Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Hybrid Nano Supports of Nano Polyaniline, Magnetic Iron Oxide Nanoparticles and Nano Clay as Matrices for The Immobilization of β-Glucosidase, Fructosyltransferase and Carboxymethyl Cellulase

Azza M. Noor El-Deen[1], Asmaa I. El-Shazly[1], Hoda M. A. H. Shata[2], Mohamed A. Farid[1*]

[1]*Chemistry of Natural and Microbial Products Department, Pharmaceutical Industries and Drug Research Institute, National Research Centre, 33 El-Buhouth Street, P.O: 12622, Dokki, Cairo, Egypt.*

[2]*Microbial Chemistry Department, Biotechnology Research Institute, National Research Centre, 33 El-Buhouth Street, P.O: 12622, Dokki, Cairo, Egypt.*

Abstract

Hybrid nanostructures of polyaniline, magnetic iron oxide nanoparticles, and nonclay have been synthesized to serve as nanocarriers for the covalent immobilization of β-glucosidase (β-Glu), fructosyltransferase (FTase) and carboxymethyl cellulase (CMCase) individually. The resulting nano polyaniline iron oxide@Clays has been characterized by scanning electron microscopy (SEM), EDX and Fourier transform infrared (FTIR). Each immobilized enzyme supported on the produced nanocarrier underwent rounds of evaluation of its activity using each enzyme substrate. The immobilization process showed that enzyme recycling was influenced by the type and properties of the nano mixture and the enzyme itself. The immobilized β-Glu was shown to be reusable and retained approximately 100% of its activity after repeated use for 20 cycles. Whereas for FTase, only 30% of the biocatalyst's activity was lost after 10 more cycles, demonstrating the remarkable operational stability of the immobilized FTase. These properties and the facile separation from the reaction medium by application of a magnetic field demonstrate that the polyaniline-iron oxide@Clays nanoparticles can be used as efficient carriers for both enzymes. On the other hand, CMCase could only be reused four times on all types of immobilizations supports.

Keywords: Enzymes immobilization, Magnetic nanoparticles, Nano clay, Nano polyaniline, Reusability

1. Introduction

The majority of enzymes are difficult to employ in an industrial setting since they are generally relatively labile, poorly stable, technically difficult to recover and reuse, and have sluggish recycling processes. The enzyme immobilization technique overcomes these limitations and is suitable for a wide range of applications due to its frequent and continuous application, which reduces process costs [1]. In recent years, new enzyme-targeted materials have become increasingly important in the field of enzyme immobilization. One such material is nanoparticles, which are new materials of both inorganic and organic nature, and they have recently been the subject of intense research as prospective carriers for immobilizing enzymes [2, 3]. Nanoparticles offer a significant surface area for enzymes binding, resulting in increased enzyme immobilization yields and more enzyme loading on the matrix surface [4]. The ability of nanoparticles to minimize diffusional constraints, however, is their main advantage. Both organic and inorganic materials have very good mechanical characteristics and outstanding thermal and chemical stability [5]. These nanomaterials, in particular, are manufactured in a number of ways, have variable particle sizes (mainly in the nano range), and can be employed with enzymes. Additionally, an unexpected broad spectrum of functional groups that match the chemical residues on proteins is present in these materials, improving enzyme binding and surface modification [5].

Much research has been done with the aim of combining both organic and inorganic nanoparticles to maximize their benefits. Hybrids and complexes typically stabilize the interactions between enzymes and supports, increase the mechanical resistance of biocatalysts, make them stable and reusable, and protect biocatalysts from conformational changes during storage under reaction conditions [6, 7]. Hybrid nanoparticles can be synthesized by combining: (i) organic-organic; (ii) inorganic-

*Corresponding author e-mail nrcfarid@yahoo.com

Receive Date: 26 September 2023, Revise Date: 24 October 2023, Accept Date: 06 November 2023

DOI: 10.21608/EJCHEM.2023.239073.8673

inorganic precursors; or (iii) organic-inorganic precursors. Organic-organic hybrids can be synthesized by combining two synthetic materials [8, 9]. Synthetic macromolecules, including biomacromolecules [10–12] or two biopolymers [13, 14]. The prepared nanoparticles, fibers, beads, tubes, membranes, and films can all be utilized for enzyme immobilization [15, 16]. Because the organic parts of both synthetic and biological polymers comprise a variety of functional units that can interact with the chemical groups of the biocatalyst, they have a better ability to bind enzymes [17]. The resultant nano supports exhibit good catalytic characteristics and are long-lastingly stable and reusable [18]. On the other hand, hybrid nano supports for immobilizing enzymes have been made by combining organic (synthetic and natural biopolymers) and inorganic (minerals, silica, carbon compounds, and magnetic nanoparticles) nanomaterials [19]. The characteristics of the inorganic nano support play a major role in the high stability and frequent chemical inertness of the final product. For the development of effective nano organic-inorganic hybrids for enzymes immobilization, several combinations of organic and inorganic materials have been employed. Due to the organic structure of these carriers, they possess a greater capacity for binding enzymes [17]. In addition to the kinds mentioned above, hybrid and composite inorganic-inorganic materials have a number of other characteristics that make them attractive prospective supports for enzymes immobilization. Inorganic hybrids frequently have strong mechanical toughness, chemical inertia, and pH and temperature stability. In addition, their manufacturing is typically straightforward and uncomplicated, making them comparatively cheap [20].

From organic nanoparticles, polyaniline nanoforms (PANFs) offer high surface area, high stability at extreme temperatures and pHs, resistance to microorganisms, and a unique electrical conduction mechanism. PANF has been used to immobilize and stabilize various enzymes [21–23]. It offers low production effort and high yield in synthetics using nanofiber matrices and high electronic conductivity [24].The use of magnetic nanoparticles as carriers for enzymes immobilization has been quite common. They are a really fascinating family of nanoscale materials. [19, 25–28].

Clay minerals have recently been exploited as appealing inorganic carriers for enzymes immobilization owing to their inexpensive nature, chemical inertness, thermal stability, clearly established layers, and ion exchange capability [29, 30]. Enzymes can be immobilized on clay minerals through covalent bonding or non-covalent adsorption (van der Waals forces, hydrogen bonds, electrostatic and hydrophobic interactions, and hydrophobic interactions) [31]. Clay minerals had been chemically modified, and attaching components were added in order to improve enzyme immobilizations and enhance the charge, activity, and stability of the enzyme. The existence of cations that can be exchanged (Na+ or Ca2+) in the interlayer cavity of clay minerals, particularly smectite, enables them to possess a significant affinity for protein adsorption. These cations can be changed by cation exchange processes into positively charged enzyme molecules [31]. Kaolin clay has also been mentioned as a potential support material for packaging purposes due to its stability, suitable degree of porosity, mechanical strength, and ability to control temperature [32].

The covalent immobilization of β-Glu, FTase, and CMCase in the current study has been achieved using hybrid nanomaterials consisting of PANI, magnetic iron nanoparticles, and nano clay hydrophilic bentonite.

2. Materials and methods

Materials

Nano clay (hydrophilic bentonite), magnetic iron oxide (Fe II, Fe III), glutaraldehyde (GA), bovine serum albumin (BSA), ammonium persulphate (APS), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) substrates and aniline were purchased from Sigma Aldrich Chemicals (St. Louis, USA). Aminopropyltriethoxysilane (APTES) from abcr GmbH - Karlsruhe, Germany. Fructosyltransferase (FTase) from a complex enzyme mixture Pectinex® Ultra SP-L, β-glucosidase and Carboxymethyl cellulase (CMCase) from cellulase *Trichoderma reesei* Novozymes were obtained from Novozymes (Bagsvaerd, Denmark). All other chemicals were of high purity available commercially.

Synthesis of polyaniline nanofibers (PANI)

Polyaniline nanofibers (PANI) were synthesized according to Zakaria et al. [33] and Zhang et al. [34], with some modifications. Aniline is often chemically polymerized in the presence of ammonium persulfate to create polyaniline. The following method was adopted for preparing PANI nanofibers: 4 mmol (0.36 mL) of aniline and 1.0 mmol (0.23 g) of APS were dissolved in two glass vials containing 10.0 mL of 1 M HCl at room temperature (25 °C). The solutions were poured rapidly into a 30 mL glass vial and shaken vigorously for about 15 seconds. One mixture was precipitated by the same chemical oxidative polymerization in the presence of Triton-X 100. The second one was made without adding Triton-X 100. Both mixtures were agitated at 100 rpm for 24 hours. To get rid of extra acid and other

byproducts, the crude product was cleaned using dialysis against deionized water for three days.

Pretreatment of nano clays

In order to remove the remaining sodium and carbon ions from the clay surface, the nano clays were activated using ultrasonication for 1 hour in a 2 M HCl solution at 30 °C. Following in-depth analysis, the nano clays were separated by gravity sedimentation, washed repeatedly with distilled water until the pH of the supernatant approached neutral, and then the activated nano clays were dried for 24 hours at 50 °C in a vacuum. After desiccating, grinding the sample in an agate mortar, and allowing it to pass through a 47µm sieve, the sample was ready to be used [35, 36].

Nano clay magnetization

Magnetic clay nanoparticles were prepared in accordance with the study by Cabrera et al. [21] with a few minor modifications. Briefly, 0.5 g of magnetic iron oxide powder was added to the nano clay preparation (0.5% w/v). The final pH magnetization was adjusted with a citrate phosphate buffer solution (50 mM, pH 5.0) up to 5.0. After this, the magnetic solution was kept in a sonicating water bath for 15 minutes. The magnetic clay nanoparticles (Fe@Clay) obtained were washed with distilled water until pH 7.0 by using a potent magnet. The Fe@Clay magnetic clay nanoparticles were stored at 4 °C until later use.

Preparation of Fe@Clay@PANI nanoparticles

The Fe@Clay@PANI was prepared according to the procedure described by Cabrera et al. [21], with some modifications. Before the PANI-coating process, Fe@Clay was washed with sufficient distilled water until the pH became neutral and was recovered using a magnet. Coating was achieved by oxidative polymerization of aniline on the Fe@Clay surface using ammonium persulfate as an oxidizing agent. Fe@Clay nanoparticles (1.0 g) were treated with 0.23 g of ammonium persulfate (10 ml 1.0 M HCl), and the magnetic suspension was immediately supplemented with 0.36 mL of aniline solution (10 ml 1.0 M HCl). The polymerization process was performed in the dark at 4°C for 30 min. It is important to note that after each step of the polymerization process, magnetic nanoparticles were successively washed (fifteen times) with distilled water to remove any residual reagent. Finally, the Fe@Clay@PANI nanoparticles were dried at 50 °C for 4 hours until later use.

Preparation of Fe@Clay-APTES@PANI

The Fe@Clay nanoparticles (0.1 g) were submerged in a silane coupling agent solution (1 mL APTES, prepared in acetone, 2.5%) and incubated in a sonicating water bath for 3 min. Next, (Fe@Clay-APTES) were washed with distilled water until the pH became neutral and recovered using a magnet. Subsequently, Fe@Clay-APTES were then coated with PANI nanoparticles to prepare Fe@Clay-APTES@PANI nanoparticles.

Scanning electron microscope analysis

Tescan SEM (Tescan Vega 3 SBU, Czech Republic) was used for the scanning electron microscopy study. Using carbon tape to mount the samples, aluminum microscope stubs were coated with gold (Au) for 150 s using a sputter coater from Quorum Techniques Ltd. (Q150t, England).

Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform infrared spectroscopy in the frequency range of 4000–400 cm^{-1} was used. to detect the major structural groups of the prepared nanocarriers. Each sample of PANI and Fe@Clay@PANI before and after enzyme loading was pressed into a KBr pellet and recorded on a FT-IR spectrophotometer (JASCO FT/IR 4600 FT-IR, Germany).

Enzyme immobilization

Firstly, glutaraldehyde, as a spacer, was utilized to activate the PANI, Fe@Clay@PANI, and Fe@Clay-APTES@PANI carriers, according to [21]. An appropriate amount of the Fe@Clay-APTES@PANI, Fe@Clay@PANI, and PANI was submerged into a GA (0.5%, v/v) solution and shaken overnight at room temperature. The active supports for Fe@Clay-APTES@PANI and Fe@Clay@PANI were eliminated by magnetic separation while PANI fibers by centrifugation (10000 rpm, 5 min). Carriers were washed several times with deionized water to remove excess GA and then washed with a citrate-phosphate buffer solution (50 mM, pH 5.0). The immobilization process for each enzyme was carried out at 30 °C in a shaking air bath overnight. After this, the immobilized enzyme was recovered by magnetic separation or centrifugation and thoroughly rinsed with a citrate-phosphate buffer solution (50 mM, pH 5.0) two times to remove the unbound enzyme. The washed solution was collected to assay the amount of residual enzyme. The resulting immobilized enzyme was stored at 4 °C prior to use. All the data used in this formula are the average of duplicate experiments.

Enzyme assay

Extracellular β-glucosidase activity

The β-glucosidase activity was determined using *p*-nitrophenyl-β-D glucopyranoside (*p*-NPG) as a substrate according to the method reported by Otieno and Shah [37] with some modifications. The reaction mixture, in a total volume of 1.5 ml, was composed

of 0.5 ml of 0.1% *p*-NPG in 0.05 M citrate phosphate buffer with pH 5.0 and 1 ml of enzyme-loaded carrier. After 30 min of incubation at 37°C, the reaction mixture was centrifuged (model 5415D; Eppendorf, Hamburg, Germany) at 10000 rpm for 5 min. 0.5 ml of 1M cold sodium carbonate was added to the supernatant to stop the reaction. The amount of released *p-nitrophenol* was measured using a spectrophotometer (SP-2000UV; Spectra, USA) at a wavelength of 401nm. A unit of enzyme activity was defined as the amount of enzyme that would liberate 1 mM *p*-nitrophenol per minute under assay conditions.

Fructosyltransferase activity

FTase activity was measured by estimating the liberated reducing sugar released from sucrose as described by Duan et al. [38], using glucose as a standard. The assay mixture contained appropriately 0.1 ml of enzyme-loaded carrier (0.15 M Mcllvaine buffer pH 5.5) and 0.4 ml of 50% sucrose solution. The reaction mixture was incubated at 55 °C for one hour. At the end of the incubation period, the reaction was stopped by adding a copper reagent. The amount of reducing sugar liberated was determined according to the Somogyi method [39]**.**

Carboxymethyl cellulase assay

CMCase activity was estimated using 1.0 ml of substrate (0.5% CMC in 50 mM citrate phosphate buffer pH 5.00) added to 0.5 ml of enzyme-load carrier and incubated at 30 ◦C for 30 min [40]. The reaction was stopped by adding 1.0 ml of DNS, and reducing sugars were estimated [41]. One unit (IU) of CMCase activity was defined as the amount of enzyme required to release 1.0 mol of glucose per minute.

Reusability of immobilized enzymes

The three immobilized carriers for each enzyme were stored in the appropriate enzyme buffer at 4 °C and were reused for repeated batches. After each batch, the enzymatic preparation was washed several times with the appropriate enzyme buffer, and a new substrate solution was added for the next activity cycle. The relative activity was determined by measuring enzyme activity, as previously mentioned.

Results and discussion

Physical and chemical characterization of nanocarriers

SEM analysis

The nanocomplex morphologies of PANI, Fe@Clay@PANI, and immobilized enzymes nanocomplexes were studied using SEM images. PANI has a rod-like shape with an average diameter of 57.88 nanometers, and the surface of the nanofibers is significantly rough (Fig. 1 a and b), where aggregation and irregular particles occur [42]. This structure also explains many of the properties that occur in many environments where polyaniline acts as an immobilization aid for enzymes. The SEM image of the modified Fe@Clay@PANI shows a PANI-coated flaky clay structure and numerous individual particles, indicating a PANI coating layer on and between the surfaces of the clay nanoparticles. Platelets are shown (Figs. 1c and d). The clay layer size range was 84–153 nm. This finding indicated that PANI easily inserted between clay layers and expanded to push the layers apart, forming intercalated, layered silicate PANI particles. In the enzymatic loading process, fructosyltransferase and β-Glu were immobilized on the porous matrix of Fe@Clay@PANI nanoparticles. SEM images of immobilized enzymes (FTase and β-Glu) were indistinguishable from those of PANI Fe@Clay. This suggests that adsorption of both enzymes significantly improved enzyme loading on PANI Fe@Clay. On the other hand, SEM images of β-Glu show thickly coated PANI and inter-fiber pores of PANI filled by enzyme aggregates (Fig. 1 e, f, g, h) [22].

EDX analysis

To find the anticipated elements, the EDX analyses of PANI, PANI Fe@Clay, PANI Fe@Clay-FTase, and PANI Fe@Clayβ-Glu have been carried out (Table 1). The EDX spectrum clearly shows that the produced nano-supports and immobilized enzymes contain elements such as carbon, nitrogen, iron, oxygen, silicon, and aluminium. Higher nitrogen levels (7.28 & 9.16% w/w) proved that the FTase and β-Glu enzymes were linked to the PANI Fe@Clay carrier, respectively.

Enzyme activity of β-Glu, FTase, and CMCase immobilized on different nano supports

The immobilized yield (Table 2) of Fe@Clay@PANI crosslinked with glutaraldehyde was higher than that of Fe@Clay-APTES@PANI and other PANI supports (PANI 1, PANI 4). The immobilization yields (%) for the biocatalysts PANI β-Glu, PANI CMCase, and PANI FTase treated with glutaraldehyde were around 202.45, 281.43, and 101.19, respectively. In the presence of Fe@Clay@PANI, the immobilized yields of β-Glu, CMCase, and FTase were 234.35, 207.05, and 100.56, respectively. The immobilized yields of β-Glu, CMCase, and FTase using Fe@Clay@PANI crosslinked with GA were 365.74, 257.13, and 99.48, respectively.

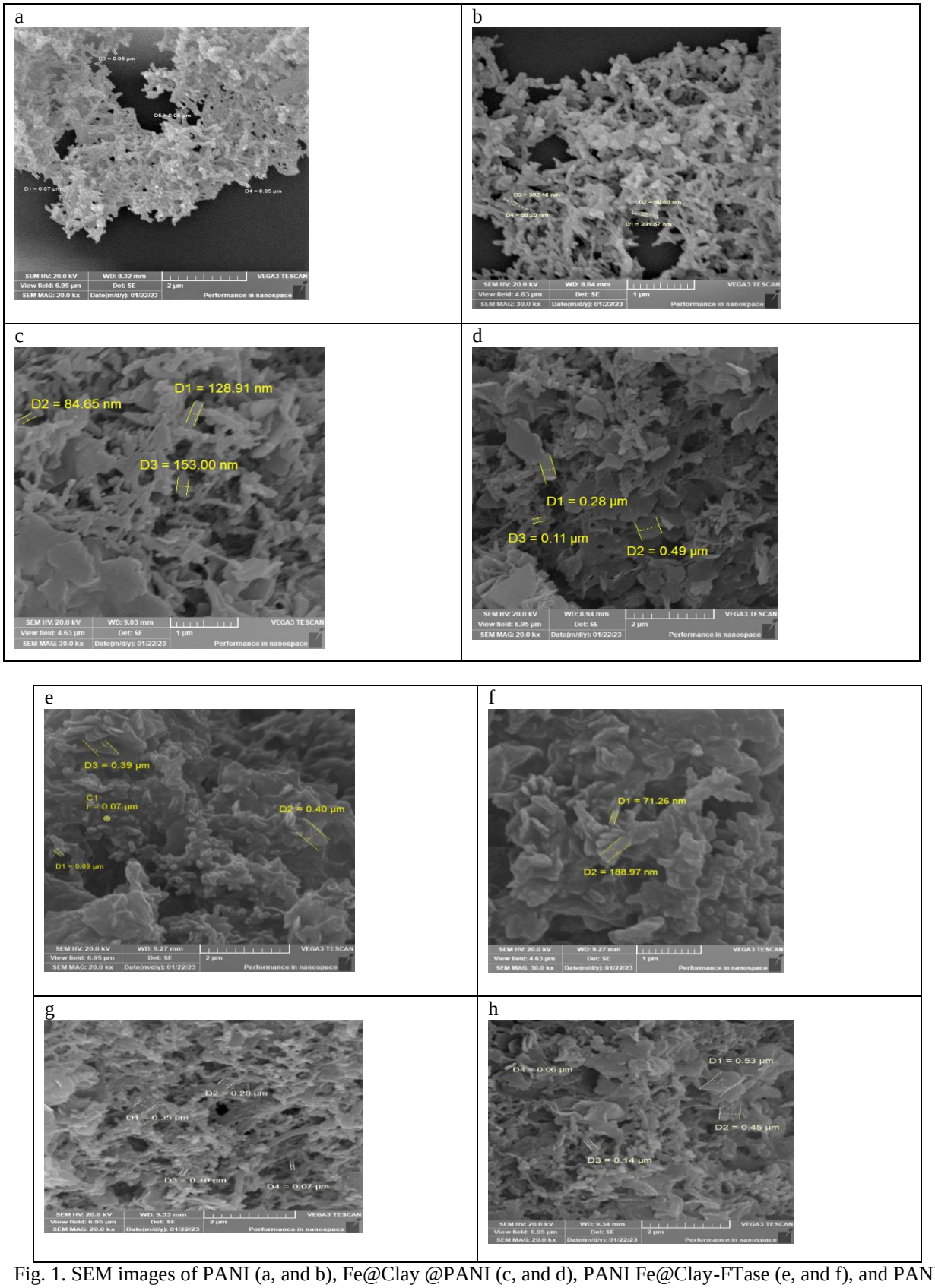


Fig. 1. SEM images of PANI (a, and b), Fe@Clay @PANI (c, and d), PANI Fe@Clay-FTase (e, and f), and PANI Fe@Clay-β-Glu (g, and h).

Table (1) Elemental composition of PANI, Fe@Clay@PANI, Fe@Clay@PANI @FTase and Fe@Clay@PANI β-Glu.

Element	PANI	Fe@Clay@PANI	Fe@Clay@PANI FTase	Fe@Clay@PANI β-Glu
	Atom (%)			
Carbon	56.90	59.85	55.62	54.82
Oxygen	24.69	26.80	33.17	32.39
Nitrogen	17.93	8.82	7.28	9.16
Iron	0.04	1.38	1.16	1.00
Silicon	0.30	1.74	1.59	1.49
Aluminium	0.13	1.40	1.18	1.13
Sum	100	100	100	100

During enzyme immobilization on clay minerals, several physical and chemical interactions take place, such as cation exchange, electrostatic interactions, hydrogen bonding, hydrophobic affinity, and van der Waals forces, which are involved in the adsorption and binding of protein molecules by clay minerals [43]. Since the surface charge of clay minerals is permanently negative, the adsorption of charged protein molecules to clay minerals via electrostatic interactions is dependent on the positively and negatively charged states of the protein molecules in adsorptive pH environments [43]. For a number of reasons, enzymes immobilized on NPs might improve their performance. These include (a) NP form, (b) interactions between surface functional groups of nanoparticles, (c) enzyme orientation for interactions with substrates, (d) mass transfer, and (e) an improvement in localized avidity and enzyme density. The increased activity of the immobilized enzymes may potentially result from conformational changes of the enzyme brought on by ionic interactions with the polyaniline nanofiber [23]. These enhancements have been noted in a number of nanoparticles, like graphene and carbon nanotubes, metal/metal oxides, polymer nanocrystals and even semiconductor nano crystals [44].

The low immobilization yield of biocatalysts may be due to the fact that supports functionalized with 3-APTES showed reduced surface area and pore volume. This is probably due to the binding of silane molecules to clay minerals in the interlayer space. The large molecule of APTES might block some pores, resulting in a reduced effective surface area and pore volume [45]. Several factors influence the immobilization yield, including mass transfer, fiber diameter, nature of enzymatic interactions, enzymatic autolysis during the immobilization process, availability of active groups on nano fibers, and compatibility of fibers with immobilization conditions [46, 47].

Table (2) Immobilization yield of β-Glu, FTase and CMCase immobilized on different supports.

Carrier With glutaraldehyde	β-Glu Immobilization yield I/ (A – B) x100	CMCase Immobilization yield I/ (A – B) x100	FTase Immobilization yield I/ (A – B) x100
PANI 1	202.45	281.43	101.19
PANI 4	234.35	207.05	100.56
Fe@Clay-APTES@PANI	251.95	207.65	101.64
Fe@Clay @PANI	365.74	257.13	99.48
Carrier Without glutaraldehyde			
PANI 1	132.0496	127.23	78.88
PANI 4	234.3502	126.62	94.03
Fe@Clay-APTES@PANI	140.1278	70.71	84.75
Fe@Clay @PANI	141.2498	113.50	79.27

FTIR analysis of PANI, iron oxide, nano clay, hybrid nano supports, and immobilized enzymes

The presence of surface functional groups was investigated by FTIR spectroscopy. The magnetic Fe@Clay@PANI composite, as illustrated in Fig. 2 a, contains the distinctive PANI infrared vibration bands as well. The large absorption bands in the range of 3400–2800 cm^{-1} are attributable to interactions between aligned polymeric chains and nitrogen-containing groups of PANI forms, such as protonated imine NH+ and secondary amine NH (46). At 2848 and 2920 cm^{-1}, the C-H bond's typical stretching vibration then takes place. A signature for the ring stretching vibrations of the hexagonal carbon rings from PANI in their respective quinoid (Q) and benzenoid (B) structures is thought to be the two small peaks located at 1631 and 1495 cm^{-1} [48].

Fig. 2a (c) represents the FTIR spectrum of the iron oxide NPs. It displays several bands at 460, 590, 1460, 1630, and 3415 cm^{-1}. Fe-O-Fe stretching vibrations (462 and 560 cm^{-1}), O=C=O stretching vibrations (1481 cm^{-1}), O-H stretching (3415 cm^{-1}), and O-H bending vibrations (1630 cm^{-1}) all can be attributed to the observed vibration bands. Due to their mesoporous nature, nanomaterials typically absorb carbon dioxide and water from the atmosphere, leading to absorption bands at 1460, 1630, and 3415 cm^{-1} [49]. The characteristic FTIR spectrum of nano clay (Fig 2 a–d) shows a doublet at 2890 and 2928 cm^{-1}, broad peaks at 1043 and 1085 cm^{-1}, and another doublet at 450–600 cm^{-1}.

A protein's peptide groups, which are structural repeating units, can give rise to up to nine distinctive bands known as amides A, B, I, II, and VII. The Fermi resonance between the first overtone of amide II and the N-H stretching vibration gives birth to the amide A band, which is 3500 cm^{-1} in wavelength, and the amide-B band, which is 3100 cm^{-1}. The amide I and amide II bands are the two major bands in the infrared spectrum of proteins. The amide I band (1600–1700 cm^{-1}) is mainly associated with the C=O stretch (70–85%) and is directly related to the backbone conformation [50]. In the current study on β-Glu enzymes, it ranges from 1612 to 1633 cm-1 for both solid and soluble enzymes. Amide II (1448–1580 cm^{-1}) arises from N-H bending and C-N stretching vibrations (Fig. 2b). Amides III and IV are highly complex ribbons resulting from a mixture of multiple coordinate shifts. Out-of-plane motion is seen in amides V, VI, and VIII [50]. Therefore, characteristic peaks of β-Glu carbonyl stretching are also shown at 1654, 2920, and 2977 cm^{-1}.

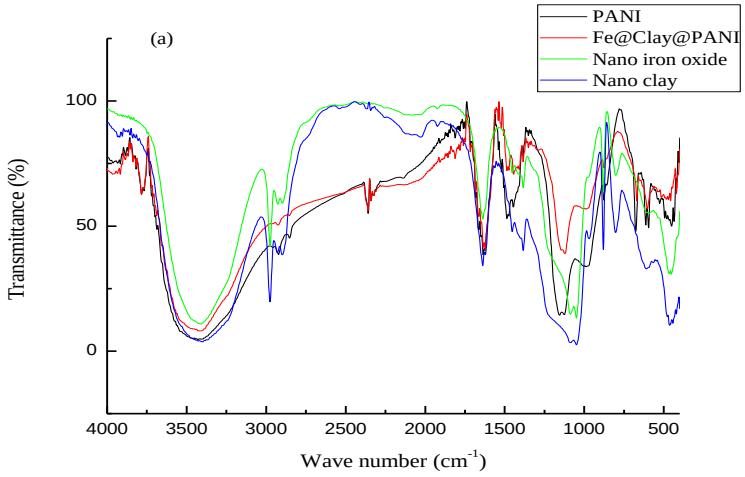
As can be seen in Fig. 2c (FTIR spectra of Glu, Fe@Clay@PANI, and Glu Fe@Clay@PANI) and 2d (Fe@Clay@PANI FTase), nanoparticles revealed iron oxide Fe3O4 by exhibiting absorption peaks about 581 cm^{-1}. This wavelength corresponds to the stretching vibration of the Fe-O bond. The presence of β-Glu on the magnetic nanoparticles was confirmed by the FTIR spectra of enzyme-magnetic nanoparticles, which displayed an absorption peak of about 1543 cm^{-1} (-N-H stretch vibrations) that could only be attributed to the β-Glu in all added materials. As a result, the FTIR spectra also display the distinctive peak of the carbonyl stretch of β-Glu at 1656 cm^{-1} [51]. From the FTIR spectra, it was shown that Fe@Clay@PANI β-Glu underwent a strong conformational modification. Bands at 1454, 2908, and 2964 cm^{-1} for β-Glu disappeared in the immobilized Fe@Clay@PANI β-Glu.

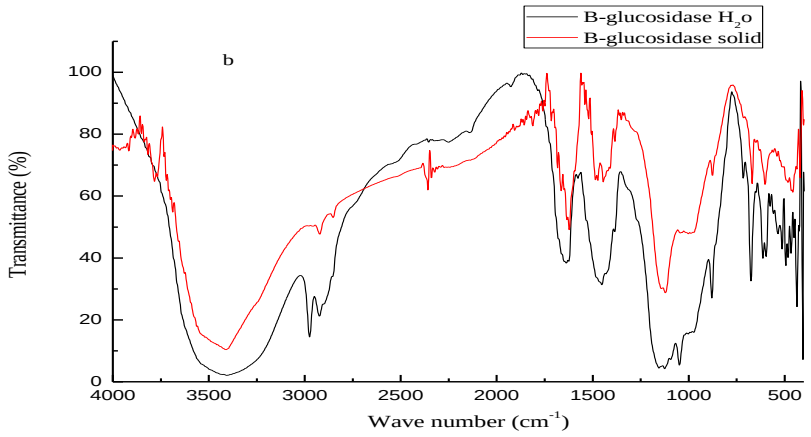
Reusability assay of immobilized enzymes

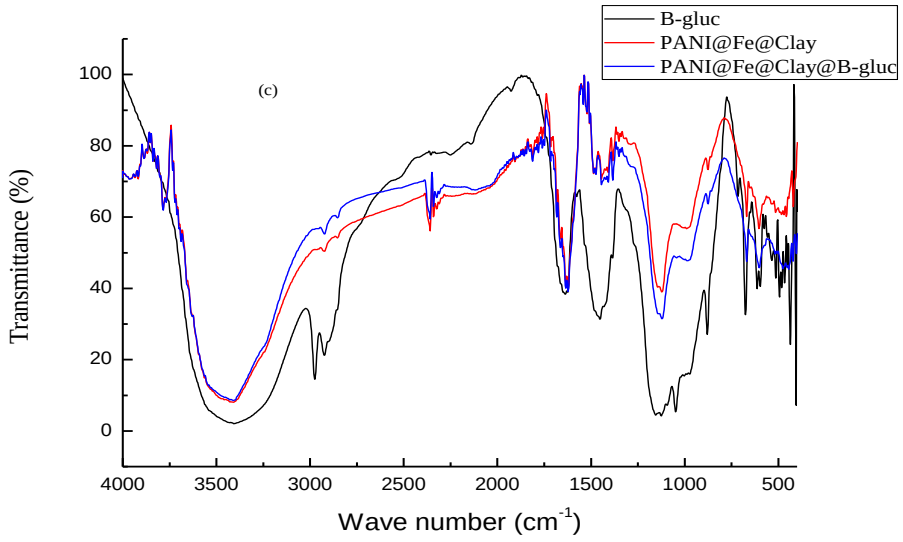
Reusability of immobilized β-glucosidase

The main purpose of enzyme immobilization is to reuse the enzyme several times without losing its catalytic activity. There are many approaches to determining enzyme stability, substrate specificity, or their successful use in industrial processes such as immobilization, modification, protein design and media engineering [2, 3, 52, 53]. The relative activity of the immobilized enzymes over successive application cycles is shown in Figures 3, 4, and 5. In the current study, catalyst reusability studies of the β-Glu loaded on PANI 1, PANI 4, Fe@Clay@PANI, and Fe@Clay-APTES@PANI, were performed under the above optimum assay conditions. At the 20^{th} cycle, the immobilized β-Glu on Fe@Clay@PANI and crosslinked with glutaraldehyde retained 108% of its original activity (Fig. 3). The total activity of the immobilized β-Glu on Fe@Clay@PANI over 20 cycles was 1935/g carrier. Consequently, compared to the other carriers, our findings turned out to be encouraging. As previously reported, the formation of a large complex consisting of interconnected hydrolytic enzyme molecules increased the stability of the generated biocatalyst by demonstrating high retention activity of β-Glu on glutaraldehyde-treated Fe@Clay@PANI [54]. As a result, an excess substrate solution (*p*NPG) microenvironment might have evolved around this complex, facilitating the diffusion of substrate molecules and increasing the likelihood of enzyme-substrate interactions.

Covalently immobilized β-Glu on the nanoparticle carrier has been studied by many authors. Singh et al. [55] demonstrated that β-Glu immobilized on fictionalized silica nanoparticles showed residual activity up to 95% after 25 cycles, significantly higher than other β-Glu/nanoparticle bioconjugates.


(a)
PANI
Fe@Clay@PANI
Nano iron oxide
Nano clay
Transmittance (%)
100
50
0
4000 3500 3000 2500 2000 1500 1000 500
Wave number (cm-1)


b
B-glucosidase H2o
B-glucosidase solid
Transmittance (%)
100
80
60
40
20
0
4000 3500 3000 2500 2000 1500 1000 500
Wave number (cm-1)


(c)
B-gluc
PANI@Fe@Clay
PANI@Fe@Clay@B-gluc
Transmittance (%)
100
80
60
40
20
0
4000 3500 3000 2500 2000 1500 1000 500
Wave number (cm-1)

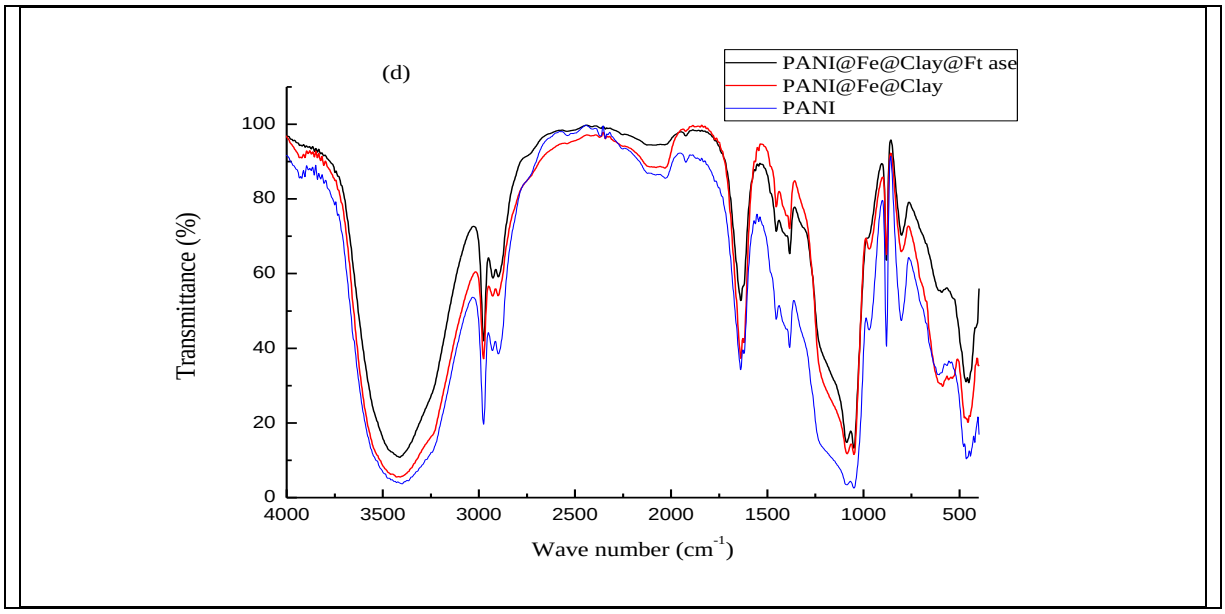


Fig. 2. (a) FTIR spectra of PANI (a); Fe@Clay@PANI (b); Nano iron oxide (c); Nano clay (d).
(b) FTIR spectra of β-glucosidase solution and solid β-glucosidase.
(c) FTIR spectra of β-glucosidase, Fe@clay@PANI and Fe@clay@PANI@B-glucosidase.
(d) FTIR spectra of PANI, Fe@clay@PANI and Fe@clay@PANI@Fr-transferase

According to Verma et al. [56], until the ninth reuse, β-Glu immobilized on magnetic nanoparticles preserved more than 80% of its original activity. After an additional eight hydrolysis cycles, Zheng et al. [57] reported that 84.4% of the initial activity of β-Glu immobilized on chitosan microspheres had been recovered. The separation of immobilized β-Glu is substantially facilitated in these situations by the magnetic beads. Instead, Agrawal et al. [58] revealed that after 10 cycles of reuse, β-Glu still had 70% of its initial activity, which is consistent with our findings.

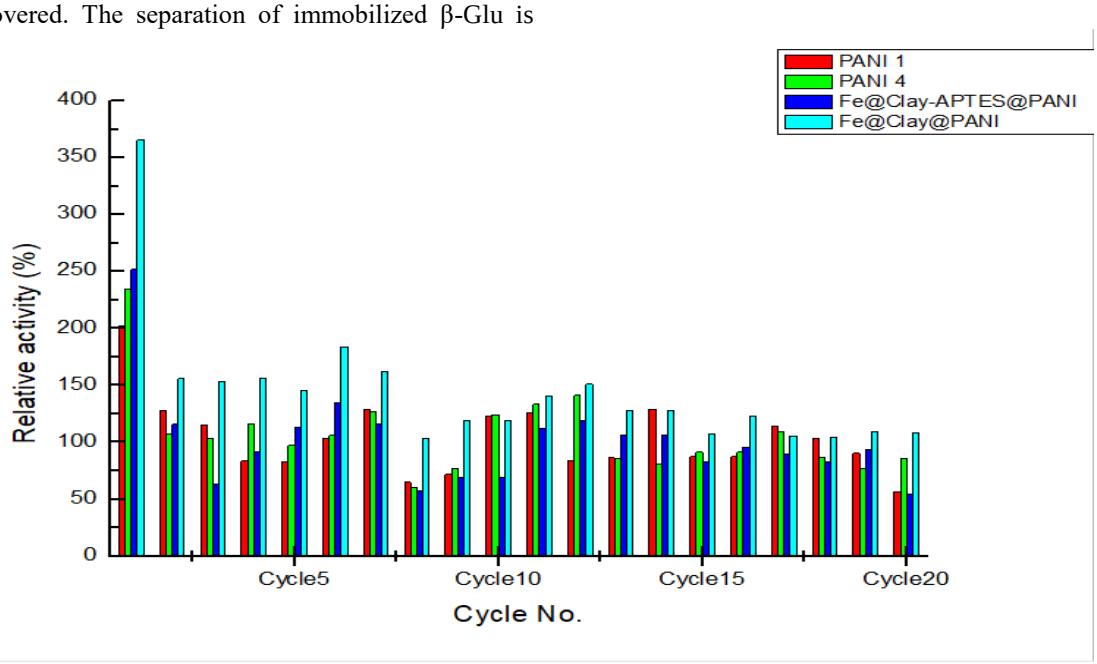


Fig. 3. Reusability of immobilized β-glucosidase on different carriers (with glutaraldehyde treatment), (PAN1) nano-polyaniline fibers No. 1, and (PAN4) nano-polyaniline fibers No. 4., Fe@Clay-APTES@PANI, and Fe@Clay@PANI

Reusability of immobilized fructosyltransferase

Pectinex Ultra SP-L with fructosyltransferase activity has been used to hydrolyze sucrose producing fructooligosaccharides [59, 60]. At the 10th cycle, FTase immobilized on PANI 4, Fe@Clay@PANI and Fe@Clay-APTES@PANI retained 105.5, 90.3 and 99.3% of its initial activity, respectively. (Fig. 4) This high performance of FTase immobilized on PANI 4 would be an advantage for industrial application. High sucrose concentrations and low water content enhance the fructosylation reaction [61]. The hydrophobic nature of the immobilization matrix, which provides low water content for the enzyme, may be to blame for the rise in product quantity.

Reusability of immobilized CMCase

By contrast, immobilized CMCase could only be reused four times with all types of immobilized supports crosslinked with glutaraldehyde (Fig. 5), achieving with the reticulated enzyme a maximum of 25% on the fourth cycle. Due to many considerations, such as deformation of the original protein structure, steric hindrance, and slow diffusion of the incidental substrate to the main surface, immobilization of an enzyme on a planar surface can limit enzyme performance [44, 62]. The loss of CMCase activity following its immobilization on PANI supports can be attributed to a number of causes, such as the potential for strong interactions between the amino acids of the enzyme which are crucial for catalysis or bonding and the surface of natural clay minerals. The disruption of the enzyme protein's three-dimensional structure, the steric restriction on the approach of the substrate (CMC), and the diffusional limitations on solute transport near particles may all contribute to the observed behavior [63]. Due to the immobilization process, the CMCase active center may be near the clay support's surface, which may result in a more hydrophobic environment around the active center than the watery environment did for the free enzyme.

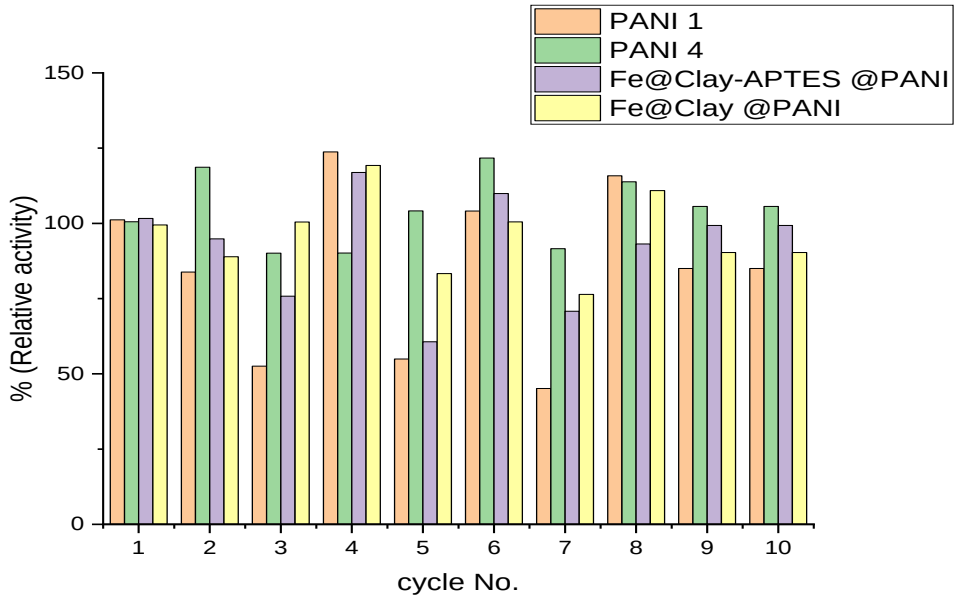


Fig. 4. Reusability of immobilized FTase on different carriers (with glutaraldehyde treatment), (PAN1) nano-polyaniline fibers No. 1, and (PAN4) nano-polyaniline fibers No. 4., Fe@Clay-APTES@PANI, and Fe@Clay@PANI.

Thus, the hydrophobicity of the support or its internal morphology might alter the CMCase's capacity for adsorption, the shape of the area around the active center, and ultimately the CMCase's final catalytic properties [64]. Because clay minerals have a persistently negative surface charge, they can bind and adsorb protein molecules via a variety of physical and chemical processes, such as hydrogen bonds, Van der Waals forces, hydrophobic affinity, cation exchange, and static electricity. In a medium with an adsorbent pH, it depends on the positively and negatively charged states of protein molecules [65]. Many of the adsorption sites on clay minerals are influenced by clay minerals as well as the structure, size, and characteristics of protein molecules. Through cation exchange, a few small, positively charged protein molecules may be adsorbed into the montmorillonite interlayers. Between layers of montmorillonite, small protein molecules associated with wine [66] and chicken egg white lysozyme [67]

are joined by cation exchange. Through hydrophobic interactions between layers, some proteins containing hydrophobic groups are incorporated into clay minerals. Therefore, the weight and size of proteins must be considered in the adsorption of proteins to clay minerals. Ralla et al. [68] mentioned that the adsorption capacity for ovalbumin (43.5–45 kDa), trypsinogen (23.9 kDa), and hemoglobin (64.5 kDa) was somewhat greater than alkaline phosphatase (AP) because the average pore diameter of the smectitic clay mineral is less than the AP hydrodynamic diameter, which led to limited access for the relatively large AP. The adsorption of three distinct proteins, bovine serum albumin (BAS, 68 kDa), α-lactalbumin (28.4 kDa), and β-lactglobulin (18.4 kDa), on the surface of kaolinite was studied by Barral et al. [69]. They mentioned that the size of the protein and the degree of adsorption were clearly correlated, with the largest BSA proteins being the least adsorbed.

Clay, on the other hand, is inherently very porous. Montmorillonite functionalized with 3-APTES and glutaraldehyde shows reduced surface area and pore volume. This is likely due to the binding of silane and glutaraldehyde molecules to clay minerals in the interlayer space [70].

Several factors influence the activity, stability, and abundance of charged enzymes. These include mass transfer [71], fiber diameter, type of enzymatic interaction, enzymatic autolysis during the immobilization process, availability of active groups on nanofibers, and compatibility of the immobilization condition with fibers [46, 47]. External mass transfer, i.e., moving the substrate from the environment to the enzyme's surface, and internal mass transfer, i.e., diffusion of the substrate into the support or carrier media, have been identified as two types of mass transfer that affect the function of an immobilized enzyme [71].

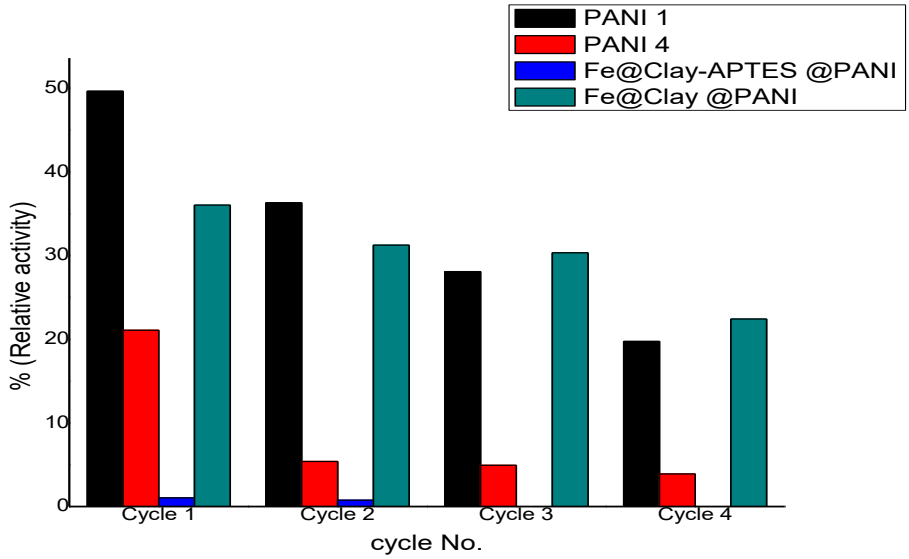


Fig. 5. Reusability of immobilized CMCase on different carriers (with glutaraldehyde treatment), (PAN1) nano-polyaniline fibers No. 1, and (PAN4) nano-polyaniline fibers No. 4., Fe@Clay-APTES@PANI, and Fe@Clay@PANI.

Another element determining the activity and stability of an enzyme is the type of interaction with nanofibers. By increasing the surface-to-volume ratio, nanofibers increased mass transfer and enzyme activity [72, 73]. Since the loaded enzyme in aggregation is more excellent than the covalent approach, Lee et al. [74] found that the stability and activity of β-glucosidase immobilized by the aggregation method are higher than those of the enzyme immobilized by the covalent method. Enzyme autolysis during the immobilization process generates amino acids, which compete for active sites on the nanofibers, resulting in reduced enzyme loading, stability, and activity. In some cases, the active groups are covered by other nanofibers, making them unavailable to the enzyme and reducing enzyme loading [47].

Conclusion

An efficient preparation of a composite based on nanomagnet oxide, nano clay, and nano polyaniline (Fe@Clay@PANI) crosslinked with glutaraldehyde was used as a matrix for immobilizing three enzymes: β-glucosidase, fructosyltransferase, and carboxymethyl cellulase. The immobilized

derivatives of β-glucosidase, fructosyltransferase, have shown improved catalytic efficiency and good reusability, making them attractive for a number of biotechnological applications. While carboxymethyl cellulase did not. Structural analysis indicates that the enzymes is intercalated in the prepared hybrid nano particles.

Acknowledgements
The authors would like to thank Chemistry of Natural and Microbial Products Department, and Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt for supporting this work.

Author contributions
Nour El-Deen A. M., El-Shazly A. I., Shata H. M. A. H., Farid M. A. conceived the experiments;
Nour El-Deen A. M., El-Shazly A. I., Shata H. M. A. H., performed the experiments;
Nour El-Deen A. M., El-Shazly A. I., Shata H. M. A. H., analyzed the data;
Nour El-Deen A. M., El-Shazly A. I., Shata H. M. A. H., writing draft preparation.
Farid M. A. writing-review and editing.

Data availability
All data used in this study are available on request from the corresponding author.

Declaration of competing interest
The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

References

[1] Maghraby Y. R., et al., *Enzyme Immobilization Technologies and Industrial Applications*. ACS omega, 2023. 8 (6): p. 5184-5196.
[2] Singh N.,Dhanya B.,Verma M. L., *Nano-immobilized biocatalysts and their potential biotechnological applications in bioenergy production*. Materials Science for Energy Technologies, 2020. 3: p. 808-824.
[3] Thakur K., Attri C., Seth A., *Nanocarriers-based immobilization of enzymes for industrial application*. 3 Biotech, 2021. 11: p. 1-12.
[4] Zdarta J., et al., *A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility*. Catalysts, 2018. 8 (2): p. 92.
[5] Singh V., et al., *Polysaccharide-silica hybrids: design and applications*. Polymer Reviews, 2016. 56 (1): p. 113-136.
[6] Liu W.,Wei L., *Research progress on carbonic anhydrase immobilization*. J. Mol. Catal, 2016. 30: p. 182-197.
[7] Yang X.Y., et al., *Immobilization technology: a sustainable solution for biofuel cell design*. Energy & Environmental Science, 2012. 5 (2): p. 5540-5563.
[8] Rajdeo K., et al., *Immobilization of pectinase on reusable polymer support for clarification of apple juice*. Food and Bioproducts Processing, 2016. 99: p. 12-19.
[9] Sui C., et al., *Immobilization of glucoamylase onto electrospun PAA/PVA microfibrous membrane by active ester method*. Chin. J. Process Eng, 2016. 16 (3): p. 494-499.
[10] Riccardi C. M., Kasi, R. M., Kumar, C. V., Nanoarmoring of enzymes by interlocking in cellulose fibers with poly (acrylic acid). In *Methods in enzymology*, Elsevier: 2017; Vol. 590, pp 475-500.
[11] Yavuz A. G.,Uygun, A., Bhethanabotla, V. R., *Preparation of substituted polyaniline/chitosan composites by in situ electropolymerization and their application to glucose sensing*. Carbohydrate Polymers, 2010. 81 (3): p. 712-719.
[12] Badgujar K. C.,Bhanage, B. M., *Investigation of deactivation thermodynamics of lipase immobilized on polymeric carrier*. Bioprocess and biosystems engineering, 2017. 40: p. 741-757.
[13] Zdarta J., et al., *Chitin-lignin material as a novel matrix for enzyme immobilization*. Marine drugs, 2015. 13 (4): p. 2424-2446.
[14] Monteiro de Paula C. R., Pessoa Andrade Feitosa, J.,Beserra Paula, H. C., *Polysaccharide based copolymers as supramolecular systems in biomedical applications*. Current drug targets, 2015. 16 (14): p. 1591-1605.
[15] Zucca P., Fernandez-Lafuente, R., Sanjust, E., *Agarose and its derivatives as supports for enzyme immobilization*. Molecules, 2016. 21 (11): p. 1577.
[16] Huang J.,Kaner, R. B., *A general chemical route to polyaniline nanofibers*. Journal of the American Chemical Society, 2004. 126 (3): p. 851-855.
[17] Zhang S., et al., *Polymer–inorganic microcapsules fabricated by combining biomimetic adhesion and bioinspired mineralization and their use for catalase immobilization*. Biochemical Engineering Journal, 2015. 93: p. 281-288.
[18] Kara F., et al., *Immobilization of heparin on chitosan-grafted polyurethane films to enhance anti-adhesive and antibacterial properties*. Journal of Bioactive and Compatible Polymers, 2016. 31 (1): p. 72-90.
[19] Bilal M., et al., *Magnetic metal-organic frameworks immobilized enzyme-based nano-biocatalytic systems for sustainable biotechnology—A review*. International Journal

of Biological Macromolecules, 2023. p. 123968.
[20] Ambrogio M. W., et al., *Mechanized silica nanoparticles: a new frontier in theranostic nanomedicine*. Accounts of chemical research, 2011. 44 (10): p. 903-913.
[21] Cabrera M. P., et al., *Polyaniline-coated magnetic diatomite nanoparticles as a matrix for immobilizing enzymes*. Applied Surface Science, 2018. 457: p. 21-29.
[22] Kim R. E., et al., *Enzyme adsorption, precipitation and crosslinking of glucose oxidase and laccase on polyaniline nanofibers for highly stable enzymatic biofuel cells*. Enzyme and microbial technology, 2014. 66: p. 35-41.
[23] Ghosh S., Chaganti S. R., Prakasham R., *Polyaniline nanofiber as a novel immobilization matrix for the anti-leukemia enzyme l-asparaginase*. Journal of molecular catalysis b: enzymatic, 2012. 74 (1-2): p. 132-137.
[24] Huang J., *Syntheses and applications of conducting polymer polyaniline nanofibers*. Pure and applied chemistry, 2006. 78 (1): p. 15-27.
[25] Valls-Chivas Á., et al., *Enzyme–Iron Oxide Nanoassemblies: A Review of Immobilization and Biocatalytic Applications*. Catalysts, 2023. 13 (6): p. 980.
[26] Sardar R. A. M., *Enzyme immobilization: an overview on nanoparticles as immobilization matrix*. Biochemistry & Analytical Biochemistry, 2015. 4 (02): p.
[27] Sardar M., et al., *Immobilization of enzymes on magnetic nanoparticles*. Encyclopedia of Nanoscience and Nanotechnology, ed. HS Nalwa (Los Angeles, CA: American Scientific Publishers), 2018. p.
[28] Lau E. C.,Yiu H. H., *Enzyme immobilization on magnetic nanoparticle supports for enhanced separation and recycling of catalysts.* In Nanomaterials for biocatalysis, Elsevier: 2022; pp 301-321.
[29] Özdemir F.,Yalcinkaya, Z., *Examination of The Immobilization and Kinetics of The Laccase Enzyme on Various Clay Minerals*. MAS Journal of Applied Sciences, 2023. 8 (2): p. 286-306.
[30] Remonatto D., et al., *Utilization of clay materials as support for Aspergillus japonicus lipase: An eco-friendly approach*. Catalysts, 2021. 11 (10): p. 1173.
[31] An N., et al., *Immobilization of enzymes on clay minerals for biocatalysts and biosensors*. Applied Clay Science, 2015. 114: p. 283-296.
[32] Chen M., et al., *The Application of Mineral Kaolinite for Environment Decontamination: A Review*. Catalysts, 2023. 13 (1): p. 123.
[33] Zakaria Z., et al., *Effect of hydrochloric acid concentration on morphology of polyaniline nanofibers synthesized by rapid mixing polymerization*. Journal of Nanomaterials, 2016. 16 (1): p. 312-312.
[34] Zhang X., Manohar S. K., *Polyaniline nanofibers: chemical synthesis using surfactants.* Chemical communications, 2004. (20): p. 2360-2361.
[35] Yang C., et al., *Surface Functionalized Natural Inorganic Nanorod for Highly Efficient Cellulase Immobilization*. RSC Adv., 2016. 6: p.
[36] Yang C., et al., *Surface functionalized natural inorganic nanorod for highly efficient cellulase immobilization*. RSC advances, 2016. 6 (80): p. 76855-76860.
[37] Otieno D., Shah, N., *Endogenous β-glucosidase and β-galactosidase activities from selected probiotic micro-organisms and their role in isoflavone biotransformation in soymilk*. Journal of Applied Microbiology, 2007. 103 (4): p. 910-917.
[38] Duan K. J., Sheu D.C., Chen J. S., *Purification and characterization of β-fructofuranosidase from Aspergillus japonicus TIT-KJ1*. Bioscience, biotechnology, and biochemistry, 1993. 57 (11): p. 1811-1815.
[39] Somogyi M., *Determination of blood sugar*. Journal of Biological Chemistry, 1945. 160: p. 69-73.
[40] Mandels M., Reese, E. T., *Fungal cellulases and the microbial decomposition of cellulosic fabric,(Volume 5)*. Journal of industrial microbiology & biotechnology, 1999. 22: p.
[41] Miller G., *Modified DNS method for reducing sugars*. Anal Chem, 1959. 31 (3): p. 426-428.
[42] Fang F. F., Dong Y.-Z., Choi H. J., *Effect of oxidants on morphology of interfacial polymerized polyaniline nanofibers and their electrorheological response*. Polymer, 2018. 158: p. 176-182.
[43] Yu W. H., et al., *Adsorption of proteins and nucleic acids on clay minerals and their interactions: A review*. Applied Clay Science, 2013. 80: p. 443-452.
[44 Ding S., et al., *Increasing the activity of immobilized enzymes with nanoparticle conjugation*. Current opinion in biotechnology, 2015. 34: p. 242-250.
[45] Chinwatpaiboon P., et al., *Modified Activated Carbon: A Supporting Material for Improving Clostridium beijerinckii TISTR1461 Immobilized Fermentation*. Bioinorganic Chemistry and Applications, 2023. 2023: p.
[46] Lee G., Kim J., Lee J. H., *Development of magnetically separable polyaniline nanofibers*

for enzyme immobilization and recovery. Enzyme and microbial technology, 2008. 42 (6): p. 466-472.
[47] Fathi M., et al., *Use of nanotechnology for immobilization and entrapment of food applicable enzymes*. In Bioactive molecules in food, Springer: 2019; pp 2037-2061.
[48] Trchová M., et al., *Spectroscopy of thin polyaniline films deposited during chemical oxidation of aniline*. Chemical Papers, 2012. 66 (5): p. 415-445.
[49] Rahman M. M., et al., *Iron oxide nanoparticles*. Nanomaterials, 2011. 3: p. 43-67.
[50] Aboul-Enein Y., Bunaciu A., Fleschin, S., *Evaluation of the protein secondary structures using Fourier transform infrared spectroscopy*. Gazi University Journal of Science, 2014. 27 (1): p. 637-644.
[51] Zhou Y., et al., *Immobilization of β-glucosidase onto Magnetic Nanoparticles and Evaluation of the Enzymatic Properties*. BioResources, 2013. 8 (2): p.
[52] Chauhan V., et al., *An insight in developing carrier-free immobilized enzymes*. Frontiers in Bioengineering and Biotechnology, 2022. 10: p. 794411.
[53] Pratush A., Seth A., Bhalla T. C., *Expression of nitrile hydratase gene of mutant 4D strain of Rhodococcus rhodochrous PA 34 in Pichia pastoris*. Biocatalysis and Biotransformation, 2017. 35 (1): p. 19-26.
[54] Öztürk H., et al., *Nano clays for lipase immobilization: biocatalyst characterization and activity in polyester synthesis*. Polymers, 2016. 8 (12): p. 416.
[55] Singh R. K., et al., *Covalent immobilization of β-1, 4-glucosidase from Agaricus arvensis onto functionalized silicon oxide nanoparticles*. Applied Microbiology and Biotechnology, 2011. 89: p. 337-344.
[56] Verma M. L., et al., *Immobilization of β-glucosidase on a magnetic nanoparticle improves thermostability: application in cellobiose hydrolysis*. Bioresource technology, 2013. 135: p. 2-6.
[57] Zheng P., et al., *Immobilized β-glucosidase on magnetic chitosan microspheres for hydrolysis of straw cellulose*. Process Biochemistry, 2013. 48 (4): p. 683-687.
[58] Agrawal R.,Verma A., Satlewal A., *Application of nanoparticle-immobilized thermostable β-glucosidase for improving the sugarcane juice properties*. Innovative Food Science & Emerging Technologies, 2016. 33: p. 472-482.
[59] Hang Y.,Woodams E., *Fructosyltransferase activity of commercial enzyme preparations used in fruit juice processing*. Biotechnology letters, 1995. 17: p. 741-744.
[60] Hang Y.,Woodams E., *Optimization of enzymatic production of fructo-oligosaccharides from sucrose*. LWT-Food Science and Technology, 1996. 29 (5-6): p. 578-580.
[61] Peña-Cardeña A., et al., *Synthesis of Fructooligosaccharides by IslA4, a truncated inulosucrase from Leuconostoc citreum*. BMC biotechnology, 2015. 15 (1): p. 1-9.
[62] Ni Y., et al., *Improved activity of immobilized horseradish peroxidase on gold nanoparticles in the presence of bovine serum albumin*. Journal of nanoparticle research, 2013. 15: p. 1-10.
[63] Sedaghat M., et al., *Enzyme immobilization. Part 4. Immobilization of alkaline phosphatase on Na-sepiolite and modified sepiolite*. Applied clay science, 2009. 46 (2): p. 131-135.
[64] Fernandez-Lorente G., et al., *Interfacially activated lipases against hydrophobic supports: Effect of the support nature on the biocatalytic properties*. Process Biochemistry, 2008. 43 (10): p. 1061-1067.
[65] Benetoli L. O., et al., *Amino acid interaction with and adsorption on clays: FT-IR and Mössbauer spectroscopy and X-ray diffractometry investigations*. Origins of Life and Evolution of Biospheres, 2007. 37: p. 479-493.
[66] Xifang S., et al., *Adsorption of protein from model wine solution by different bentonites*. Chinese Journal of Chemical Engineering, 2007. 15 (5): p. 632-638.
[67] Johnston C. T., et al., *Interaction of biological molecules with clay minerals: a combined spectroscopic and sorption study of lysozyme on saponite*. Langmuir, 2012. 28 (1): p. 611-619.
[68] Ralla K., et al., *Adsorption and separation of proteins by a smectitic clay mineral*. Bioprocess and biosystems engineering, 2010. 33: p. 847-861.
[69] Barral S., et al., *Interactions between whey proteins and kaolinite surfaces*. Acta Materialia, 2008. 56 (12): p. 2784-2790.
[70] Gopinath S., Sugunan S., *Enzymes immobilized on montmorillonite K 10: Effect of adsorption and grafting on the surface properties and the enzyme activity*. Applied Clay Science, 2007. 35 (1-2): p. 67-75.
[71] Drauz K.,Gröger, H.,May, O., *Enzyme catalysis in organic synthesis, 3 volume set*. John Wiley & Sons: 2012; Vol. 1.
[72] Verma M. L., Puri M., Barrow C. J., *Recent trends in nanomaterials immobilised enzymes*

for biofuel production. Critical reviews in biotechnology, 2016. 36 (1): p. 108-119.

[73] Seenuvasan M., et al., *Review on surface modification of nanocarriers to overcome diffusion limitations: An enzyme immobilization aspect*. Biochemical engineering journal, 2020. 158: p. 107574.

[74] Lee S.M., et al., *β-Glucosidase coating on polymer nanofibers for improved cellulosic ethanol production*. Bioprocess and biosystems engineering, 2010. 33: p. 141-147.