Cellulose-deconstruction potential of nano-biocatalytic systems: A strategic drive from designing to sustainable applications of immobilized cellulases

Sarmad Ahmad Qama1, Mahpara Qamar1, Muhammad Bilal2,*, Ram Naresh Bharagava3, Luiz Fernando Romanholo Ferreira4,5, Farooq Sher6, Hafiz M.N. Iqbal7,*

1Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.
2School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huaiian, 223003, China.
3Laboratory of Bioremediation and Metagenomics Research (LBMR), Department of Microbiology (DM), Babasaheb Bhimrao Ambedkar University (A Central University), Vidya Vihar, Raebareli Road, Lucknow 226 025 U.P., India.
4Waste and Effluent Treatment Laboratory, Institute of Technology and Research (ITP), Tiradentes University, Farolândia, Aracaju-SE, 49032-490, Brazil.
5Graduate Program in Process Engineering, Tiradentes University (UNIT), Av. Murilo Dantas, 300, Farolândia, 49032-490, Aracaju-Sergipe, Brazil.
6Department of Engineering, School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, UK.
7Tecnologico de Monterrey, School of Engineering and Sciences, Monterrey, 64849, Mexico.

*Corresponding authors emails: bilaluaf@hotmail.com (M. Bilal); hafiz.iqbal@tec.mx (H.M.N. Iqbal).

Abstract

Nanostructured materials along with an added value of polymers-based support carriers have gained high interest and considered ideal for enzyme immobilization. The recently emerged nanoscience interface in the form of nanostructured materials combined with immobilized-enzyme-based bio-catalysis has now become research and development frontiers in advance and applied bio-catalysis engineering. With the involvement of nanoscience, various polymers have been thoroughly developed and exploited to nanostructured engineer constructs as ideal support carriers/matrices. Such nanotechnologically engineered support carriers/matrix possess unique structural, physicochemical, and functional attributes which equilibrate principal factors and
strengthen the biocatalysts efficacy for multipurpose applications. In addition, nano-supported catalysts are potential alternatives that can outstrip several limitations of conventional biocatalysts, such as reduced catalytic efficacy and turnover, low mass transfer efficiency, instability during the reaction, and most importantly, partial, or complete inhibition/deactivation. In this context, engineering robust and highly efficient biocatalysts is an industrially relevant prerequisite. This review comprehensively covered various biopolymers and nanostructured materials, including silica, hybrid nanoflower, nanotubes or nanofibers, nanomembranes, graphene oxide nanoparticles, metal-oxide frameworks, and magnetic nanoparticles as robust matrices for cellulase immobilization. The work is further enriched by spotlighting applied and industrially relevant considerations of nano-immobilized cellulases. For instance, owing to the cellulose-deconstruction features of nano-immobilized cellulases, the applications like lignocellulosic biomass conversion into industrially useful products or biofuels, improved paper sheet density and pulp beat in paper and pulp industry, fruit juice clarification in food industry are evident examples of cellulases, thereof are discussed in this work.

**Keywords:** Cellulose-deconstructing enzymes; Immobilization; Nanostructured carriers; Polymeric supports; Bio-catalysis; Sustainability; Food industry

### 1. Introduction

Enzymes are extremely efficient biocatalysts being extensively employed in various industrial and biotechnological processes. Cellulase (EC 3.2.1.4) is one of the important enzymes, frequently used for the hydrolysis of 1,4-glycosidic linkages among cellulose molecules to synthesize monosaccharides subunits. Cellulases exhibit numerous applications in pharmaceutical, pulp & paper, detergents, chemical, food, and biomedical sectors [1-4]. Moreover, cellulases could be employed for the fermentation of lignocellulosic biomass to produce biofuels [5-8]. However, much concern has been devoted towards the stability and reusability of cellulases that restricted its industrial applications [9-13]. Recently, the microbial production of cellulases using cost-effective methodologies, and to overcome the research challenges has been remarkably considered for the cellulases to improve industrial applications [14,15]. Although cellulase enzyme exhibit several applications in industrial and biomedical sectors, majority of these
cellulase types suffer from low pH and thermal stabilities in various media. Moreover, their utilization is also restricted because of their lack of recyclability. Various methods e.g., chemical modifications, protein engineering, and their immobilization in different biopolymers and nanomaterials could be used to enhance enzymatic stability and recyclability potential [10,11,13,14,16]. Figure 1 shows significant potential of nanomaterials for enzyme immobilization [17]. Among all, immobilization provides more benefits regarding heterogeneous catalytic reactions and to overcome the lack of recyclability [18,19]. Immunization methodologies could be divided into three major classes such as (i) surface immobilization [20,21]; (ii) self-immobilization [22]; and (iii) entrapment [23-25]. These methodologies are extensively adopted for the immobilization of biocatalysts that could be inorganic or organic, e.g., polymers, polysaccharides, proteins, activated carbon, and metal nanoparticles [13,16,26]. However, the efficiency of immobilized biocatalyst on its stability, support separation from product, extent of recyclability, and the activity after immobilization are the key factors which are carefully considered while using a support material [10]. Among all supports, nanostructured materials have special place regarding their distinguished high surface area along with other characteristic features that enable higher enzyme loading efficacy (Figure 2) [17,22,27]. However, the enzymes immobilization using nanostructured materials may be restricted due to the issues with recovery, such as filtration or centrifugation for industrial biotechnological applications, which have created serious issues with the processes e.g., extraction and purification of enzymes. These limitations could be removed by employing magnetic nanoparticles as a promising support material for biomolecules e.g., nucleic acids, antibodies, enzymes, and peptides, with high recovery and recyclability potential [9,28]. The convenient process for the recovery of magnetic nanocarriers from the reaction mixture could be the employment of magnetic field and their less toxic nature make these nano-supports as promising materials for enzyme immobilization [9,22,29]. Except these several biopolymer and nano-structured supports have also been employed for the immobilization of cellulases for functional applications. Typically, there are four different immobilization modes of enzymes e.g., entrapment, covalent attachment, physical adsorption, and crosslinking. Attachment of enzymes and
support matrix can be covalently or weakly and physical or chemical [10]. Physical attachment of enzyme with support matrix is relatively weaker than covalent binding, and it has lower capability of keeping enzyme fixed to the support matrix. The support materials are generally polymers/biopolymers, and nanocarriers. In entrapment, the enzymes are integrated on a membrane apparatus such as polymer network e.g., an organic polymer or a silica sol-gel, desolate fiber, or a microcapsule. The formation of a polymer network is required for entrapment in the presence of enzyme. The formation of enzyme aggregates or crosslinking crystals by the utilization of crosslinking or bifunctional agent is another mode of immobilization of enzymes [30]. Carrier-binding or physical adsorption mode of immobilization uses water-insoluble carriers e.g., synthetic polymers, polysaccharide derivatives, and glass [31,32]. In covalent binding or crosslinking, crosslinker reagent e.g., hexamethylene diisocyanate, bisdiazobenzidine, and glutaraldehyde are required [33]. Different polymers such as carrageenan, collagen, and cellulose are used by entrapment mode. However, membrane confinement require the formation of microcapsules and liposomes [34,35].

Immobilization of enzymes using membrane entrapment method is reported to be an excellent biochemical engineering approach because it enable continuous operations, stability enhancement, and retention in bioreactors. In membrane bioreactor, physical adsorption immobilization reduce the loss of catalytic activity of enzymes and enhance the recyclability potential ultimately decreasing the bioprocess cost [36]. The enzyme immobilization is preferred over free enzymes due to its long-term availability that reduce the excessive purification methodologies. The enzyme recovery method could be the ultrafiltration which require the utilization of membrane systems allowing the passage of small molecules and keeping the enzyme in bioreactors. Membrane fouling is another important factor to consider while the operation and development of membrane system because it effects the performance, operational cost, cleaning needs, and pretreatment requirements [37]. There are few other modes of enzymatic immobilization which could be the combination of above-mentioned processes, which are particular for specific enzyme or matrix. Nevertheless, not a single method or support matrix is efficient for all enzyme kinds and their usage because of array of different product utilization, characteristics of enzymes, substrates, and products, and the chemical composition.
This review discusses the current advancements in engineering aspects of novel nanostructured and biopolymer-based support materials for their effective deployment for cellulase immobilization (Figure 3). Moreover, the exceptional features of various nanomaterials such as silica, hybrid nanoflower, carbon nanotubes/nanofibers, nanomembranes, graphene oxide nanoparticles, and metal-oxide frameworks have also been reviewed as robust matrices for cellulase immobilization. Several biotechnological applications of immobilized cellulases such as cellulose hydrolysis, pulp and paper industry, food industry, and other potential multifunctional applications have also been reviewed in this article.

2. Immobilization of cellulases on polymers-based supports

2.1 Cellulases immobilization on Ca-alginate

Ca-alginate entrapment is an important strategy for the immobilization of enzymes [25]. Alginates are biopolymers which are commonly present in the market as water soluble Na-alginates. Ca-alginate entrapment is recognized as inexpensive, non-toxic, rapid, and versatile technique for the immobilization of various cells and enzymes [38]. Alginates are biopolymers obtained from marine plants, composed of unbranched linear polymer chains consisting of α-(1,4)-linked L-guluronic acid, and β-(1,4)-linked D-mannuronic acid residues. It results in the development of thermostable and biocompatible hydrogels in the occurrence of Ca$^{2+}$ ions [39,40]. The use of alginate as immobilization support for bioprocessing application provides high thermo- and pH stable gels to use at room temperature. Alginates synthesize gels with most divalent and multivalent cations. In addition, the gelation could not be induced by Mg$^{2+}$ and monovalent ions, however, Sr$^{2+}$ and Ba$^{2+}$ induce stronger alginate gels than Ca$^{2+}$. However, the use of Ca-alginate gels for enzymes immobilization has commonly been reported [25,41-43].

Andriani et al. [41] studied the immobilization of cellulase produced by *Bacillus subtilis* strain isolated from puffer fish, using carboxy methyl cellulase (CMC) as substrate and Ca-alginate as support-material. Different factors were optimized such as calcium chloride concentration, sodium alginate concentration, pH, and temperature. The enzyme showed high stability at pH 6, however, no changes were observed at maximum pH before and after immobilization. The enzyme with greater stability was achievable at the 2% sodium alginate and 0.15 M calcium chloride solution. As compared to the free
enzyme, the slight increase in $K_m$ and $V_{\text{max}}$ was attained by kinetic studies. The immobilized enzyme showed high recyclability up to four times without significant loss in initial activity. The enzyme lost its activity at 30 °C within three days but the storage stability was more efficient at 4 °C and remained active up to 12 days [41]. Similarly, Viet et al. [44] studied cellulase immobilization using Ca-alginate entrapment. The 2% of sodium alginate content was reported efficient for the formation of stable alginate beads. The CMC was used as a substrate to analyze the cellulase activity. The beads with 3 mm diameter and 30 min immobilized time showed the maximum (83.645%) efficiency for enzyme immobilization. The optimum pH 4.5 was observed for cellulase immobilization. As compared to the free cellulase, the highest optimum temperature of 55 °C and 60 °C was calculated from immobilized enzyme. The 69.2% activity retention after 5 consecutive cycles indicated good storage stability of immobilized enzyme [44].

Sankarraj and Nallathambi, [45] immobilized cellulase enzyme on to hybrid Con-A (concanavalin A) covered by the calcium alginate–starch beads. The jack beans were used to isolate the Con-A and SDS-PAGE analysis was used for the determination of crude protein. The high storage stability and mechanical properties were observed by the immobilized cellulase that even after a month of incubation, showed 100% and 85% activities at 4 °C and 30 °C, respectively. The free form of enzyme maintained 20% of its activity even after 5 consecutively repeated experiments, however, the immobilized cellulase retained 70% of its activity that demonstrates its better thermal stability after immobilization. The research provided a facile process for the immobilization of cellulase for improved stability and recyclability perspective [45]. Another study conducted by Abdel-Sater et al. [46] reported cellulase production by Penicillium brevicompactum specie and the enzyme was immobilized in Chitosan-alginate beads using magnetic nanocarriers and glutaraldehyde as crosslinking agents. The optimizational results indicated pH 6 and 30 °C temperature to achieve the maximum cellulase activity in the medium containing sodium nitrate and palm date leaves incubated for 9 days. The high structural stability was observed after the ammonium sulfate precipitation due to the two-folded increase in enzymatic activities. It was observed that acidic pH and high temperature favors the precipitated enzyme. The maximum activity at pH 5.5 and 50 °C temperature was achieved by immobilized cellulase which was remained excellent up to
80 °C. Different conditions such as resistant to microbial invade, nontoxicity, biocompatibility, easy synthesis, and moderate gelatin conditions play important role for the desirability of enzyme encapsulated within alginate beads. For different biotechnological and industrial demands, the immobilized enzyme shows the usefulness proven by the study [46].

In a recent report, Imran et al. [42] studied the production of cellulase from *Aspergillus tubingensis*, and the enzyme was immobilized using Ca-alginate as support material. The excellent increase in catalytic activity and stability was determined. As compared to the free enzyme after 26 h incubation, the immobilized cellulase showed 82% thermostability at high temperature (75 °C). For both free and Ca-alginate immobilized cellulase, the enzymatic activities were decreased after the 20th day of incubation. The activity of cellulase (179 ± 0.4 UmL⁻¹min⁻¹) for xerogel matrix was obtained at 45 °C and it exhibited the activity of (174 ± 0.4 UmL⁻¹min⁻¹) at pH 4.5. The highest $K_m$ values were noticed for the immobilized enzyme as compared to the free cellulase; however, lowest $K_m$ was observed by xerogel immobilized enzyme. The enhanced tolerance capacity of 75–82% was observed for the immobilized cellulase on Ca-alginate and xerogel matrix in opposition of activators and or inhibitors like EDTA, SDS, Hg²⁺, Co²⁺, and Ca²⁺ [42]. These studies proved the immobilized cellulase as an excellent candidate for industrial and biotechnological uses due to enhanced fruit juice saccharification.

### 2.2 Cellulases immobilization on chitosan

Chitosan as a functional material, offers various desirable characteristics including hydrophilicity, gel forming properties, heavy metal ions chelation, antibacterial properties, physiological inertness, nontoxicity, biodegradability, biocompatibility, and remarkable affinity to proteins. Due to these novel features, chitosan-based materials are yet understudied, and can be expected to be frequently explored in near future for various bioprocessing applications including immobilization supports [25,47,48]. For instance, Abd El-Ghaffar and Hashem, [49] studied the immobilization of cellulase enzyme onto chitosan, chitosan–4-amino butyric acid, and chitosan–L-glutamic acid supports using covalent crosslinking approach. The assay was performed at 25 °C and pH 7, and retention in cellulase activities were observed for the chitosan, chitosan–4-aminobutyric acid crosslinked with 1% of glutaric dialdehyde, and chitosan–L-glutamic acid as 65.52%,
As compared with free enzyme, immobilized enzyme exhibited better pH, thermal, and storage stability profiles. The immobilized enzyme maintained 60% of its initial activity 6-times from its original activity after the immobilization on chitosan-GDA (1%). The change was not observed even after the 10th cycle for chitosan-glutamic acid-GDA (1%) and chitosan-4-aminobutyric acid–GDA (1%) immobilized cellulase. For above-mentioned carriers, the 70% and 50% of activities were maintained after the 25 consecutively repeated experiments [49]. Similarly, Miao et al. [50] used Fe₃O₄ nanoparticles onto chitosan for direct immobilization of cellulases via glutaraldehyde crosslinking to form nano-supports of magnetic chitosan microspheres. Different conditions for enzyme immobilization were also optimized which indicated 5 h incubation, 15 mL (0.1 mg/mL) enzyme, temperature 30 °C and pH 7. The enzymatic recovery was 73.5 mg/g (71.6%) of maximum solid loading rate was observed for medium-chain triglycerides (MCTs) at optimized conditions. The immobilized cellulase can be regenerated and reused without significant loss in activity for 3 consecutive experiments. However, the better storage stability and thermal optima were observed for the immobilized cellulase as compared with free cellulase [50].

Sánchez-Ramírez et al. [28] studied the production and immobilization of Trichoderma reesei cellulase using chitosan-coated magnetic nanocarriers as support material via glutaraldehyde as coupling agent. Magnetic nanocarriers (around 10 nm diameter) were formed after cellulase immobilization however, 8 nm diameter was observed before immobilization. The enhanced thermal and storage stability was analyzed for immobilized cellulase along with the 37% retention of initial activity. The magnetic field was applied to separate the cellulase and after 15 cycles of CMC hydrolysis, immobilized enzyme maintained around 80% of its initial activity. Kinetic studies indicated about 8 times increase in $K_m$ value of immobilized enzymes as compared with free enzyme. The hydrolyzed Agave atrovirens leaves-based lignocellulosic material showed the ability to reuse in material hydrolysis up to four consecutively repeated experiments with 50 % of activity retention. Lignocellulose hydrolysis showed the yield near to the activity obtained from free enzyme [28]. Similarly, Díaz-Hernández et al. [51] studied cellulase and xylanase immobilization by chitosan coated magnetic iron oxide nanoparticles produced in single step via alkaline precipitation to get maximum enzyme loading. Overall, 93%
magnetic saturation of the magnetite was achieved by the crosslinking of chitosan-coated magnetite particles (Fe$_3$O$_4$@chitosan) with cellulase and xylanase enzymes. The characterizational analysis indicated that the 12 mg enzyme per 1 g of magnetic support, and 162 mg of chitosan was coated on 1 g of nanocomposite. The crosslinking between cellulase and Fe$_3$O$_4$@chitosan support was confirmed by characterization analysis. The average particle size of 230–430 nm was reported for supports before and after immobilization [51].

Mo et al. [52] used sugarcane bagasse to prepare the porous biochar which was covered with varying quantities of chitosan for cellulase immobilization using glutaraldehyde as the crosslinker (Figure 4). Characterizational analysis indicated that high thermal and pH stability after immobilization. Furthermore, the good reusability and activity was also observed for these three types of immobilized cellulases. For cellulase@CS25, the support maintained the better morphology of porous biochar with the feeding ratio (biochar: chitosan, 0.5 g:25 mg). The associated immobilized cellulase demonstrated the 90.8 % of glucose production even after 10 repeated experiments and maintained 67 % activity of free enzyme at pH 4 and 60 °C [52]. In another study, Mo and Qiu, [53] prepared porous biochar by pyrolyzing sugarcane bagasse followed by calcination for the magnetization with γ-Fe$_2$O$_3$. The synthesized chitosan/magnetic porous biochar was employed as an immobilization support material for cellulase by covalent bonding after fabrication with chitosan activated by glutaraldehyde. The pH 5 and temperature 25 °C for 12 h of incubation showed 80.5 mg cellulase/g support amount for efficient immobilization of cellulase. Varying pH and temperature were used to study the CMC hydrolysis Both free and immobilized enzymes showed optimum values as pH 4 and temperature of 60 °C. The relatively high enzyme recovery of 73.0% was recorded for the immobilized cellulase. Moreover, the slower maximum reaction velocity ($V_{\text{max}}$) and higher $K_m$ values were reported than free cellulase [53].

2.3 Cellulases immobilization on hybrid polymers-based supports

Various hybrid polymer-based support matrices have been reported for the immobilization of cellulases for bioprocessing applications. For instance, the use of reversible insoluble-soluble enteric polymer coupled with carbodiimide to perform covalent immobilization of a commercial cellulase was performed by Yu et al. [54]. The binding efficiency (81.08%)
of covalently immobilized Eudragit-cellulase was greater as compared with non-covalent Eudragit-cellulase (56.83%). The optimum pH 5 and temperature 50 °C caused the increase in the relative activity of both immobilized and free cellulase; however, more increase in pH and temperature showed the negative impacts on the activity of both native cellulase and covalent Eudragit-cellulase. The higher pH and temperature showed higher stability for the covalent Eudragit-cellulase. The free cellulase had the $K_m$ value of 2.89 g/L, less than that of covalent Eudragit-cellulase (4.78 g/L). The immobilization on the Eudragit S-100 tend to increase the affinity of the cellulase to its substrate [54].

Ince et al. [55] used surface initiated-atomic transfer radical polymerization for grafting the poly(styrene-divinylbenzene) (PS-DVB) microspheres with the polystyrene. In next step, sulfuric acid in the existence of $P_2O_5$ was used to proceed the sulfonation of the grafted polystyrene chains. Aniline (4.8 mmol/g) was applied on the surface to neutralize the sulfonic acid groups. The oxidation of potassium persulfate was carried out to provide the self-doped and thick (16 μm) PANI layers on the microstructures. The oxidized potassium persulfate was further used to polymerize the adsorbed aniline achieved by previous stage. The adsorption/crosslinking methodologies were used to immobilize the cellulase on the polyaniline coated PS-DVB-g-PS micro-spheres. As compared with free enzyme, the immobilized cellulase had the excellent storage stability, higher maintenance of activities relative to the temperature and pH [55]. Similarly, Romo-Sánchez et al. [56] studied immobilization of two enzymes (cellulase and xylanase) on two polymeric support matrices (alginate-chitin and chitosan-chitin) via different chemical ways such as crosslinking-adsorption, reticulation, and adsorption to improve stability and recyclability of enzymes. The chitosan polymer was proved as an ideal support by giving 170 μg/mL of optimal enzyme concentration for cellulase. However, 127.5 μg/mL for the xylanase. Moreover, the optimal pH binding of cellulase was 4.5 and for xylanase was 5.0. The immobilization procedure showed the better stability after the application of lower amounts of glutaraldehyde. The use of glutaraldehyde enabled the activity retention up to 64% after immobilization of cellulase for 19 cycles [56].

The use of core–shell polymer–protein nanocarriers for cellulase immobilization was reported by a recent study [57]. The immobilization of His6-tagged cellulases with controlled spatial orientation of enzymes was achieved through the synthesis of functional
polymeric micelles that collectively potent towards the hydrolysis of cellulose known as cellulosomes. The one-pot reversible addition–fragmentation chain-transfer polymerization was used for the formation of poly(styrene)-b-poly(styrene-alt-maleic anhydride), followed by the usage of nitrilotriacetic acid (NTA) to attain amphiphilic block copolymer. The Ni-NTA-functionalized micelles were prepared by the mixing of self-assembled polymer with the solution of NiSO₄. These functionalized micelles were able to synthesize core–shell nanostructures with cellulases as the immobilized biocatalyst after the capturing of His6-tagged cellulases. Synergistic analysis has been achieved in this study resulting from over twofold activity enhancement because of the site specificity and close proximity of site particular oriented enzymes [57].

2.4 Cellulases immobilization on other polymers
The development of cellulase bioconjugates with N-isopropyl methyl acrylamide with N-(Hydroxymethyl) acrylamide and methyl acrylate for recyclable thermo-responsive immobilization support was studied by Ding et al. [58]. The process of construction of bioconjugate is shown in Figure 5 [58]. Small-molecular quenching was applied for the adjustments of the LCST by the aminooxy polymerization of N-isopropylmethacrylamide (PNMN). PNMN by carbodiimide bioconjugate (PNMN-C) was covalently linked with the cellulase. The highest immobilization yield was 83.2% for the polymer-cellulase bioconjugate construction under the optimized conditions. The free cellulase revealed the maximum activity at 55.0 °C (pH 5.0) as compared to the polymer-cellulase bioconjugates at 50.0 °C (pH 5.0). After repeated five hydrolysis experiments, 85.2% of initial activity was maintained for polymer-cellulase bioconjugate. As compared to the LCST, PNMN could be collected as precipitate after dissolving and efficient use at 50.0 °C [58].

The impactful carriers favorable for the cellulase immobilization were investigated by Tata et al. [59] by free radical cross-linking co-polymerization in reverse suspension to prepare the copolymers of divinylbenzene (DVB) and N-vinylformamide (NFV). The variation of spherical and crosslinking nanoparticles with variable sizes were used for the synthesis of nanocarriers types based on P(NVF-co-DVB). The three (VAM-co-DVB) polymers with vinylamine units were achieved after the hydrolysis of the formamide carrier group into the amino groups. The vinyl formamide groups (without glutaraldehyde) and VAM (with glutaraldehyde) were used for cellulase immobilization. The efficient immobilization of
cellulase was achieved by tested carriers that act as excellent support materials. But as compared to the native enzyme, the enzyme immobilized on the P(VAM-co-DVB0.27)/2000/350 carrier showed the highest catalytic activity [59]. The production of cellulose-derived bioethanol was studied by cellulase enzyme immobilization to enhance the catalytic productivity and cellulase reusability [60]. The visible light induced graft polymerization on low-density polyethylene films, fabricated by a layered structure with a thin poly(ethylene glycol) gels as the inner layer and sodium polyacrylate (PAANa) brush as the outer layer. This hierarchical support showed for the immobilization of two enzymes i.e., cellulase and β-glucosidase. The β-glucosidase from the LDPE surface was in situ entrapped into inside hydrogels layer during the polymeric grafting to improve the catalytic efficiency additionally to cellulase. The cellulase was covalently immobilized on to the outer PAANa brush layer during the reinitiating of sodium acrylate after its polymerization on the PEG hydrogel layer. The β-glucosidase could attain the high activity after the graft polymerization because of the slight reaction such as visible-light irradiation. The optimal temperature of cellulase and the β-glucosidase or the optimal pH did not change during the immobilization. But after the immobilization, the sudden shift of pH 5.0 was observed in case of cellulase. The dual enzyme system showed the 82% and 20% enhanced enzymatic efficiency contrasting with the original activity of isolated BG/cellulase immobilization system and single cellulase system. The repeated experiments up to 10 cycles of CMC hydrolysis relative to original activity shows high stability and recyclability of enzyme after immobilization [60].

3. Cellulases immobilization on nanosupports

3.1 Cellulases immobilization on silica-based supports

The electrostatic interaction is an important factor to consider for the intensification of adsorption rate, while dealing with immobilized cellulases and other enzymes. Therefore, the development of opposite surface charges on the enzyme and the carrier is considered as basic factor [61]. Secondly, the adjustment of pore size for the entrapment of enzymes is essentially must not be so large that it will cause desorption. Therefore, the similarity is necessarily required in the mesopores size and the molecular dimensions of the biocatalysts [25,62]. Cellulases exhibit high affinity with hydrophobic surfaces, therefore, the hydrophobic groups of silica surfaces also have significant role in enzyme adsorption
and desorption. Poorakbar et al. [63] developed mesoporous silica-magnetic Au-NPs core-shell for the immobilization of cellulase enzyme. Santa Barbara Amorphous-15 (SBA-15) was used for the early immobilization of cellulase on mesoporous silica-based nano-support [64,65]. The accommodation of bulky enzymes was carried on the SBA-15 because of its larger pores size. Takimoto et al. [66] studied the cellulase produced from \textit{Trichoderma viride}, immobilized on SBA-15 nanosupports with different pore sizes of 4, 8.9, and 11 nm. The SBA-15 (isoelectric point (pI) = 3) was negatively charged and the cellulase (pI = 4.9) was positively charged. The electrostatic interactions were considered as the driving force for the enzymatic adsorption on nano-supports [66]. The measurements were taken at pH 4.0 and 37 °C for cellulase activity determination based on the hydrolysis of crystalline cellulose. The highest activity was reported by the use of intermediate pore sized support, regardless of conviction that the largest pore size support indicated the smallest increase in the amount of absorbed enzyme. The smaller pores of silica were not enough to penetrate the large microcrystalline cellulose. The cellulase was primary entrapped in the interior of the pores (11 nm sized) support, however, in case of .9 nm pore size, the cellulase molecules were located at or very close to the entrance of the pores. The immobilized nanobiocatalyst showed improved storage stability and recyclability [66].

Hartono et al. [67] studied the immobilization of cellulase via physical adsorption using organo-operational FDU-12 type mesoporous silica-based supports. The immobilization showed favorable behavior towards FDU-12 materials with larger pore size and high pore connectivity. However, the desired interaction between enzyme and silica support was achieved by surface modification through selective functionalization. This development was carried out by the co-condensation of organosilanes (trimethylbenzene), vinyl-(VTMS) trialkoxysilane, 3-mercaptopropyl (MPTMS), 3-aminopropyl- (APTES) and TEOS. S-APTES and S-VTMS were selected for further studies. The loading capacity of S-APTES (21.80 mg/g) was higher than the S-VTMS (18.19 mg/g). While, the FDU-12 had the 10.35 mg/g of support loading capacity, which was less than both functionalized nanocomposites. The adsorption pH 4.8 gave the negative charge to the support matrices and the enzymes which contributed to the loading capacity of S-VTMS to provide the hydrophobic interactions between the vinyl group and the enzyme. The CMC hydrolysis
revealed higher activity retention (up to 70% of the free enzyme) for S-VTMS, while the S-APTES showed less activity (3.4%) of the free enzyme. The formation of amide bonds at the enzyme active site imparted less S-APTES activity due to the active site of cellulase which contained these residues. The benign microenvironment for cellulase activity was developed due to the presence of hydrophobicity in S-VTMS. The S-VTMS reattained 100% of its initial activity after 15 days with very minute leaching [67].

Harmoko et al. [68] investigated the co-condensation optimization of tetraethyl orthosilicate (TEOS) and conc. vinyltrimethoxysilane (VTMS) in term of particle size for cellulase immobilization. The nano and micro particles were synthesized by varying the VTMS/TEOS ratio along with pore entrance of 5-6 nm and pore size of 9-10 nm. This study revealed the higher activity for the cellulase immobilized on silica nanoparticles in contrast to microparticles with immobilized cellulase. This feature was explained by higher microparticle channel length that caused the inactivity of enzyme. The efficient contact between enzyme and substrate was observed due to the short channel length of nanoparticles which prevent the formation of inactive site along the pore channels [68].

Similarly, Chang et al. [69] developed silica nanoparticles of ultra large pore (20–40 nm) and small pore size of 2-5 nm. Dimethyl phthalate as pore expander was utilized for the formation of larger pore sized materials by co-condensation with 3-aminopropyltrimethoxysilane. They immobilized the enzyme on large porous silica by both physical adsorption and covalent binding. The functionalized silica was prepared by covalent crosslinking of cellulase to (3-trietoxysylilpropyl) succinic acid anhydride (TESP-SA). High immobilization efficiency was reported by large pore sized silica as compared with smaller pore size. The presence of both Si-OH and Si-NH₂ groups provided the larger pore size to the silica supports than cellulase molecule size. Therefore, electrostatic interaction between cellulase and Si-NH₂ enabled easy physical adsorption [69].

The ionic liquid method was applied to synthesize the oligomers of cellulose. The glucose yield of free cellulase was approximately 85% which plotted further against the glucose yields of the three biocatalysts were 33.30%, 77.89%, and 83.79%, respectively. The importance of pore size of the host material was proved by the results [70]. The carboxylic groups showed the binding with the cellulose-binding domain. The covalent crosslinking was shown by the storage stability of TESP-SA that hinder enzyme leaching, however, it
showed 86.56% of glucose yield after 23 days’ storage at room temperature. The direct linkages between carboxylic acids possibly present in the active site of the enzyme and –NH₂ of APTES was avoided by the operated silica-surface with APTES, followed by glutaraldehyde crosslinking. The steric constraints are avoided by the glutaraldehyde acting as a spacer arm between the matrix and the enzyme. In another study, Kannan and Jasra, [71] studied the *Penicillium funiculosum* cellulase immobilization on meso-cellular foams via covalent linking. The operated reactions was consisted of APTES with amino functionalization and glutaraldehyde crosslinking. The pore size was decreased from 21.8 nm to 10.8 nm by the crosslinking of meso-cellular foams; however, the pores had sufficient vacuum for cellulase shelter. The modified meso-cellular silica with surface-functional groups for CMC hydrolysis was shown with greater activity of the immobilized enzyme. Furthermore, the immobilized enzyme showed higher $V_{\text{max}}$ (9.8 U/mg) in contrast to free enzyme (5.3 U/mg). The enzyme and meso-cellular silica surface revealed opposite charges at pH 5. However, the diffusion of substrate molecules and enzyme was easy due to larger pore size. Moreover, 66% of the initial activity was retained after 15 reaction cycles which exhibited excellent stability of immobilized cellulase [71].

Yin et al. [72] studied the immobilization of cellulase enzyme using mesoporous silica (SBA-7) as support material without the NaBH₄ reducing. They found 8-fold increase in $V_{\text{max}}$ assigned to the stability enhancement after immobilization. Limited substrate diffusion was observed inside the pores due to increased $K_m$ value. The support materials maintained the enzymatic tertiary structure at high temperature. The multi-point attachment caused the higher activity of immobilized cellulase in broad range of pH and increase in thermal stability at 60 °C. After 11 cycles of reaction, the 88% of initial activity was conserved for immobilized cellulase. Similarly, Zhang et al. [73] studied immobilization of cellulase on silica gel by covalent linking. Herein, the used surface functionalization reduced the industrial-silica pore size from 10.6–16.2 nm to 7.7–10.6 nm. The loaded cellulase retained 7% of its initial activity in CMC hydrolysis with quantity of 18.8 mg/g of silica gel. The activity loss was observed in three steps during reuse for immobilized enzyme. The 82-100% of activity was reattained from 1st to 7th cycle, 60-48% from 8th to 13th cycle, and 23-36% from 14th to 26th cycle. It was observed that the enzyme desorption by support caused decomposition of outer surface and denaturation
in the vicinity of the pores at the 2nd stage. The conformational structure shifting was reported to protect the cellulase inside the pores. The storage of immobilized enzyme at 4 °C for 32 days retained the 92.4% of its initial activity and high storage ability [73]. Ungurean et al. [74] studied Trichoderma reesei cellulase immobilization using binary and tertiary mixtures of tetramethyl orthosilicate (TMOS) with methyl-(MeTMOS), and phenyl-trimethoxysilane (PhTMOS) for the development of nanobiocatalyst using sol–gel encapsulation method. MeTMOS/TMOS with 3:1 molar ratio and no additives were used to derive the best operating materials at 4.8 pH in CMC hydrolysis experiment. This study reported more than 90% of total enzyme recovery. The hydrolysis of microcrystalline cellulose (Avicel PH101) was used to study the catalytic efficiency of the entrapped enzyme. The decrease of the kinetic nature was observed by immobilized enzyme and longer reactions; however, after 24 h reaction, the immobilized enzyme showed the less glucose yield than the free one. The immobilized enzyme showed the 10–20% higher thermal stability as compared to free cellulase and increase in pH stability was observed in the pH domain 5.5–7.0. The rigidity provides the protection against undesirable modifications by preventing the denaturation and microenvironment inside the porous structure. An enhancement of enzyme/substrate affinity was used to explain the half $K_m$ for the immobilized cellulase as compared to free one. The mass transfer resistance within the sol–gel matrix showed threefold decrease in $V_{max}$. The 20% leaching after the 6th cycle described the effective reusability potential for various applications [74]. Chen et al. [75] studied the synthesis of two mesoporous silicates having pore size of 3.8 and 17.6 nm, and the cellulase immobilization was performed by pure physical adsorption method. The pore size of the mesostructured support was associated with the enzyme loading. The 1.2-times higher cellulase loading was observed for MS-17.6 pore size. Some cellulase molecules of MS-3.8 played role in blocking the pore entrance. However, the cellulase molecules were easily well managed and adjustable into the MS-17.6 due to larger space. The opposite trend with respect to loading was observed during the measurement of activity of the two biocatalysts, in the CMC hydrolysis at 50 °C and pH 5.0. The MS-17.6 showed the less specificity of 26.6% as compared to the MS-3.8 displayed a higher specific activity (63.3% of free cellulase). The MS-3.8 was observed to increase the availability of active site, trapping of molecules in the pore entrance and
conserving the native structure of cellulase. The conformational flexibility of cellulase lowered its activity because of the obstruction caused by the dense and ordered arrangement of MS-17.6. However, the interaction of substrate with the enzyme requires the conformational change [75].

3.2 Cellulases immobilization on non-magnetic magnetic nanostructures

Different methodologies have been designed to gain wide range of enzymatic applications following low toxicity, enzyme recovery, excellent separation from the reaction mixture, and improved stability for cellulase immobilization on magnetic nanoparticles (MNPs). The cellulase immobilization on MNPs was proceeded by both the nonspecific physical adsorption and covalent binding [20]. Different binding types have been observed such as hydrophobic or stacking interactions, van der Walls, and electrostatic forces during the enzyme’s interaction with the surface of nanomaterials by non-covalent binding [76]. The protein leakage from the surface of nanomaterial was observed as the major drawback from the non-covalent immobilization. The leakage from the carrier and high operational stability was observed during the covalent binding of enzymes [77]. The nanomaterials characteristics such as size, functionalization and structure greatly influence the catalytic behavior and stability of cellulase to determine the effect of magnetic nanoparticles [78]. The conformation and biological function of conjugated enzymes, adsorption effect, and nanomaterial interaction with protein molecules are greatly influenced by the surface chemistry of these nanomaterials [79]. For example, aiming to increase the enzymatic stability, the immobilization was performed on superparamagnetic nanoparticles through ionic linking [80]. Figure 6 shows cellulase immobilization onto iron oxide nanoparticle surfaces [78].

In different experiment to increase the magnetization of nanoparticles and saturation, an activated magnetic support using zinc doping was applied for cellulase immobilization by Abraham et al. [81]. The loading of the enzyme was increased by series of porous terpolymers with crosslinking through suspension and polymerization [82]. In a recent study, Abbaszadeh and Hejazi, [83] immobilized Aspergillus niger cellulase using amine functionalized Fe₃O₄ magnetic nanoparticles via metal binding affinity immobilization. The nano-biocatalytic characterization was performed for the cellulase immobilization by the addition of any intermediate, and copper was selected as ligand for enzyme loading on
magnetic nano-supports in buffering surroundings. The relative enzyme activity 91% was determined, and the amount of enzyme 164 mg/g of magnetic nano-supports, under the optimized conditions. The immobilized enzyme exhibited more stability than free enzyme tested by CMC hydrolysis at 1% concentration. Moreover, after five cycles of reusability, immobilized enzyme reattained 73% of its initial activity. After 8 days of storage at 4 °C, the immobilized cellulase reattained 84% of their initial activity and 70% of initial activity for free cellulase [83].

Mo et al. [84] studied the cellulase immobilization using porous biochar-based support material obtained from lignocellulose biomass due to its attractive properties i.e., poly-porous structure and high specific surface area. The preparation of γ-Fe₂O₃ combined with poly-porous biochar was performed by calcination which was used as support material for the immobilization of cellulase. The highest immobilization capacity (266 mg/g) was achieved for cellulase immobilization with relative 73.6% activity as compared with free enzyme. The results indicated that by increasing temperature, endothermal process was occurred, which resulted high cellulase adsorption. Similarly, Paz-Cedeno et al. [85] studied the immobilization of cellulase and xylanase enzymes using graphene oxide-magnetic nanoparticles (GO-MNPs) as support material for efficient synthesis of cellulosic ethanol and other useful compounds. Homogeneous distribution of MNPs onto the graphene oxide nanosheets was observed. The nanobiocatalysts were developed by covalent crosslinking using hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The designed nanobiocatalyst showed enhanced efficacy for sugarcane bagasse hydrolysis and showed relative activities 66%, 70%, 70%, 88% after ten consecutively repeated experiments for xylanase, β-xylosidase, endoglucanase, and β-glucosidase, respectively. The 80% and 50% nanobiocatalysts efficiency was reported for cellulose and xylan hydrolysis, respectively. The results indicated it as a potent candidate for cellulosic ethanol production.

3.3 Cellulases immobilization on cross-linked enzyme aggregates

The attractive concept that offers valuable technology is explained by the cross-linked enzyme aggregates (CLEAs). Potential advantages of CLEAs are shown in Figure 7 [86]. Generally, the procedure of making CLEAs include physical precipitation followed by glutaraldehyde crosslinking [87,88]. The resulting CLEAs exhibit improved enzymatic
activity and recyclability up to several folds than native enzyme [89]. However, the optimum recovery and handling of CLEAs is difficult because they are mechanically fragile [90]. Kim et al. [91] reported the formation of CLEAs entrapped in mesoporous silica, which did not show leaching through narrow channels. Moreover, significantly high enzyme loading, and improved activity was reported after CLEAs immobilization. The one-pot bioconversion of lignocellulosic biomass to fermentable sugars was achieved through the preparation of CLEAs with xylanase, cellulase and β-1,3-glucanase [92]. The development of CLEAs was carried out by three-phase partitioning (TPP) method. The crosslinking time of 7.5 h was given with glutaraldehyde (100 mM) as a chemical crosslinker. The initial 70% of activity was reattained at 70 °C compared to 30% for the free enzyme indicating good thermal stability of CLEAs. After the incubation for 11 weeks at 4 °C, more than 97% of activity was observed indicating excellent storage stability of CLEAs in contrast to 65% of initial activity for free enzymes. The reuse of CLEAs was made possible due to the presence of free enzymes in the hydrolysate inhibiting their restoration. The CLEAs caused the maximum hydrolysis of ammonia about 83.5% in 48 h while the free enzymes hydrolyzed the sugarcane bagasse about 73% [92]. Similarly, Perzon et al. [93] studied the formation of cellulase-CLEAs via precipitation and crosslinking method which proved as rapid and multifunctional way. There is still needed to elucidate the association between the process parameters and cellulase-CLEA final activity. The CLEAs made from cellulase (EC 3.2.1.4) were optimized for various factors. The different temperature, crosslinking time, and crosslinking concentrations were used for three types of participants such as ammonium sulfate, polyethylene glycol, tert-butyl alcohol. The polyethylene glycol and ammonium sulfate-CLEAs were recovered 29% and 17% of the free enzyme activity, respectively. However, the CLEAs synthesized with tert-butyl alcohol were inactive. The ammonium sulfate-CLEA only recovered 10% of its activity after one cycle whereas the polyethylene glycol-CLEA recovered 40% of the initial activity after four cycles which demonstrated the significance of precipitant on final CLEA activity instead of enzymatic activity in re-solubilization. The ammonium sulfate showed better performance in CLEAs while PEG was not capable to precipitate enzyme [93]. In another study, the supercritical carbon dioxide was used for the activation of cross-linked cellulase aggregates by Podrepšek et al. [94]. Several precipitating reagents such
as propanol, tetrahydrofuran, 2-propanol, acetone, ammonium sulphate, ethanol, and methanol were used to analyze the enzyme precipitation. The highest enzyme activity was achieved by the immobilized enzyme using optimized enzyme concentration of BSA and glutaraldehyde. This study presented the efficient and cost-effective biosynthetic process using 0.0625% glutaraldehyde concentration, and precipitant ethanol. The enhanced level of reusability and stability of immobilized cellulase was reported. This study also indicated more catalytic sites on spherical structure of CLEAs with high surface area. The introduction of new catalytic sites proved beneficial for the nanobiocatalyst [94]. Due to increased depletion of fossil fuels, an alternative energy production way was investigated using enzymatic conversion methodologies using renewable biomass resources and formation of high-value chemicals are being promoted [95-100]. The efficient pH and thermal stability, and recyclability have been reported by the enzymes after immobilization. Jia et al. [101] studied the novel magnetic CLEAs development for the immobilization of cellulase. High thermal and pH stability was observed by the CLEAs immobilized enzyme as compared to free enzyme. The immobilized cellulase maintained the 74% of its initial activity along with the successful magnetic separation after the continual six repeated cycles of CMC hydrolysis. The immobilized enzyme showed the high potential for biomass conversion, indicated by reusability (38% activity retention) up to 4 cycles of biomass conversion and 21% yield during the hydrolyze of bamboo biomass [101].

Jafari Khorshidi et al. [102] conducted study on the amine-functionalized Fe₃O₄@silica core-shell magnetic nanoparticles for the immobilization of cross-linked aggregates (CLEAs) of cellulase aiming to increase usability for the industrial bioconversion of lignocellulosic materials to glucose and other renewable biomaterials. The significant change was not observed in optimum temperature during the acidic behavior switched by the optimum pH of the cellulase cocktail upon immobilization (cellulase CLEA–MNP). The free cellulase lost all of its activity while the cellulase-CLEAs–MNP maintained about 45% of its initial activity. However, at 80 °C, immobilized cellulase maintained 65% of highest activity than the free enzyme. The highest thermal stability was developed at 65°C. Cellulase CLEAs–MNP reattained 30% of its initial activity through six cycles of reusability after the acute decrease during two cycles of CMC hydrolysis [102]. Similarly, Li et al.
[103] synthesized a new carrier-free cross-linked aggregates of cellulase (CLEAs-C) through (NH₄)₂SO₄ precipitation and glutaraldehyde crosslinking. As a precipitant, 95% of ammonium sulfate was used to prepare cellulase-CLEAs. The 50 mg/mL cellulase concentration and 3% (v/v) glutaraldehyde was used in order to acquire the excellent enzymatic activity. The optimum temperature was found to be 60 °C, while the pH 3.0 was found most effective. The CLEAs maintained the 80% of initial activity during the storage for 28 days at 4 °C [103].

3.4 Cellulases immobilization on metal oxide nanoparticles
Different kinds of metal oxides (TiO₂, ZnO, Fe₂O₃/Fe₃O₄, Bi₂O₃, CeO₂, SiO₂, MoO₂) are important for various applications including usability in gas sensors, dye sensitized solar cells, and their catalytic, antimicrobial, electronic, electrical conductivity, and high optical characteristics [104-106]. However, the high cost and environmental factors influence the recovery and reusability of nanostructures. Therefore, immobilization and incorporation are carried out by distinct type of substrates but finding of an appropriate substrate is still a major concern. Various metal oxide nanostructures have been immobilized due to its natural biopolymer properties. In paper matrices, the retention issues are resolved by using the retention aids, binders, and appropriate linkers to conduct the immobilization and incorporation [107,108]. The use of metal oxide nanoparticles for catalytic immobilization purpose have widely been investigated [108-110]. For instance, Jordan et al. [109] studied the immobilization of cellulase enzyme onto magnetic iron oxide (Fe₃O₄) nanoparticles via carbodiimide activation and covalent binding. After the binding of complex, no significant change in size was observed in the magnetic particles, and SEM micrographs revealed a mean diameter of 13.3 nm. Enzyme was supported on the saturation point occurred at a weight ratio of 0.02 and low enzyme loadings demonstrated the maximum binding of 90%. The relative peak enzyme activity was analyzed at 50 °C and enhanced stability was observed over the boarder range of temperature by thermal measurements of nanoparticles. The shift in optimum pH from 4.0 to 5.0 was observed by the ionic forces between the enzyme and support surface [109].
Xu et al. [111] studied cellulase immobilization on magnetic Fe₃O₄ nanoparticles through glutaraldehyde crosslinking. No structural or particle size changes were observed by binding step, and the mean diameter of 11.5 nm was observed in all the nanosized
particles of the magnetic particles with or without bound cellulase. The covalent binding was observed between residual amine groups on magnetic Fe₃O₄ nanoparticles and amine groups of the cellulase efficiently controlled the binding capacity of cellulase. As compared to the free enzyme, improved storage stability and wider ranges of pH and temperature was observed by immobilized cellulase. Immobilized cellulase showed the greater affinity for cellulosic substrate than the free enzyme determined by the enzyme kinetics. The hydrolysis of steam-exploded corn stalks and bleached sulfa the bagasse pulp demonstrated the efficient performance for the immobilized cellulase [111]. Han et al. [112] conducted a study for cellulase immobilization using the surface of magnetic-Fe₃O₄ nano-supports modified by dendritic polymer 4-arm-PEG-NH₂. The covalently immobilized cellulase was prepared by the glutaraldehyde that act as coupling agent for the magnetic supports. Different characteristics such as reusability, storage stability, optimum temperature, Michaelis constant, thermal stability and PH were analyzed. Results indicated 132 mg/g loading ability of cellulase with wider range of pH, temperature, storage, and functional stability as compared to the free cellulase. The 76% increased catalytic activity was observed by immobilized cellulase as compared to the free cellulase [112].

Abbaszadeh and Hejazi, [83] conducted metal affinity immobilization of cellulase on the amine functionalized Fe₃O₄ magnetic nanoparticles (MNPs). The process was carried out without any addition of intermediates, and copper was chosen as ligand and loaded on to magnetic nanoparticles in buffering solution. The relative enzyme activity (91%) was reported, and the amount of immobilized enzyme was 164 mg/g of MNPs under optimized conditions (Cu/MNPs = 1, E/MNPs = 0.11, pH = 6). In contrast to the free enzyme, the immobilized cellulase showed more stability tested by repeated CMC hydrolysis at 1% concentration. Furthermore, 73% of initial activity of immobilized cellulase was reattained after the 5 cycles of usability. The storage step at 4 °C showed the 70 and 84% of initial activity for free and immobilized cellulase after the 8 days storage. This study proved as an excellent candidate for various biotechnological and industrial sectors [83].

3.5 Cellulases immobilization on carbon nanotubes/nanorods

Different strategies have been applied for the synthesis of nanotubes of transition metal chalcogenide materials such as the chemical vapour deposition, use of solid templates
and chalcogenization, etc. Carbon nanotubes formed by such transition metal chalcogenide materials are maybe single walled (SWCNT) or multi walled (MWCNT). Significant research has been conducted to immobilize biocatalysts on these carbon nanotubes and nanoroads. For instance, Mubarak et al. [113] studied the immobilization of cellulase enzyme on functionalized-MWCNT using physical absorption process to overcome the catalytic stability and efficiency issues. The optimum enzyme immobilization percentage of 97% was attained by the usage of 4 mg/mL enzyme concentration. The optimum reaction conditions were reported as 50 °C temperature and pH 5. Characterizational results indicated high efficiency of nanobiocatalyst because cellulase-MWCNT nanocomposite retained 52% of its initial activity after six repeated experiments of CMC hydrolysis. The convenient separation and high stability make it a robust candidate for various applications [113].

Ahmad and Khare, [114] reported the immobilization Aspergillus niger cellulase onto functionalized-MWCNT by carbodiimide crosslinking. MWCNT impart useful characteristics including rapid electrode kinetics, high edge-to-plane ratio, enhanced electronic properties and improved tensile characteristics because of structural arrangements. The nanobiocatalyst designed under optimized conditions exhibited high thermal and pH stability, with up to 85% activity retention. The half-life of nanobiocatalyst was 4-folds higher than free enzyme at 70 °C temperature. Two folds increase in $K_m$ value of resulted nanobiocatalyst towards the substrate was reported. High reusability potential was reported by 10 consecutively repeated experiments without much actual enzymatic activity loss, which make it potential candidate for effective cellulose hydrolysis. Similarly, Ma'an et al. [115] studied the production of cellulase from Trichoderma reesei and the enzyme was immobilized on functionalized-MWCNTs via covalent crosslinking. Different parameters were optimized to get efficient immobilization yield which indicated three most influential parameters i.e., temperature, pH, and EDC concentration. The optimized conditions were 30°C temperature, 4.5 pH, and 1 mL (10 mg/mL) of EDC. The highest immobilization yield (98%) was achieved using above-mentioned optimized conditions [115].

Li et al., [116] reported novel method for immobilization of cellulase using combined sodium alginate and MWCNT. The optimizational results indicated temperature 40 °C and
pH 3.0. Cellulase activity retention (71.2% of its initial activity) was reported after 1 month of storage at 4 °C temperature. The nanobiocatalyst showed up to 70% of its initial activity after 7 consecutively repeated experiments of cellulose hydrolysis. Moreover, high thermal and pH stability, storage stability, and recyclability was reported which showed potential for biotechnological applications. Similarly, Azahari et al. [117] reported cellulase production from *Trichoderma reesei*, and its successful immobilization was performed using MWCNTs by physical absorption. The nanobiocatalyst showed enhanced pH and thermal stability profiles as compared with free cellulase at pre-optimized conditions of pH 5 and temperature 50 °C. After consecutive 3 experiments up to 60% of cellulase activity retention was demonstrated by nano-conjugates [117]. The easy separation, high thermal and pH stability, and excellent reusability of CNT immobilized enzyme make them robust catalyst for various biotechnological and industrial applications.

### 3.6 Cellulases immobilization on graphene oxides nanoparticles

Cellulase immobilization was performed by the development of graphene-based nano-supports with controlled pH and temperature and magnetoresponsive properties [118]. The 2D immobilization supports created the issue of geometric drawback which was resolved by the synthesis of closed copied free functionalized biocatalyst under similar reaction environment. The covalent immobilization showed the betterment in the bio-receptivity of graphene supports and supramolecular assembly of oppositely charged quenched polyelectrolytes and maghemite–magnetite nanoparticles on 2D graphene supports. The chances of recovery and reuse of the enzyme over multiple cycles were achieved by the incorporation of magnetic nanoparticles. The 55% of initial activity was exhibited by immobilized enzymes after four repeated experiments. The effective tool to control the activity of immobilized enzymes was achieved through the modified degree of polyelectrolyte swelling by the controlled temperature and pH. In contrast to the immobilized enzymes without the brushes, the immobilized enzyme with stiffed polyelectrolyte brushes showed the 1.5-fold betterment in the activity at pH 5.1 and 50 °C temperature [118].

Gao et al. [119] used the etherification and diazotization for the synthesis of functionalized graphene oxide and implantation with hydrophobic spacer P-β-sulfuric acid ester ethyl sulfone aniline. The immobilization of cellulase through covalent bonding was attained by
the functionalized graphene oxide as a nano-support. The high immobilization yield and efficiency of above 90% were observed after the optimization of reaction parameters. The significant betterment was observed in thermal and functional stabilities of immobilized cellulase as compared to the free cellulase. The increase of six-fold higher thermal stability was observed by immobilized enzyme (533 min) in contrast to the half-life of free cellulase (89 min) at 50 °C. Furthermore, the immobilized cellulase showed the highest catalytic activity due to linkage between substrate and immobilized enzyme \((K_m = 2.19 \text{ g/L})\) as compared to the free cellulase \((K_m = 3.84 \text{ g/L})\). Similarly, Dutta et al. [119] used the graphene oxide as nano-support reinforced with magnesium oxide nanoparticles (MgN). The \textit{Bacillus subtilis} cellulase was immobilized on GO nano-support crosslinked with glutaraldehyde which increased 3.5-folds increase in enzyme activity at 90 °C and 2.98-folds increase in enzymatic activity at 8 °C. In contrast to the untreated enzyme, the MgN-cellulase graphene oxide showed 5-folds and 4.7-folds increase in \(V_{\text{max}}\) at 8 °C and 90 °C and 6.7-folds decrease in \(K_m\) at 8 °C and 34-folds at 90 °C was reported. In contrast to the natural enzyme, GO-MgN-cellulase showed the half-life of 41.6-folds at 8 °C while 72.5-fold half-life at 90 °C. The storage stability of GO-MgN-cellulase was observed at 4 °C for more than 120 days and the enzymatic activity was maintained even after 12 repeated uses [119].

Paz-Cedeno et al. [85] studied the immobilization of cellulase on magnetic graphene oxide nanoparticles (GO-MNP) as support-material. The immobilized biocatalysts were designed by carbodiimide crosslinking. The developed nanobiocatalyst showed enhanced efficacy for sugarcane bagasse hydrolysis and showed relative activities 66%, 70%, 70%, 88% after ten consecutively repeated experiments for xylanase, \(\beta\)-xylosidase, endoglucanase, and \(\beta\)-glucosidase, respectively. The 80% and 50% nanobiocatalysts efficiency was reported for cellulose and xylan hydrolysis, respectively. The results indicated it as a potential candidate for cellulosic ethanol production. Similarly, Zhang et al. [120] studied the co-immobilization of glucose oxidase and cellulase using graphene oxide as support-material. The one-pot modification of gluconic acid from CMC due to feasible control of loading enzymes with different sorts was reported. The multi enzyme systems had the superficial pH 5 and temperature 40 °C. The values of kinetic constants were \(V_{\text{max}} = 0.18 \pm 0.01 \mu\text{mol.L}^{-1}\text{s}^{-1}, K_{\text{cat}}/K_m = 24.12 \pm 0.52 (17.74 \pm 0.85) \text{s}^{-1} \text{mmol}^{-1}\text{L and}
\[ K_m = 0.15 \pm 0.02 \ (0.43 \pm 0.09) \ \text{mmol.L}^{-1}. \] The loading abilities of cellulase and glucose oxidase on nanobiocatalyst were 49.07 ± 7.47 mg/g and 10.22 ± 2.03 mg/g. After seven cycles, almost 65% of the initial activity was reattained by immobilized catalysts. Remarkably, the 63.82 ± 8.03% conversion of gluconic acid was observed within 2 h of treatment [120].

### 3.7 Cellulases immobilization on nanostructured hybrid organic-inorganic nanosupports

There has been a growing interest in designing hybrid organic-inorganic nanosupports for potential applications in biocatalytic immobilization with the aim to improve recyclability and stability for bioprocessing applications [121,122]. The development of organic-inorganic hybrid nanostructure is quite convenient, but it requires up to three days, which restrict their workability. Therefore, Batule and coworkers designed a sonochemical method, which can rapidly (within 5 min) synthesize organic-inorganic hybrid nanoflowers, apparently due to sonication method causing quick self-assembly of copper phosphate, delivering high energy to the structure [123]. These newly designed hybrid nanoflowers exhibited improved stability and recyclability with similar morphology to those synthesized by conventional method. Studies have primarily reported copper ions for the synthesis of hybrid nanomaterials; however, various other inorganic ions have also been used for this purpose [124]. The immobilization of cellulase using TiO₂-lignin hybrid support via physical absorption was reported by Zdarta et al, [125]. The immobilized cellulase was precipitated by the physical adsorptions on the inorganic–organic hybrid matrix. Different parameters were chosen for the early immobilization such as the 5 mg/mL enzyme solution, 6 h process time, and pH 5. The free and immobilized cellulase were analyzed and compared in terms of storage stability, impacts of pH and number of catalytic cycle sequences. The thermal and chemical stability, immobilization time and amount of enzyme solution were improved during this study evaluated by the dependence of catalytic activity of the immobilized enzyme on the early immobilization factors. The immobilized cellulase retained over 80% of its initial activity after 3 h at 50 °C and pH 6.0. The free enzyme showed the half-life of 63 min while the nanobiocatalyst showed the half-life of 307 min. The immobilized cellulase maintained over 90% of its initial catalytic characteristics after the ten repeated cycles. This novel study illustrated the convenient
and excellent mode for the production of hybrid titanium dioxide–lignin material and its utilization for the immobilization of cellulase as a support material. Over the several cycles, this method proved as efficient way to utilize commercially without any expiration of characteristics. Other biocatalysts could also apply this strategy for the immobilization [125].

Dragomirescu et al. [126] immobilized the cellulase produced from Aspergillus niger by the entrapment in the Na-alginate gels and in Na-alginate/silica gel-hybrid materials. Sol-gel method was used to attain silica gel by using two precursors tetra ethoxy silane and tetra methoxy silane. The results for the similar loadings showed that the mixed organic-inorganic nano-supports showed the less CMCase activities, as compared to the CMCase activities obtained by Na-alginate which was 1.12-1.17-times higher noticing by comparing the enzymatic activities of the immobilized products. The 13% activity of the cellulase was maintained after 4 cycles for the cellulase immobilized in three types of aforementioned organic-inorganic gel matrices. The relative activity was 98% more than the initial for the immobilized Aspergillus niger CMCase after one hour of storage at 37 °C and pH 3.0 [126]. In addition, the immobilization of enzymes has also been studied by the new type of nanomaterial known as nanoflowers that acts as novel nano-support. These are hybrid in nature because of their organic and inorganic combination. The organic portion is formulated by DNA and protein; however, the inorganic portion is made up with metal ion such Cu, Mn, or Ca. The analytical science and catalysis have reported to use the inorganic nanoflowers until the introduction of organic–inorganic nanoflowers. These hybrid nanoflowers are reported to have superior features over the free or immobilized enzymes due to the different properties such as higher stability and catalytic activity, simple production, and greater surface area than the spherical nanoparticles. The five different types of hybrid-nanoflowers are capsular nanoflowers, protein manganese, copper-DNA, protein-copper, and calcium-protein nanoflowers [127,128].

### 3.8 Cellulases immobilization on metal organic frameworks

The metal organic frameworks are synthesized from the particular metal ions and certain organic linkers. Further, the metal organic frameworks are species of highly ordered microporous crystalline hybrid materials and identified as the porous coordination polymers. Mostly used metal ions are actinide elements, alkaline-earth metals, transition
metals and p-block elements for the construction of metal organic framework [129]. But the included organic linkers are sulfonates, carboxylates, amines, nitrates, and phosphates. The magnetic organic frameworks demonstrate the unique features such as plentiful binding interactions for the selection of reactant such as uniform aperture size, comparatively high thermal, mechanical, and chemical stability, adjustable topological structure, large particular area, intrinsic crystalline structure, adjustable ultrahigh porosity, pore volumes and eximious optoelectronic characteristics (Figure 8) [130,131].

The development of novel cellulase immobilized magnetic organic framework composite system with increased reusability and stability for cellulose hydrolysis was performed by Ahmed et al. [132] using physical absorption method. The extra anchoring sites of NH\textsubscript{2} groups showed higher protein loading by NH\textsubscript{2}-functionalized metal organic framework as compared to the precursor UiO-66. Moreover, pH tolerance and increased thermostability were also shown by the immobilized cellulase. The abundance of NH\textsubscript{2} and COOH functional groups on the MOFs increase the stability of cellulase after its absorption and chances of composite recovery were achieved through the mild centrifugation because of the heterogeneity offered by the NH\textsubscript{2} and COOH groups. The maximum activity gained was 85% at 55 °C while utilized at 80 °C and the residual activities were 72% after ten cycles and 65% after 30 days storage. The development of cellulase-MOF composite with ultrahigh operations and durability for research revealed the auspicious future by this study [132].

Qi, Luo & Wan, [133] prepared UIO-66-NH\textsubscript{2} metal organic framework for cellulase immobilization purposes. The highest enzymatic recovery and protein loading efficiency of 78.4% was exhibited by as-prepared immobilized nanobiocatalyst. As compared to the free form, the immobilized cellulase showed high catalytic efficiency, pH stability, and thermal stability on the magnetic organic framework of UIO-66-NH\textsubscript{2}. The good recycling ability for 5 consecutive runs was determined by the immobilized enzyme. Moreover, better tolerance towards two inhibitors (formic acid and vanillin) present in lignocellulosic pre-hydrolysates was shown by the immobilized cellulase in contrast to the free one. The immobilized cellulase showed 16.8% and 21.5% higher activity than free enzyme in the presence of 5 g/L of formic acid and vanillin. The hydrolysis showed the betterment in yield which was 18.7% and 19.6% for the aforementioned amounts of formic acid and
vanillin. This study suggested that the inhibitory impacts of several pretreatment inhibitors on cellulase can be enhanced by the immobilization [133].

Zhou et al. [12] studied cellulase immobilization to attain high ionic liquid tolerance and development of enzymatic hydrolysis biomass in situ. The study used four kinds of organic metal frameworks including PCN-250, ZIF-8, UIO-66-NH₂, and MIL-100-Fe. Physical adsorption method was used for immobilization. The largest enzyme adsorption capacity (176.16 mg/g) was exhibited by ZIF-8 nano-supports. The activity of immobilized cellulase was analyzed using CMC and filter paper as substrates in the presence of ethyl-3-methylimidazolium diethyl phosphate ([Emim]DEP). As compared to the free cellulase, the superior ionic liquid tolerance was achieved by the immobilized cellulase (0% to 50%, v/v). The activity of the CMCase and filter paper cellulase were enhanced the by 112.59% and 59.86% in 50% (v/v) [Emim]DEP by the specific demonstration in ionic liquid tolerance of ZIF-8-immobilized cellulase. The involved ionic liquid showed that the immobilized cellulase can cause the decrease of cellulase inactivation and was linked to the kinetic parameters as the immobilized cellulase had a lower equilibrium dissociation constant value and a higher final enzyme plateau activity value in a reaction system. As compared to the free cellulase, 50% (v/v) [Emim]DEP, the ultimate 92.92% increase was observed in the ZIF-8-immobilized cellulase with in situ hydrolysis of bagasse [12].

4. Biotechnological applications of cellulases

4.1 Applications in cellulose hydrolysis

The current scenario showed the significance of lignocellulosic biomass conversion into industrially useful products or biofuels. The municipal wastes, industrial waste materials, and agricultural byproducts are considered as the major forms of cellulosic biomass [134,135]. Bioconversion of lignocellulosic biomass to fermentable sugars by immobilized magnetic cellulolytic enzyme cocktails is illustrated in Figure 9 [135]. Relevant to these industries, the major concern is to eliminate these wastes from the environment. These industrial wastes are converted into different forms like biohydrogen, biomethane, bioethanol, and sugars with the assistance of cellulose digesting enzymes. Different factors are responsible for affecting the enzymatic hydrolysis such as enzyme linked factors (enzyme compatibility, product inhibition, thermal sensitivity, specificity, origin of enzyme and enzyme processibility) and structural properties of solid substrate [136]. The
crude oil prices are indicating the increasing for the worldwide demand of fuels. The facility of fossil fuel is diminishing at highest speed. The modification of lignocellulosic biomass to bioethanol is possible due to the action of cellulase [137,138]. Plants have a defense barrier named lignin for the enzymes to perform on cellulososes. Therefore, the modification of hemicellulose and cellulose biomass into smaller size sugars by the action of cellulase is achieved by the pretreatment on the plants to remove the lignin from them. Then fermentation is proceeded further to convert the sugars into the ethanol [139]. The biomass conversion into ethanol was more conveniently obtained from *Penicillium* cellulase [140]. In solid waste management, cellulase enzyme is being used to convert the agricultural solid wastes into beneficial products [141,142]. The wastes lignocellulosic materials are also reported to produce renewable energy sources such as bio-methane and bio-hydrogen [143]. Lignocellulose hydrolysis have been showed by the immobilized enzyme nearly equal to the activity obtained from free enzyme; however, high stability and recyclability potential have been reported after immobilization [28]. Similarly, Ingle et al. [144] studied the lignocellulosic biomass conversion by immobilized and free cellulases for bioethanol production. Comparative evaluation of biomass hydrolysis from both free and immobilized cellulase showed that free enzyme converted 78% cellulose to glucose after 24 hours at 40 °C while, immobilized enzyme showed 72% activity in similar environment. Furthermore, efficient recovery by magnetic field and recyclability up to 3rd cycle was noticed which suggested 68% and 52% hydrolysis after second and third cycle, respectively. These findings suggest the convenient recovery of cellulase after immobilization and high reusability with improved thermal and pH stability profiles which make this process useful in biotechnological sectors.

4.2 Applications in pulp and paper industry

The demands of paper and pulp have been enhanced by the enzyme cellulase due to the different requirements of daily uses such as production of paper towels and sanitary pads, bio-modification of fibers, betterment wastewater of the papermills, removing of toners and ink coating from papers and for bio-mechanical pulping [145]. The woody raw material along with stiffness, bulk, and high number of fines are achieved by mechanical pulping such as grinding and refining of woody raw materials [146]. The downside of mechanical pulping is high expenditure of energy, although the attained fibers are used
to produce papers of different quality. Moreover, the efficient hand sheet strength properties and substantial energy savings (20–40%) are observed during refining of cellulase pulping from white-rot fungi by the biochemical pulping as compared to the mechanical pulping [147]. The lower degree of hydrolysis and reduce viscosity of pulp were observed in endoglucanase [148]. The high productivity and trouble-free printing procedure are acquired by the bio-modification of fibers that uses cellulase and hemicellulose and enhances the paper sheet density and pulp beat ability [147]. The recycling of waste papers such as books, magazines and newspapers can be accomplished by cellulases. Thereafter, fibers could be reused in ethanol synthesis and in manufacturing newspapers through bleaching. The discoloration of different sorts of paper wastes have been carried out by cellulase alone or in combination with xylanases. There are few benefits of enzymatic bleaching such as reduced fine particles, strength enhancement, better fiber brightness and prevention of alkali [149]. Yang et al. [150] reported the catalytic degradation potential of methyl orange using Ag-Pt nanoconstructs. The enzymatic bleaching by cellulase at acidic pH decrease the environmental pollution, modification in ink particle size distribution, facilitates the bleaching step and prevention of alkaline yellowing [151,152]. Yassin et al. [153] studied both immobilized and free cellulases for the development of cellulose nanofibers. The results indicated high thermal and mechanical stability by gel-immobilized cellulase. In addition, high activity retention (85%) after six repeated experiments showed good recyclability potential after immobilization. The immobilized cellulose showed high potential to disintegrate cellulose into nanofibrils of diameter (15-35 nm) with varying length. These results indicated high applicability for paper and pulp industry and packaging industry applications.

4.3 Applications in food industry

In food industries cellulase are used for several purposes. The nutritive juice yield with better stability and less processing time is achieved by the maximum liquefaction of smashed fruit pulps which continuous crushing by macerating enzyme having cellulase with pectinases and hemicellulases. The decrease in viscosity, texture and cloud stability of purees and juices are upgraded by macerating enzymes [154]. The higher levels of antioxidants and vitamin E is observed in olive oil extracted by macerating enzymes with
slower initiation of rancidness [9]. The extraction of olive oil was improved by an enzyme 
olivex which was attained by the intermixing of cellulases and hemicellulases from 
*Aspergillus aculeatus* along with pectinase [155]. The coloring agent production for food 
can also be achieved by cellulase [156]. The carotenoids are responsible for providing 
the colors for many plants from red to yellow and these are also considered as the major 
group of coloring substances in nature. The carotenoids have continuous demand in 
market, and they are used as food colorants due to their null toxicity, natural sources, 
high versatility, alluring characteristics and lipo- and hydro soluble colorants [157].

### 4.4 Applications in biofuel production

Enzyme immobilization using variety of different biopolymers and nanostructured 
materials for biofuels production using biomass hydrolysis strategies have widely been 
reported. For this purpose, various nanomaterials including nano-porous silica, carbon 
nanotubes, graphene, nanocomposites, nanofibers, and others provide excellent support 
material for biocatalytic immobilization. Immobilized enzymes show high operational and 
thermal stability, and convenient recyclability using various simple chemical and physical 
methodologies. For biofuel production, two enzymes namely, lipases and cellulases are 
key candidates for biofuel production through various production methodologies. 
Environmentally friendly biomass hydrolysis can be improved in terms of reusability, 
efficiency, thermal and pH stability using enzymes immobilization technology [25]. 
Cellulases immobilization have been studied using variety of different biopolymers and 
nanostructured materials which have previously been discussed in section 2 and 3. For 
biofuel production applications, cellulase immobilization has been performed on silica 
[6,36], polymeric nanostructures [158]. Affinity-tagged cellulases were investigated for co-
immobilization using magnetic silicon nanoparticles doped with gold using one-pot 
cellulose hydrolysis [159]. Approaches to enhance biofuels production in the presence of 
cellulase are shown in Figure 10 [6].

### 5. Challenges, prospects, and conclusions

Nanobiotechnological advancements are the important part of human life, especially in 
the field of industrial. Due to the increase in human and environmental population, nano-
biotechnology manipulates biomaterials to improve the product yield. Cellulase is the 
most frequently employed enzyme that convert cellulosic biomass into monosaccharide
building blocks which are further used for the synthesis of value-added products e.g.,
biofuels. Although extensive developments have been made in the immobilization of
enzymes using variety of different polymeric/biopolymeric, and nanostructured supports,
few critical issues are needed to be addressed before the industrial-scale applications of
nanomaterials-immobilized enzymes. It is demanded to obtain deep acquaintance and
insight on the influence of nanocarriers on enzymes and other biomolecules. For instance,
in depth studies on structural influences on nano-constructs, type of activation agent on
enzymatic loading, functionalization, orientation of bounded proteins could assist in
designing the optimized enzymatic systems. Moreover, such enzymatic methodologies
need to be cost-effective and highly efficient. Therefore, the major challenge for the
scientists is to design and optimize novel processes with improved enzymatic stability,
activity, recyclability, and cost issues, along with easy down-stream processing from the
reaction mixture.

The recent advancements in nanostructured immobilization methodologies have shown
high potential as novel nanobiocatalysts, which can further be optimized in terms of
catalytic efficiency. The re-designing and engineering of novel nanomaterial-based
systems for enzyme immobilization with finely tuned functionalities and structural
features, exhibiting high biocompatibility, minimal toxicity, and insignificant
environmentally hazardous influences accompanied by the choice of appropriate
immobilization protocol might lead to the development of functionalized nanobiocatalytic
systems in the field of energy and biofuel production, biosensing, organic synthesis,
biotransformation, and industrial biocatalysis [122]. The use of magnetic nanocarriers is
becoming interesting due to easy enzyme separation from the reaction mixture which
significantly reduce the catalytic reutilization cost. Different nanostructures having
diversified functional groups as immobilization support exist i.e., inorganic-organic
hybrids, silica-based, metal oxide-based, CLEAs, magnetic, carbon nanotubes (SWCNT
and MWCNT), graphene oxide and others. Despite current advancements, novel
methodologies are still required to achieve highly efficient nanobiocatalysts for bioprocess
applications. Since the recyclability is the major concern to reduce the production cost;
therefore, convenient down-stream processing without actual loss in catalytic efficiency
could be the interesting research area in this regard. Studies show that immobilization have potential to impart such useful characteristics to biocatalysts.

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Conflict of interests

The author(s) declare no conflicting interests.

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hydrolysates and eventually the ethanol fermentation by Saccharomyces cerevisiae. Biomass and Bioenergy, 34(8), 1189-1194.


Figure captions

**Figure 1** Significant potential of nanomaterials for enzyme immobilization. Reprinted from Ref. [17] with permission from Elsevier. License Number: 5081540394562.

**Figure 2** Advantages of nanomaterials as enzyme immobilization platforms.

**Figure 3** Stepwise illustration of cellulose-deconstruction potential of cellulase based nano-biocatalytic systems as a strategic drive from designing to sustainable applications.

**Figure 4** Schematic illustration of the preparatory method of chitosan/magnetic porous biochar as support for cellulase immobilization in the presence of glutaraldehyde (GA) as a cross-linker. Initially, sugarcane bagasse was used to prepare biochar via pyrolysis in the presence of potassium hydroxide (KOH). In the following step, calcination was performed to engineer magnetic biochar which was subjected to chitosan coating and used for cellulase immobilization by GA activation. The bar graph given in red color at the bottom left corner shows the effect of recycling on the glucose productivity of immobilized cellulase. Reprinted from Ref. [53] with permission under the terms and conditions of the Creative Commons Attribution (CC BY) license.

**Figure 5** The process of construction of bioconjugates. Reprinted from Ref. [58] with permission from Elsevier. License Number: 5081660389521.

**Figure 6** Cellulase immobilization on iron oxide nanoparticle surfaces. Reprinted from Ref. [78] with permission from Springer Nature. License Number: 5081660693327.

**Figure 7** Potential advantages of CLEAs. Reprinted from Ref. [86] with permission from Taylor & Francis. License Number: 5081660850455.

**Figure 8** Diagrammatic sketches of enzyme immobilization onto MOF surface by covalent grafting. (A) Direct covalent grafting between MOF and enzyme; (B) A dye-tagging strategy for enzyme immobilization on MOF surface. Reprinted from Ref. [130] with permission from Elsevier. License Number: 5081660986472.

**Figure 9** Bioconversion of lignocellulosic biomass to fermentable sugars by immobilized magnetic cellulolytic enzyme cocktails. Reprinted from Ref. [135] with permission from American Chemical Society.

**Figure 10** Approaches to enhance biofuels production in the presence of cellulase. Reprinted from Ref. [6] with permission from Elsevier. License Number: 5081661163485.
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**Table 1** Polymer-based supports for cellulase immobilization with binding method, stability/recyclability, and industrial applications.

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<th>Stability/Recyclability potential</th>
<th>Industrial applications</th>
<th>References</th>
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<td>Ca-alginate</td>
<td>Entrapment</td>
<td>High activity retention up to four cycles</td>
<td>-</td>
<td>[41]</td>
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<td>Ca-alginate-xerogel matrix</td>
<td>Entrapment</td>
<td>-</td>
<td>Fruit juice clarification</td>
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<td>Na-alginate polyethylene</td>
<td>Glutaraldehyde crosslinking</td>
<td>Up to 53% activity after 3 cycles</td>
<td>Enzymatic MCC hydrolysis</td>
<td>[43]</td>
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<tr>
<td>Ca-alginate</td>
<td>Entrapment</td>
<td>45% retention after 3 days, with stability at 4 °C for 12 days and at 30 °C for 3 days</td>
<td>Enzymatic CMC hydrolysis</td>
<td>[41]</td>
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<td>Ca-alginate</td>
<td>Entrapment</td>
<td>69.2% activity retention after 5 cycles</td>
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<td>Chitosan-Magnetic NPs</td>
<td>Entrapment</td>
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<td>Chitosan amino condensation adduct</td>
<td>Crosslinking and covalent attachment</td>
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<td>Enzymatic CMC hydrolysis</td>
<td>[53]</td>
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</table>
Table 2  Nanocarriers for cellulase immobilization with binding method, stability/ recyclability, and industrial applications.

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<th>Immobilization support</th>
<th>Binding method</th>
<th>Stability/Recyclability potential</th>
<th>Industrial applications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWCNT</td>
<td>Covalent binding</td>
<td>-</td>
<td>Fruit bunches degradation</td>
<td>[115]</td>
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<tr>
<td>GO@CMC-g-poly(AMPS-co-AAm)</td>
<td>Physical crosslinking</td>
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<td>Lignocellulosic biomass hydrolysis</td>
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<td>Saccharification of lignocellulose</td>
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<td>Physical adsorption</td>
<td>High recyclability</td>
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<td>[165]</td>
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<td>Mesoporous silica (SBA-15)</td>
<td>Encapsulation</td>
<td>Up to 70% activity retention after four weeks incubation</td>
<td>-</td>
<td>[66]</td>
</tr>
<tr>
<td>Mesoporous silica (FDU-12)</td>
<td>Physical adsorption</td>
<td>Almost 100% of its activity retention after 15 days incubation</td>
<td>-</td>
<td>[67]</td>
</tr>
<tr>
<td>Iron oxide nanoparticles</td>
<td>Physical adsorption</td>
<td>High recyclability</td>
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<td>[78]</td>
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</tbody>
</table>