0.87 mg	86.9	65.4
7.0			0.86 mg	86.0	59.0
5.0	5°C	0.25 mg	0.24 mg	95.1	62.9
		0.5 mg	0.48 mg	95.1	98.7
		1.0 mg	0.92 mg	91.8	80.8
		2.0 mg	1.67 mg	83.5	48.9

The PCN@cellulase was crosslinked using different concentrations of glutaraldehyde to elucidate the effect of the amount of glutaraldehyde on immobilized cellulase activity. As presented in Fig. 1, the highest activity was observed in the non-crosslinked PCN@cellulase preparation. A slight decrease in relative activity was noted at a glutaraldehyde concentration of 0.625% (w/v). However, the relative

activity further declined to 65%, 32.1%, and 10.7% at glutaraldehyde concentrations of 1.25%, 2.5%, and 5%, respectively. These results indicate that increasing the glutaraldehyde concentration increases the rigidity of the immobilized cellulase, which in turn reduces its enzymatic activity (32, 1).

Table 2 presents the results of covalent immobilization of cellulase on glutaraldehyde-modified PCN supports at different pH values and initial protein concentrations. The IY value was approximately 87% at both pH 7.0 and 8.0. Increasing the immobilization pH to 10.0 resulted in a slight reduction in IY%. The RA value was about 70% at pH 7.0. A slight decrease in RA% was observed at pH 8.0, while a further increase in pH led to a significant drop in RA%. At higher pH values, conformational changes in the enzyme’s tertiary structure may compromise the integrity of the active site, thereby reducing its catalytic efficiency. As shown in Table 2, the amount of bound protein increased with the rise in initial protein concentration from 0.25 to 2 mg/mL. However, the highest RA value of 80.0% was achieved at an initial protein concentration of 0.5 mg/mL. Increasing the concentration further to 2 mg/mL led to a decrease in RA to 42.4%. At higher protein concentrations, excessive enzyme molecules may be adsorbed onto the surface of the PCN support, leading to molecular crowding. This crowding can result in improper enzyme orientation, restricted substrate access to active sites, and increased steric hindrance—all of which contribute to reduced catalytic activity (12, 9).

Table 2
Effect of immobilization pH and initial added protein on IY (%) and RA (%) values for the covalent immobilization of cellulase on glutaraldehyde modified PCN support.

Immobilization pH	Immobilization temperature	Added protein amount	Bound protein amount	IY (%)	RA (%)
7.0	5°C	1 mg	0.872 mg	87.2	70.2
8.0			0.870 mg	87.0	68.0
10.0			0.815 mg	81.5	46.4
7.0	5°C	0.25 mg	0.23 mg	93.6	77.5
		0.5 mg	0.46 mg	91.3	80.0
		1.0 mg	0.87 mg	87.2	70.2
		2.0 mg	1.46 mg	73.0	42.4

Figure 2 shows the SEM images of bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase. As demonstrated in Fig. 2, bare PCN had a typical agglomerated surface appearance of graphitic carbon nitride materials (Fig. 2a-c) (41). After cellulase immobilization, the surfaces of PCN supports were clearly different from those of bare PCN, and the roughness of the PCN surfaces increased as a result of the cellulase immobilization (Fig. 2d-f). After glutaraldehyde crosslinking of the adsorbed cellulase, the surface structure is considerably altered (Fig. 2g-i) and

fracture structures have increased. For PCN/Glu@cellulase, the roughness of the surfaces was highly increased after glutaraldehyde modification and covalent immobilization (Fig. 2j-l).

The results of EDX analysis of bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase are shown in Fig. 3. After cellulase immobilization, the presence of oxygen atom in PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase approved the immobilization of cellulase.

TEM images of bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase are shown in Fig. 4. The bare PCN support, and PCN@cellulase are spherical in shape and their surfaces are smooth and homogeneous. However, PCN@cellulase/Glu, and PCN/Glu@cellulase have increased surface roughness, and irregularities. The crosslinking of PCN@cellulase and modification of bare PCN with glutaraldehyde followed by covalent bonding may disturb the structure of PCN and this can result in more amorphous appearances or less distinct edges in TEM images compared to the bare PCN support and PCN@cellulase samples. The particle sizes of bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase are measured to be 375 ± 0.31 , 228 ± 0.75 , 226 ± 0.74 , and 219 ± 0.65 nm, respectively.

FT-IR spectra of bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase are shown in Fig. 5. A sharp peak at 805 cm^{-1} corresponds to the triazine/heptazine units of PCN (21). The peaks observed in the range of $1230\text{--}1650\text{ cm}^{-1}$ are attributed to the skeletal vibrations of aromatic C–N heterocycles. For bare PCN, the peak at 1630 cm^{-1} is associated with the conjugated imine groups in the heptazine rings (23, 10). The broad band between 3000 and 3300 cm^{-1} is due to N–H and O–H stretching vibrations (4). There is no obvious change in the spectra for PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, suggesting the intact structure of PCN (38). In contrast, the peak at 1637 cm^{-1} in PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase corresponds to the amide I band.

The optimum pH of the free and immobilized cellulase preparations was studied in the pH range of 4.0–6.5 and the results obtained were presented in Fig. 6A. All cellulase preparations had an optimum pH at 5.5. As shown in Fig. 6A, free and immobilized cellulase preparations showed nearly similar behaviour against pH change. This result indicates that the three-dimensional structure of the cellulase is protected upon immobilization on PCN. The effect of temperature on the free and immobilized cellulase preparations was determined in the temperature range of 35°C – 65°C of pH 5.5. The free cellulase had an optimum at 45°C , whereas the optimum temperature increased for the immobilized cellulases. All immobilized cellulases showed an optimum at 55°C (Fig. 6B). Moreover, the free cellulase showed 60% of its maximum activity at 65°C , however; the immobilized cellulases displayed at least 80% of their initial activity at the same temperature. Ahmad and Khare reported that the optimum pH of free cellulase from *Aspergillus niger* and its covalently immobilized form on carbon nanotubes were both 5.0. Moreover, both cellulase forms had an optimum temperature of 50°C (2). Zdarta et al. (40) immobilized *A. niger* cellulase on a TiO_2 –lignin hybrid support and determined the maximum activity of free and

immobilized cellulase preparations to be 5.5 and 6.0, respectively. The free enzyme showed its maximum activity at 50°C, while the maximum activity of the immobilized cellulase increased to 55°C.

Thermal stability of the free and immobilized cellulase preparation was investigated at 45°C and 55°C over 24 h. The initial activity of the free cellulase was decreased with the incubation time and its remaining activity was determined to be 60% after 24 h. Under the same conditions, the remaining activities were 76, 80, and 86% for PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, respectively (Fig. 7A). The k_d values were calculated to be 27.9×10^{-3} , 18.7×10^{-3} , 14.0×10^{-3} , and $10.0 \times 10^{-3} \text{ h}^{-1}$ for the free, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, respectively. The corresponding $t_{1/2}$ values were 24.8, 37.0, 49.4, and 69.4 h. According to these results, SF values were calculated to be 1.5, 2.0, and 2.8 for PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, respectively (Table 3). At 55°C, the remaining activities were determined to be 35, 66, 76, and 82% for the free, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, respectively after 24 h of incubation (Fig. 7B). The k_d values were found to be 50.2×10^{-3} , 24.3×10^{-3} , 18.8×10^{-3} , and $13.4 \times 10^{-3} \text{ h}^{-1}$ for the free, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, respectively. The corresponding $t_{1/2}$ values were 13.8, 28.5, 36.9, and 51.7. According to these results, SF values were calculated to be 2.1, 2.7, and 3.7 for PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase, respectively (Table 3). Ahmad and Khare reported that the $t_{1/2}$ value of *A. niger* cellulase immobilized on COOH-functionalized MWCNTs was 4-fold higher than free enzyme at 70°C (2). Li et al. immobilized *Candida rugosa* lipase on glutaraldehyde modified C_3N_4 nanosheets ($\text{C}_3\text{N}_4\text{-NS@CRL}$) and reported that $\text{C}_3\text{N}_4\text{-NS@CRL}$ preserved 67% of its initial activity after 180 min of incubation time at 55°C (16).

Table 3
Thermal stability parameters of free cellulase, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase at 45°C and 55°C.

Biocatalyst	45°C	k_d	$t_{1/2}$	SF	55°C	k_d	$t_{1/2}$	SF
		(h^{-1})	(h)			(h^{-1})	(h)	
free		0.0279	24.8	1.0		0.0502	13.8	1.0
PCN@cellulase		0.0187	37.0	1.5		0.0243	28.5	2.1
PCN@cellulase/Glu		0.0140	49.4	2.0		0.0188	36.9	2.7
PCN/Glu@cellulase		0.0100	69.4	2.8		0.0134	51.7	3.7

The apparent K_M and $V_{\max}$ values of each cellulase sample are given in Table 4. The apparent K_M values of free cellulase, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase were determined to be 1.14 ± 0.14 , 1.39 ± 0.18 , 1.52 ± 0.21 , and $0.84 \pm 0.10 \text{ mg/mL}$ for CMC (**Fig. S1**). The alteration in the K_M value may be attributed to changes in the enzyme–substrate affinity resulting from conformational modifications of cellulase induced by immobilization, the onset of substrate diffusional constraints, or a synergistic effect of both phenomena. The corresponding $V_{\max}$ values were found to be 1.25 ± 0.09 , 1.77

± 0.22 , 1.17 ± 0.09 , and 1.30 ± 0.11 U/mg protein. The elevated $V_{\max}$ values observed for PCN@cellulase and PCN/Glu@cellulase compared to free cellulase may be attributed to the favorable orientation of enzyme molecules on the support surface, which facilitates improved substrate access to the active site. According to these results CER ratios were calculated to be 1.2, 0.7, and 1.4. These results indicate that PCN@cellulase and PCN/Glu@cellulase exhibit 1.2- and 1.4-fold higher activity, respectively, compared to free cellulase. The CER value of cellulase immobilized on COOH-functionalized MWCNTs is approximately 1.66-fold higher than that of free cellulase (2). The K_M and $V_{\max}$ values of free cellulase from *A. niger* were determined to be 2.06 ± 0.85 mM and 159 ± 11 U/mg protein for cellulose, respectively. The corresponding values for its immobilized counterpart on TiO₂-lignin hybrid support were 2.63 ± 0.96 mM and 125 ± 19 U/mg protein. In addition, CER value was calculated to be 0.62 (40).

Table 4
The apparent K_M and $V_{\max}$ values of free cellulase, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase for CMC under the optimum conditions of each preparation.

Biocatalyst	K_M (mg/mL)	$V_{\max}$ (U/mg protein)	CER
free	1.14 ± 0.14	1.25 ± 0.09	1.0
PCN@cellulase	1.39 ± 0.18	1.77 ± 0.22	1.2
PCN@cellulase/Glu	1.52 ± 0.21	1.17 ± 0.09	0.7
PCN/Glu@cellulase	0.84 ± 0.10	1.30 ± 0.11	1.4

The reuse stability of immobilized cellulase preparations was investigated in a batch type reactor for 10 cycles. Among the tested immobilized cellulases, the covalently immobilized PCN/Glu@cellulase retained 80% of its initial activity, while PCN@cellulase/Glu and PCN@cellulase retained 72% and 50% of their initial activities, respectively (Fig. 8). These results highlight that PCN, particularly when modified through covalent attachment, provides a durable and reusable platform for enzyme immobilization, offering great potential for cost-effective and sustainable industrial bioprocesses. Cellulase immobilized onto carbon coated Fe₃O₄ nanoparticles protected 80% of its initial activity after 9 reuses (45). Rashid et al. modified nickel nanoparticle with 3-APTES to generate free -NH₂ group onto the support surface and immobilized cellulase from *Aspergillus niger* by adsorption followed by glutaraldehyde crosslinking. After 10 reuses, the immobilized cellulase retained 84% of its initial activity after ten reuses (27). C₃N₄-NS@CRL retained 72% of the initial activity after 10 reuses (16). Shangguan et al. reported that CALB covalently bound to g-C₃N₄ maintained 65% of its initial activity after 9 cycles (29).

Conclusion

In this study, PCN synthesized from melamine was successfully employed as a sustainable and metal-free support for cellulase immobilization. Three different immobilization strategies - simple adsorption,

adsorption followed by glutaraldehyde crosslinking, and covalent binding via glutaraldehyde activation - were comparatively evaluated. All immobilized cellulase preparations retained their catalytic activity and exhibited improved thermal stability compared to the free enzyme. In particular, the covalently bound PCN/Glu@cellulase demonstrated the highest stabilization factor, extended half-life and better reusability, highlighting the effectiveness of covalent immobilization in enhancing enzyme performance. These findings confirm that PCN provides a promising platform for enzyme immobilization due to its biocompatibility, high surface area, and abundance. The results open up new perspectives for the application of PCN-based biocatalysts in sustainable bioprocesses, especially for biomass conversion and other industrial applications requiring robust and reusable cellulase systems.

Declarations

Credit author statement

Nuri Gulesci: Conceptualization, Methodology, Writing – original draft. Orhan

Altan: Conceptualization, Methodology, Data curation, Formal analysis. Ali

Toprak: Conceptualization, Methodology, Data curation, Formal analysis. Mustafa Serkan Yalcin:

Methodology, Data curation, Formal analysis. Ramazan Bilgin: Writing – review & editing. Deniz Yildirim:

Methodology, Writing – original draft, Writing – review & editing.

Conflict of interest

The author declares that they have no conflict of interest.

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Data availability

Data will be made available on reasonable request.

References

1. Assis Modenez, I., Sastre, D. E., Moraes, C., F. and, & Marques Netto, C. G. C. (2018). Influence of glutaraldehyde cross-linking modes on the recyclability of immobilized lipase B from *Candida antarctica* for transesterification of soy bean oil. *Molecules*, 23, 2230.
2. Ahmad, R., & Khare, S. K. (2018). Immobilization of *Aspergillus niger* cellulase on multiwall carbon nanotubes for cellulose hydrolysis. *Bioresource Technology*, 252, 72–75.
3. Alagöz, D., Toprak, A., Yildirim, D., Tükel, S. S., & Fernandez-Lafuente, R. (2021). Modified silicates and carbon nanotubes for immobilization of lipase from *Rhizomucor miehei*: Effect of support and

- immobilization technique on the catalytic performance of the immobilized biocatalysts. *Enzyme And Microbial Technology*, 144, 109739.
4. Alwin, E., Kočí, K., Wojcieszak, R., Zieliński, M., Edelmannová, M., & Pietrowski, M. (2020). Influence of high temperature synthesis on the structure of graphitic carbon nitride and its hydrogen generation ability. *Mater*, 13, 2756.
 5. Bhardwaj, S., Agrawal, D., Ghosh, D., & Sinha, A. K. (2025). Magnetically separable graphene oxide as a promising matrix for cellulase immobilization: enhanced activity and stability with high glucose yield. *Biomass Convers Biorefin*, 15, 3043–3056.
 6. Bilgin Simsek, E., & Saloglu, D. (2021). Exploring the structural and catalytic features of lipase enzymes immobilized on g-C₃N₄: A novel platform for biocatalytic and photocatalytic reactions. *J M Liq*, 337, 116612.
 7. Boudrant, J., Woodley, J. M., & Fernandez-Lafuente, R. (2020). Parameters necessary to define an immobilized enzyme preparation. *Process Biochemistry*, 90, 66–80.
 8. Cavalcante, F. T. T., Cavalcante, A. L. G., de Sousa, I. G., Neto, F. S. and dos, & Santos, J. C. (2021). S. Current status and future perspectives of supports and protocols for enzyme immobilization. *Catalysts*. 11, 1222.
 9. Diamanti, E., Santiago-Arcos, J., Grajales-Hernández, D., Czarniewicz, N., Comino, N., Llarena, I., Di Silvio, D., Cortajarena, A. L., & López-Gallego, F. (2021). Intraparticle kinetics unveil crowding and enzyme distribution effects on the performance of cofactor-dependent heterogeneous biocatalysts. *Acs Catalysis*, 11, 15051–15067.
 10. Fdez-Sanromán, A., Lomba-Fernández, B., Sanromán, A., Pazos, M., & Rosales, E. (2024). Enhancing stability and immobilization techniques for graphitic carbon nitride in photocatalytic applications. *Journal Of Molecular Liquids*, 405, 125005.
 11. Hadadi, M., & Habibi, A. (2025). Improved activity and stability of cellulase by immobilization on Fe₃O₄ nanoparticles functionalized with Reactive Red 120. *International Journal Of Biological Macromolecules*, 295, 139624.
 12. Homaei, A. A., Sariri, R., Vianello, F., & Stevanato, R. (2013). Enzyme immobilization: an update. *Journal Of Chemical Biology*, 6, 185–205.
 13. Jiang, J., Ou-yang, L., Zhu, L., Zheng, A., Zou, J., Yi, X., & Tang, H. (2014). Dependence of electronic structure of g-C₃N₄ on the layer number of its nanosheets: A study by Raman spectroscopy coupled with first-principles calculations. *Carbon*, 80, 213–221.
 14. Kessler, F. K., Zheng, Y., Schwarz, D., Merschjann, C., Schnick, W., Wang, X., & Bojdys, M. J. (2017). Functional carbon nitride materials – design strategies for electrochemical devices. *Nat Rev Mater*, 2, 17030.
 15. Lau, V. W., & Lotsch, B. V. (2022). A tour-guide through carbon nitride-land: Structure- and dimensionality-dependent properties for photo(electro)chemical energy conversion and storage. *Adv Ener Mater*, 12, 2101078.

16. Li, Y., Ruan, Z., Zheng, M., Deng, Q., Zhang, S., Zheng, C., Tang, H., Huang, F., & Shi, J. (2018). *Candida rugosa* lipase covalently immobilized on facilely-synthesized carbon nitride nanosheets as a novel biocatalyst. *Rsc Advances*, 8, 14229–14236.
17. Lowry, O., Rosebrough, N., Farr, A. L., & Randall, R. (1951). Protein measurement with the Folin phenol reagent. *Journal Of Biological Chemistry*, 193, 265–275.
18. Ma, B., & Nussinov, R. (2013). In J. Klinman, & S. Hammes-Schiffer (Eds.), *Dynamics in Enzyme Catalysis* (pp. 123–137). Springer Berlin Heidelberg.
19. Ma, X., Li, S., Tong, X., & Liu, K. (2024). An overview on the current status and future prospects in *Aspergillus* cellulase production. *Environmental Research*, 244, 117866.
20. Miller, G. L. (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426–428.
21. Narkbuakaew, T., & Sujaridworakun, P. (2020). Synthesis of tri-S-triazine based g-C₃N₄ photocatalyst for cationic Rhodamine B degradation under visible light. *Topics In Catalysis*, 63, 1086–1096.
22. Ong, W. J., Tan, L. L., Ng, Y. H., Yong, S. T., & Chai, S. P. (2016). Graphitic carbon nitride (g-C₃N₄)-based photocatalysts for artificial photosynthesis and environmental remediation: Are we a step closer To achieving sustainability? *Chemical Reviews*, 116, 7159–7329.
23. Parga, B., Vázquez, A., Ruiz-Gómez, M. A., Rodríguez-González, V., & Obregón, S. (2023). Enhancing fluorescence sensing of metal species by g-C₃N₄ prepared by co-polymerization of melamine and urea precursors. *Mater Sci Eng B*, 293, 116493.
24. Piraman, Z., & Kalhor, H. R. (2025). Cascade chemo-enzymatic synthesis of disubstituted 1,2,3-triazole derivatives: Enhancing the activity by immobilized enzyme on g-C₃N₄. *Mol Catal*, 585, 115361.
25. Prabhakar, T., Giaretta, J., Zulli, R., Rath, R. J., Farajikhah, S., Talebian, S., & Dehghani, F. (2025). Covalent immobilization: A review from an enzyme perspective. *Chemical Engineering Journal*, 503, 158054.
26. Ranjan, R., Rai, R., Bhatt, S. B., & Dhar, P. (2023). Technological road map of cellulase: A comprehensive outlook to structural, computational, and industrial applications. *Biochemical Engineering Journal*, 198, 109020.
27. Rashid, S. S., Mustafa, A. H., Rahim, M. H. A., & Gunes, B. (2022). Magnetic nickel nanostructure as cellulase immobilization surface for the hydrolysis of lignocellulosic biomass. *International Journal Of Biological Macromolecules*, 209, 1048–1053.
28. Robescu, M. S., & Bavaro, T. (2025). A comprehensive guide to enzyme immobilization: All you need to know. *Molecules*, 30, 939.
29. Shangguan, H., Zhang, S., Li, X., Zhou, Q., Shi, J., Deng, Q., & Huang, F. (2020). Synthesis of lutein esters using a novel biocatalyst of *Candida antarctica* lipase B covalently immobilized on functionalized graphitic carbon nitride nanosheets. *Rsc Advances*, 10, 8949–8957.

30. Sharma, A., Tewari, R., Rana, S. S., Soni, R., & Soni, S. K. (2016). Cellulases: classification, methods of determination and industrial applications. *Applied Biochemistry And Biotechnology*, 179, 1346–1380.
31. Shen, X., Zhong, L., Li, L., Zou, B., Suo, H., & Yan, L. (2024). Coupling chemical modification and immobilization of cellulase improves thermal stability and low-melting mixture solvent tolerance for in situ saccharification of bagasse. *Journal Of Molecular Liquids*, 398, 124253.
32. Silva, D. F., Carvalho, A. F. A., Shinya, T. Y., Mazali, G. S., Herculano, R. D., & Oliva-Neto, P. (2017). Recycle of immobilized endocellulases in different conditions for cellulose hydrolysis. *Enzyme Res.* 2017, 4362704.
33. Singh, A., Bajar, S., Devi, A., & Pant, D. (2021). An overview on the recent developments in fungal cellulase production and their industrial applications. *Bioresour Technol Rep*, 14, 100652.
34. Suhag, S., & Hooda, V. (2025). Chitosan-enhanced zirconium-based metal-organic framework: Enabling cellulase immobilization for improved cellulose breakdown. *Biocatalysis And Agricultural Biotechnology*, 66, 103599.
35. Tian, K., Liu, H., Dong, Y., Chu, X., & Wang, S. (2019). Amperometric detection of glucose based on immobilizing glucose oxidase on g-C₃N₄ nanosheets. *Colloids And Surfaces A*, 581, 123808.
36. Wahba, M. I., Saleh, S. A. A., Wahab, W. A. A., & Mostafa, F. A. (2025). Studies on the preparation of a sufficient carrier from egg protein and carrageenan for cellulase with optimization and application. *Scientific Reports*, 15, 3868.
37. Wang, S., Zhao, B., Cao, S., Wang, G., Dong, Z., Yang, Z., Niu, X., Zhao, Q., Wang, Y., & Ma, Y. (2025). Efficient immobilization of cellulase on polydopamine-modified nickel foam for enhanced lignocellulose conversion. *Bioresource Technology*, 426, 132361.
38. Wang, Y., Zhang, N., Hübner, R., Tan, D., Löffler, M., Facsko, S., Zhang, E., Ge, Y., Qi, Z., & Wu, C. (2019). Enzymes immobilized on carbon nitride (C₃N₄) cooperating with metal nanoparticles for cascade catalysis. *Advanced Materials Interfaces*, 6, 1801664.
39. Yildirim, D., Alagöz, D., Toprak, A., Tükel, S., & Fernandez-Lafuente, R. (2019). Tuning dimeric formate dehydrogenases reduction/oxidation activities by immobilization. *Process Biochemistry*, 85, 97–105.
40. Zdarta, J., Jędrzak, A., Kłapiszewski, Ł., & Jesionowski, T. (2017). Immobilization of cellulase on a functional inorganic–organic hybrid support: Stability and kinetic study. *Catalysts*, 7, 374.
41. Zedan, M., Zedan, A. F., Amin, R. M., & Li, X. (2022). Visible-light active metal nanoparticles@carbon nitride for enhanced removal of water organic pollutants. *J Environ Chem Eng*, 10, 107780.
42. Zhang, S., Deng, Q., Shanguan, H., Zheng, C., Shi, J., Huang, F., & Tang, B. (2020). Design and preparation of carbon nitride-based amphiphilic Janus N-doped Carbon/MoS₂ nanosheets for interfacial enzyme nanoreactor. *Acs Applied Materials & Interfaces*, 12, 12227–12237.
43. Zhang, Z., Xing, J., Li, X., Lu, X., Liu, G., Qu, Y., & Zhao, J. (2024). Review of research progress on the production of cellulase from filamentous fungi. *International Journal Of Biological Macromolecules*, 277, 134539.

44. Zhu, X., Qiang, Y., Wang, X., Fan, M., Lv, Z., Zhou, Y., & He, B. (2024). Reversible immobilization of cellulase on gelatin for efficient insoluble cellulose hydrolysis. *Int J Biol Macromol*, 273, 132928.
45. Zhu, Y., Han, J., Wu, J., Li, Y., Wang, L., Mao, Y., & Wang, Y. (2021). A two-step method for the synthesis of magnetic immobilized cellulase with outstanding thermal stability and reusability. *New Journal Of Chemistry*, 45, 6144–6150.

Figures

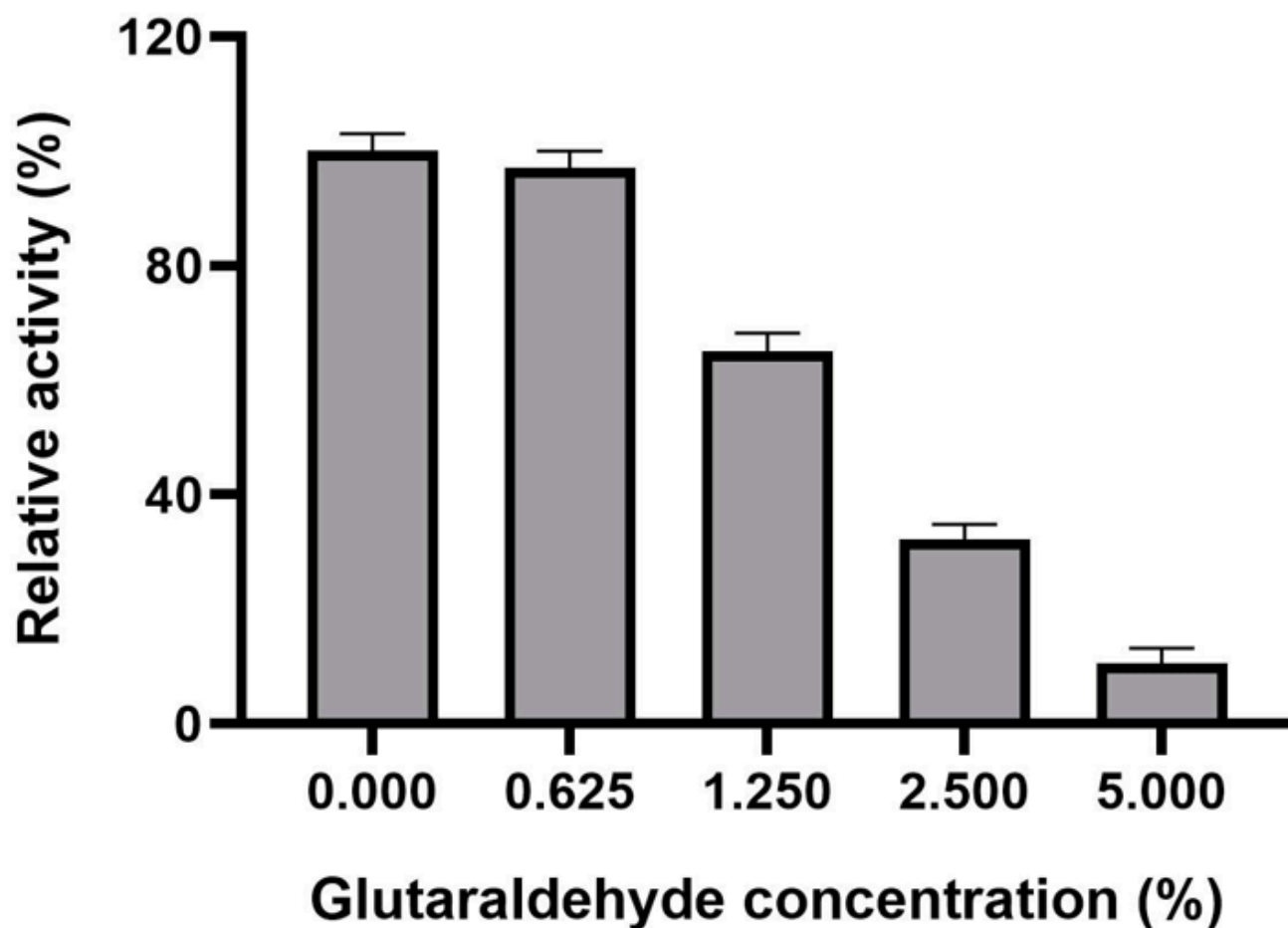


Figure 1

Effect of glutaraldehyde concentration on the activity of PCN@cellulase.

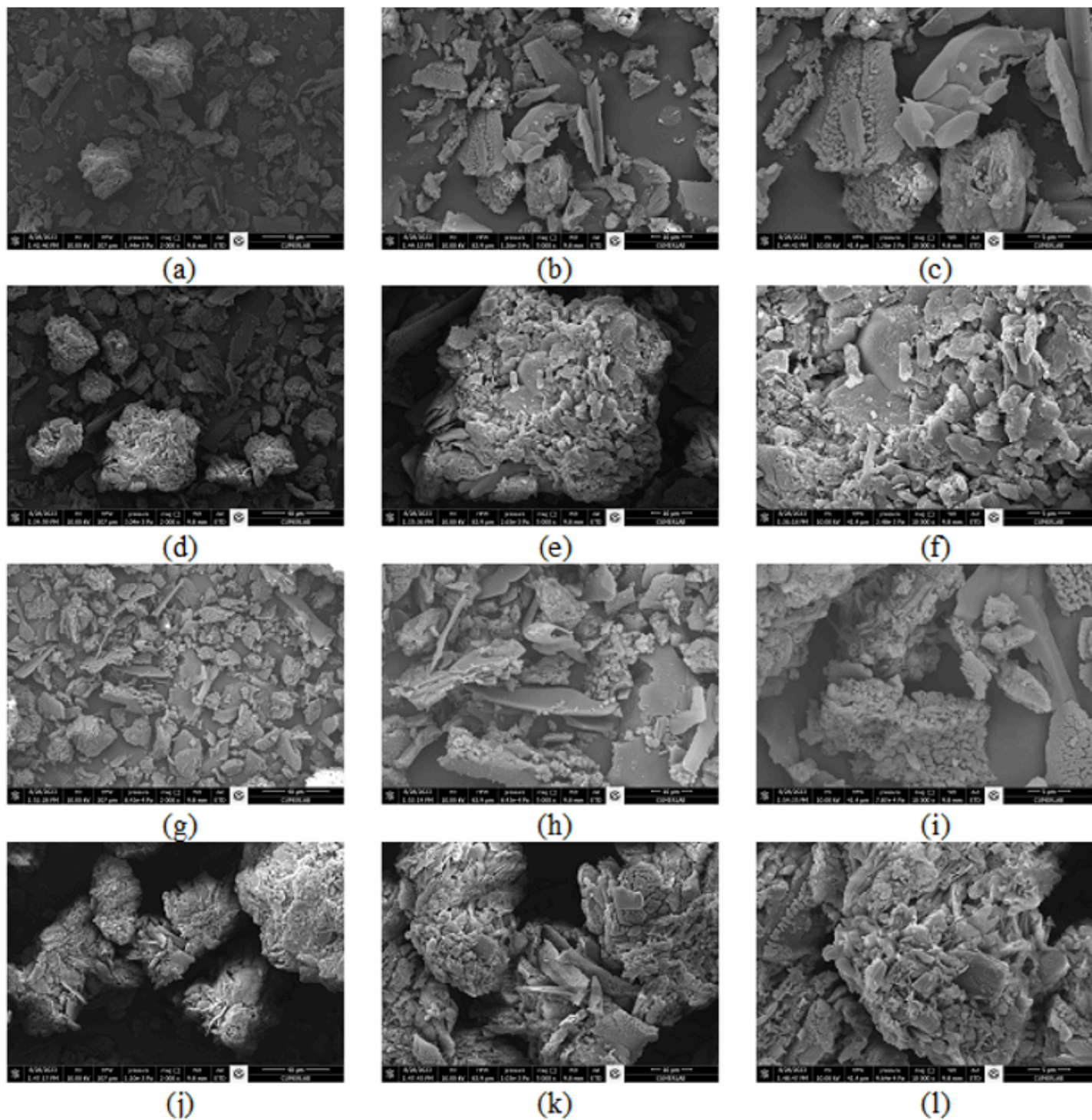


Figure 2

SEM images of bare PCN support (a-c), PCN@cellulase (d-f), PCN@cellulase/Glu (g-i), and PCN/Glu@cellulase (j-l) with different magnifications.

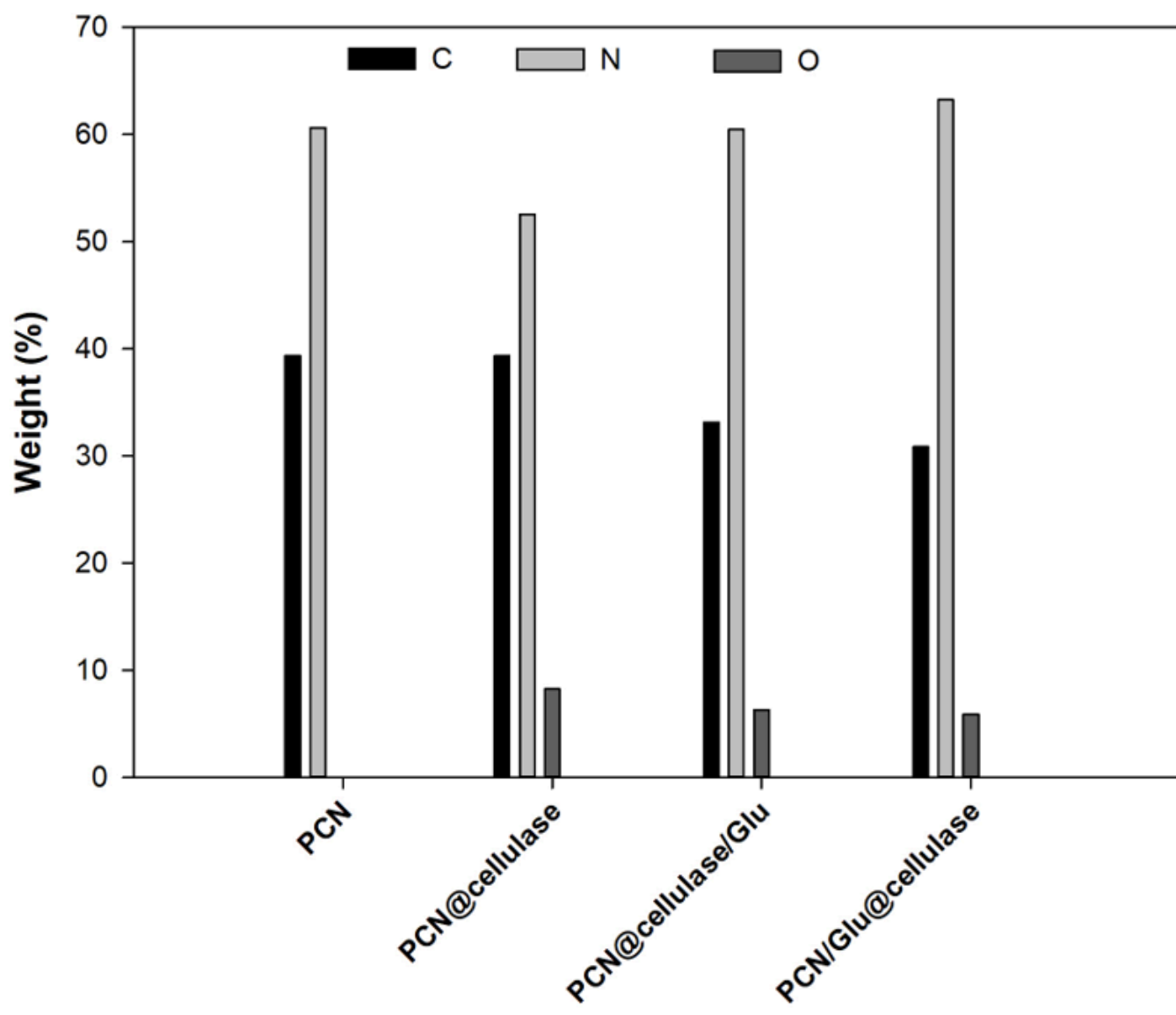


Figure 3

SEM-EDX analysis bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase.

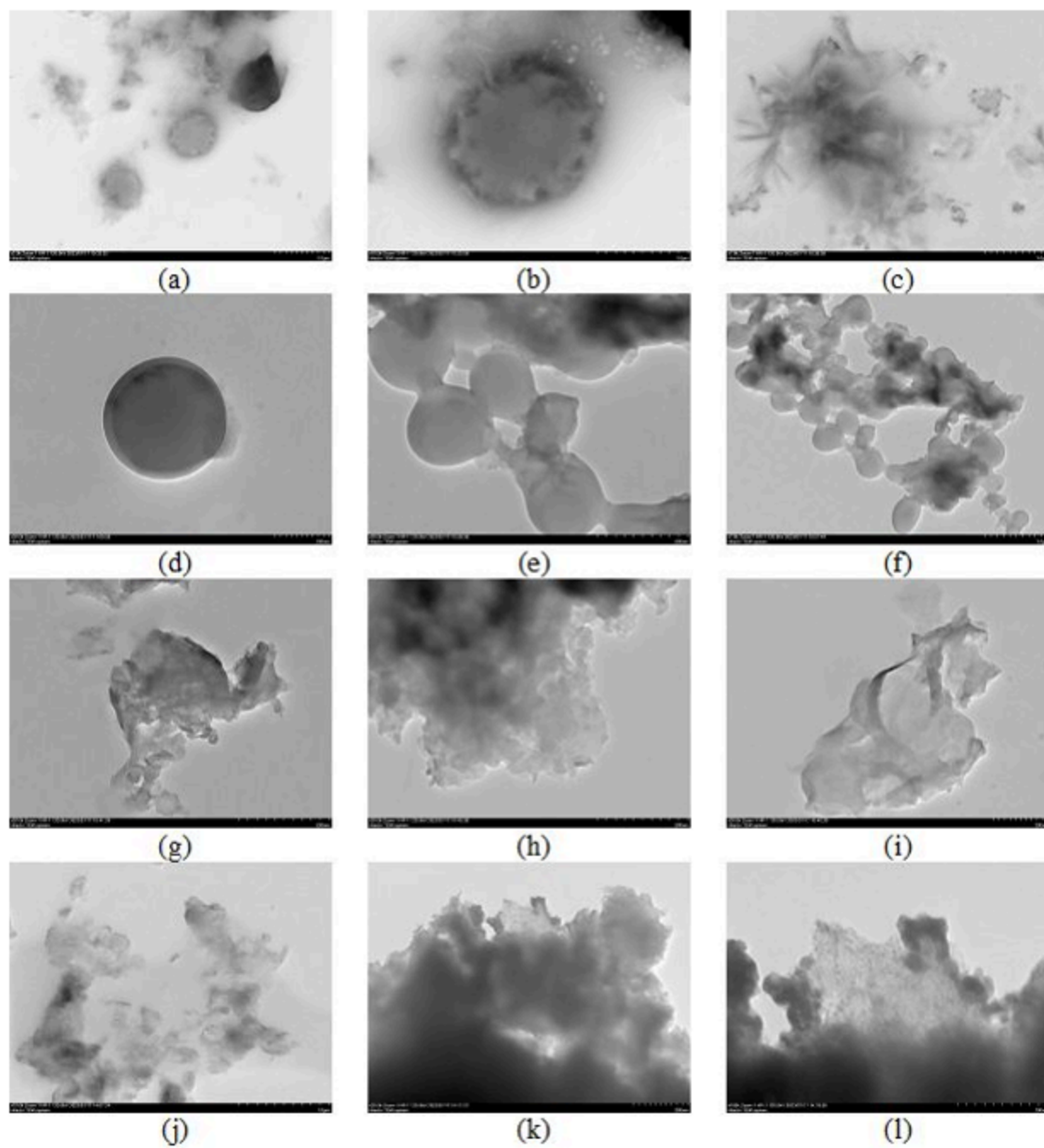


Figure 4

TEM images of bare PCN support (a-c), PCN@cellulase (d-f), PCN@cellulase/Glu (g-i), and PCN/Glu@cellulase (j-l).

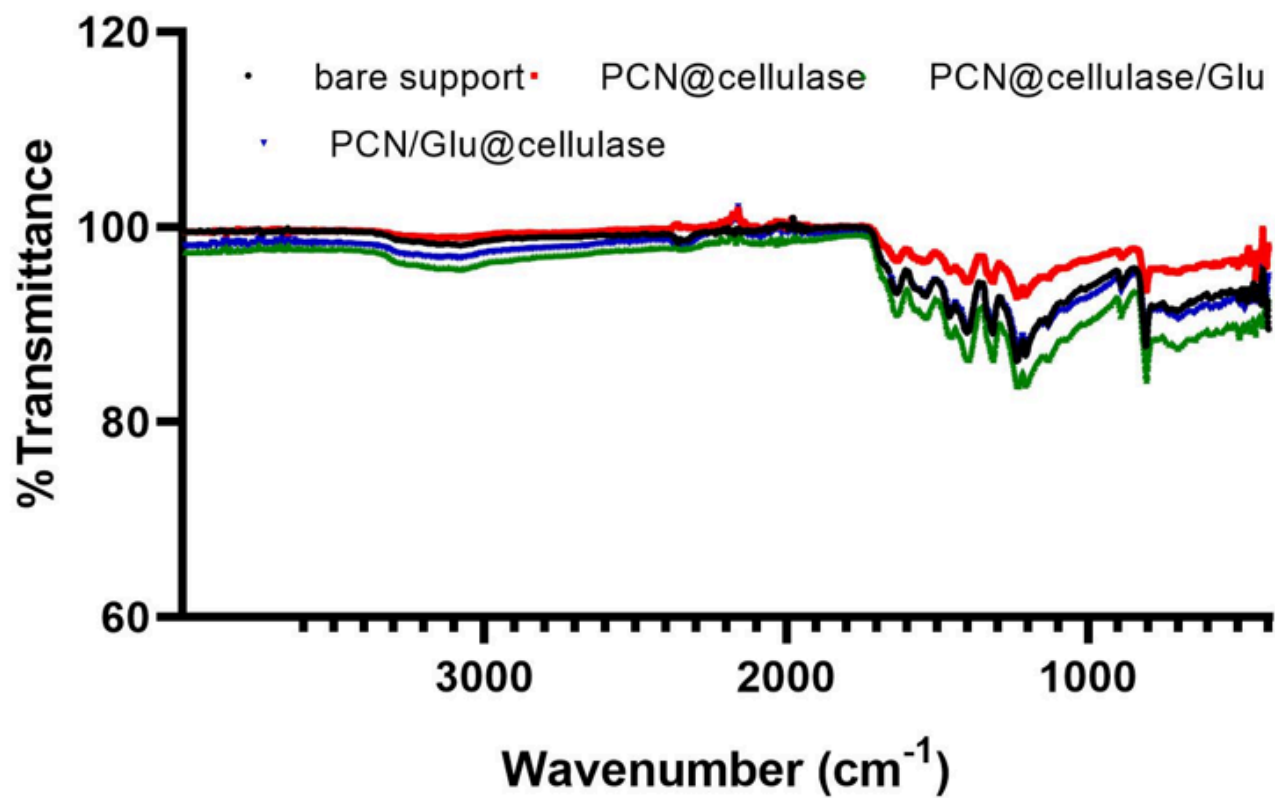


Figure 5

FT-IR spectra of bare PCN support, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase.

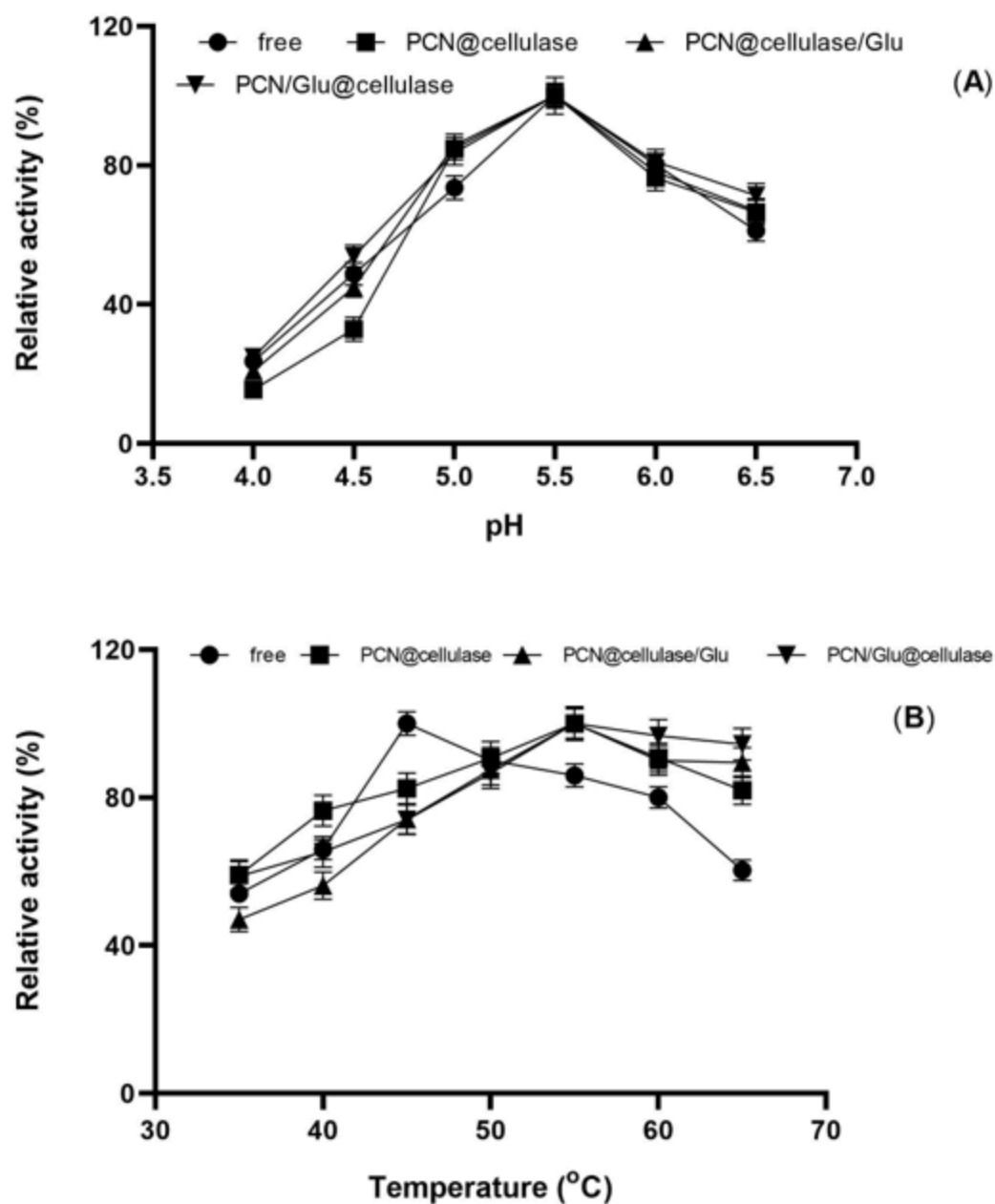


Figure 6

Effect of pH (A) and temperature (B) on the activity of free cellulase, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase. The maximum activity was taken as 100% with the relative activities (%) expressed as the ratios of the activity at the measured pH or temperature to the maximum activity.

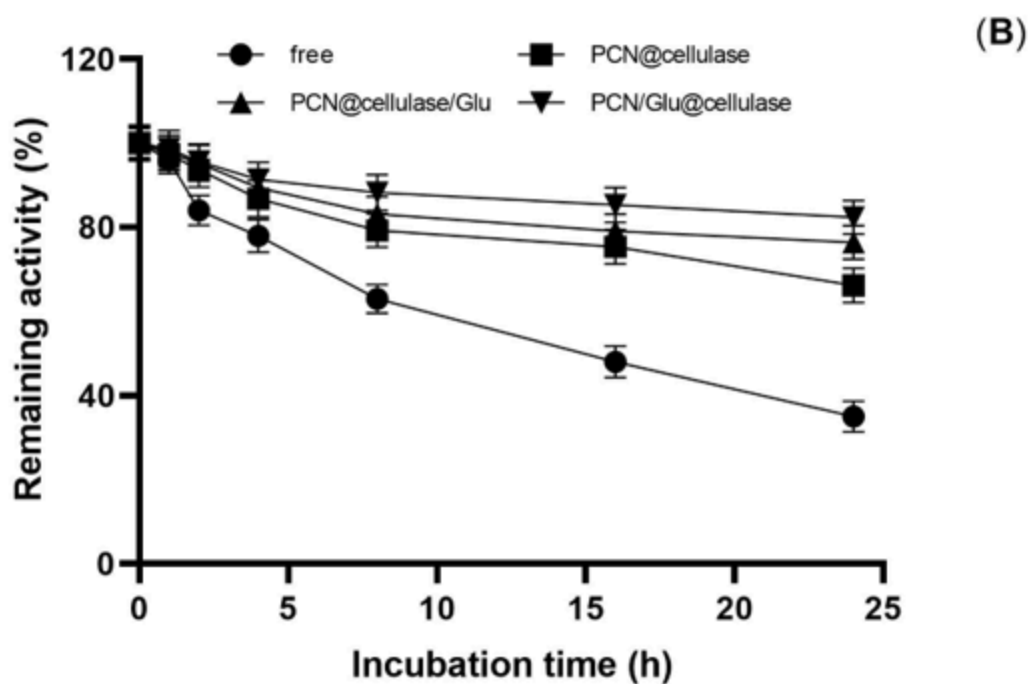
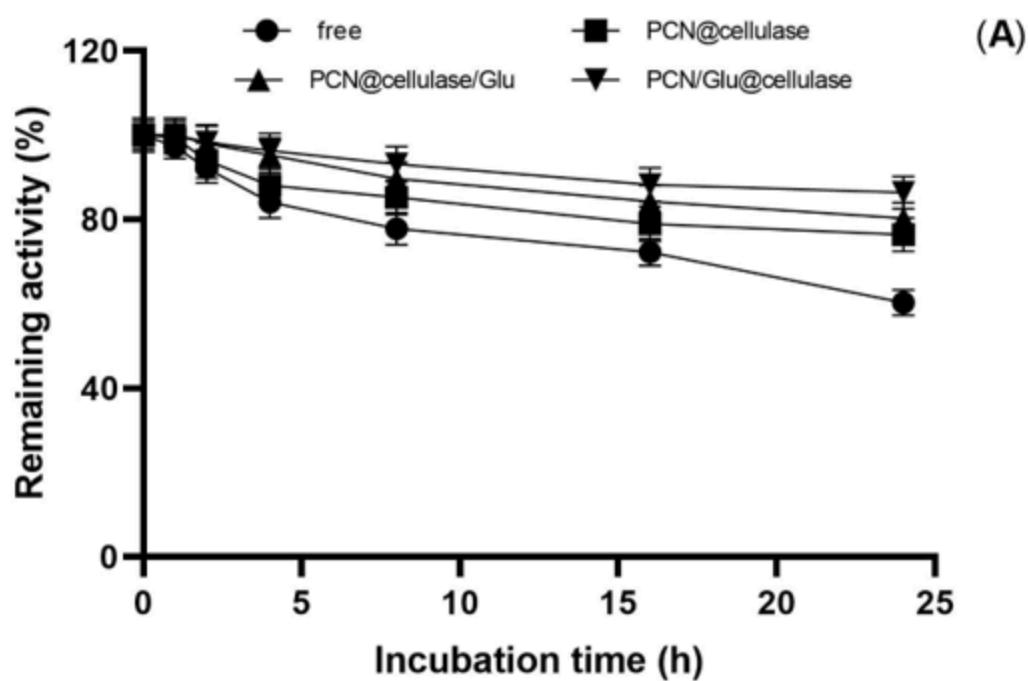


Figure 7

Thermal stability of free cellulase, PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase at 45°C (A) and 55°C (B). The residual activity was calculated as the ratio between the activity at a given time and the activity at the beginning of incubation.

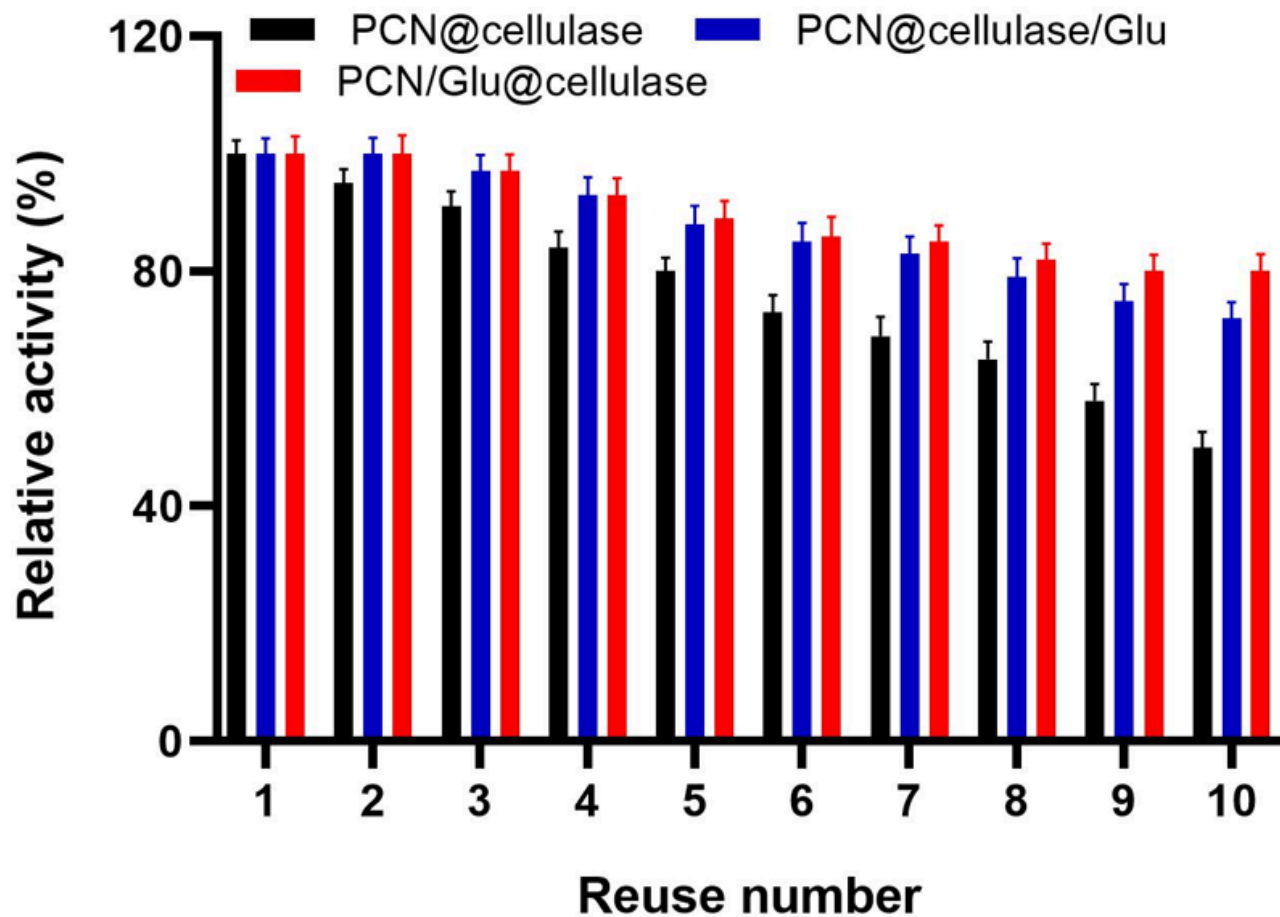


Figure 8

Reuse stability of PCN@cellulase, PCN@cellulase/Glu, and PCN/Glu@cellulase.

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