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A Review: Potential Usage of Cellulose Nanofibers (CNF) for Enzyme Immobilization via Covalent Interactions

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Abstract Nanobiocatalysis is a new frontier of emerging nanosized material support in enzyme immobilization application. This paper is about a comprehensive review on cellulose nanofibers (CNF), including their structure, surface modification, chemical coupling for enzyme immobilization, and potential applications. The CNF surface consists of mainly –OH functional group that can be directly interacted weakly with enzyme, and its binding can be improved by surface modification and interaction of chemical coupling that forms a strong and stable covalent immobilization of enzyme. The knowledge of covalent interaction for enzyme immobilization is important to provide more efficient interaction between CNF support and enzyme molecule. Enzyme immobilization onto CNF is having potential for improving enzymatic performance and production yield, as well as contributing toward green technology and sustainable sources.

Keywords Nanobiocatalysis · Chemical modification · Chemical coupling · Surface modification · Enzyme immobilization · Nanostructured materials · Cellulose nanofibers (CNF)

Introduction

There are high expectations for nanotechnology to play an important role in biotechnology field. Nanobiotechnology, a branch of nanotechnology involved with biological and biochemical elements, is based on work at the atomic and molecular level such as creation and utilization of materials, devices, and even a system (in range of 1 to 100 nm) to fabricate structures by combining the biological materials especially in integration of physical sciences, molecular engineering, biology, chemistry, genomics, and proteomics [1–3]. Development of

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nanobiotechnology leads to a better perspective in enzyme technology and has a good significance toward commercialization of enzymes, as well as a further innovation in improvement of catalytic performance [4].

Nanobiocatalysis, a common approach used in nanobiotechnology can be defined as a process based on incorporation of enzymes onto nanostructured material. Enzymes are remarkable biocatalysts and have been used in the industrial biotechnology because of their interesting characteristics, such as green chemistry, substrate and product specificity, and ease of preparation [5]. The catalytic activity of enzymes implies that they accelerate reactions by decreasing the activation energy [6]. In biocatalyst fields, major objectives are to stabilize and to recover the enzymes, since they are too costly in the current market. Achieving good stability, enzyme separation, recovery, and life-time cycle rate are successful keys in enzymatic production and commercialization [7]. Native enzymes are commonly applied as industrial biocatalysts due to their higher enzyme activity. Unfortunately, native enzymes often lack long-term stability in operational conditions and pose difficulties for recovery and reuse [8]. For example, capillary gel electrophoresis can be used for separation between enzyme and product [9], but it requires high energy consumption, is expensive, and is not applicable (difficult and time consuming) for large-scale operation.

Enzyme immobilization is a special technique to solve enzymatic problems (especially in the application of native enzyme), such as stability, reusability, and decline of activity due to inhibition either by medium or products [10]. There are two main reasons why enzyme immobilization has the potential to overcome those limitations in an effective way. First, immobilization facilitates separating between enzyme molecule and product, and thus it provides a reliable and efficient condition toward reaction technology [11]. Second, immobilization improves enzyme reusability, since insoluble enzymes are much easier in recycling compared with soluble enzymes [12]. There are several immobilization techniques such as entrapment, encapsulation, adsorption, cross-linking, and covalent binding.

In practice, immobilization of enzyme would cause lower activity or retention activity due to inactivation or denaturation of enzyme, which cannot be completely avoided. However, the level of enzyme inactivation or denaturation depends on the types of enzyme immobilization techniques, structured support, medium conditions (e.g., buffer solution, pH, and temperature), and reaction during preparation of enzyme immobilization [13]. Therefore, the best case scenario to minimize the loss of enzyme activity and to overcome the poor retention is by selecting a proper immobilization technique, supporting selection and optimum immobilization conditions, in order to preserve the enzyme activity as close as to its original performance level by not changing the chemical nature or reactive groups in the enzyme's binding site. Other reasons are to ensure biomolecule stability and to minimize cost [14, 15].

This present paper provides a comprehensive review of the covalent immobilization technique of enzymes onto cellulose nanofiber (CNF) support. Unlike other techniques (adsorption, entrapment, encapsulation, cross-linking), covalent binding has been demonstrated as the most stable interaction for immobilization of enzyme molecules to the support [16]. In order words, covalent binding will minimize the leakage of enzyme from its support into the reaction medium [7]. Covalent immobilization of enzyme also provides an unlimited contact between enzyme molecules and substrates, since there has no barrier between them and localization of enzyme onto surface support, which attributes to the stability of a biocatalyst system [17]. Therefore, a lot of approaches can be applied to derive this method, depending on the type of support materials and substrates that are used in the covalent immobilization of enzyme [18].

In addition, stability affects covalent interaction due to the fact that an immobilized enzyme is not able to undergo any additional intramolecular process, whereas the soluble enzyme is

able to undergo aggregation and interaction via a hydrophobic interface [19]. Greater stability of covalent immobilization of enzyme also induces higher resistance to temperature, decomposition, pH, and organic solvents in several cases. However, the extent of these improvements also depends on the detailed characteristics of enzyme and support that are usually dictated in immobilization system. Covalent binding is relatively a complicated procedure due to addition of a chemical coupling reagent, especially one that involves nanosized support materials [20]. In normal cases, the immobilization leads to lower residual enzyme activity as mentioned previously due to the tendency of some of amino acid residues, which are essential for catalytic activity to form covalent linkage onto the nanostructured support [21]. Sometimes, the center of active site of enzyme could be modified or altered through chemical coupling reaction, since most chemical coupling agents are toxic and very reactive [7], leading to inactivation of the enzyme molecule. Thus, appropriate selection of type and method of a chemical coupling agent needs consideration. Therefore, in-depth study of chemical coupling in covalent interaction does not only help in minimizing all these problems but also increases the efficiency of enzymatic system that could be applied for commercialization purposes [22].

This article also summarizes in detail the relationship among chemical couplings in the interaction between support and enzyme molecules for covalent immobilization. Chemical coupling agents such as ligands and spacer arms are very important in covalent interaction, which can improve binding efficiency, provide a greater mobility, and minimize steric hindrance. The use of nanofibers is potentially advantageous in this covalent immobilization of enzyme. In fact, CNF provide a great potential to be interacted, modified, or activated with functional groups by the use of specific reagents to render them more suitable for enzyme immobilization. Natural type of CNF will be discussed as a support due to their good mechanical properties and characteristic in providing functional group on the surface for immobilization purpose, as good as polymer-based nanofibers.

Current Trend in Enzyme Immobilization

A number of research findings have been published, especially for the application of nanostructured materials in biocatalysts systems. Figure 1 shows the cumulative publications about enzymes immobilization from year 2000 to 2013. There is a growing publication in enzyme immobilization onto nanostructure materials as well as publication in general enzyme immobilization. The study on enzyme immobilization onto nanostructured material increased drastically from 2004, and then the number increased steadily year by year due to a greater development on nanostructure materials, which then particularly contributed to the improvement of enzyme immobilization performance and production yield.

Cellulose Nanofibers as a Natural Nanostructure Support

As discussed previously, the performance of enzyme immobilization strongly depends on the properties of support, such as material type, composition, structure, and mechanical properties [12]. Better support properties provide a good mechanical strength, which can contribute to stability and reusability. The use of nanosized supports in enzyme immobilization is not only to enhance the stability and reusability of immobilized enzyme but also to overcome lower immobilized enzyme activity due to the presence of high surface area per volume ratio [23]. In order words, high surface area of nanosized support provides a high number of functional group on the surface support. Thus, CNF have more chance of interacting with enzyme molecules [4, 24–27]. A unique behavior of nanoscale support would distinguish them from

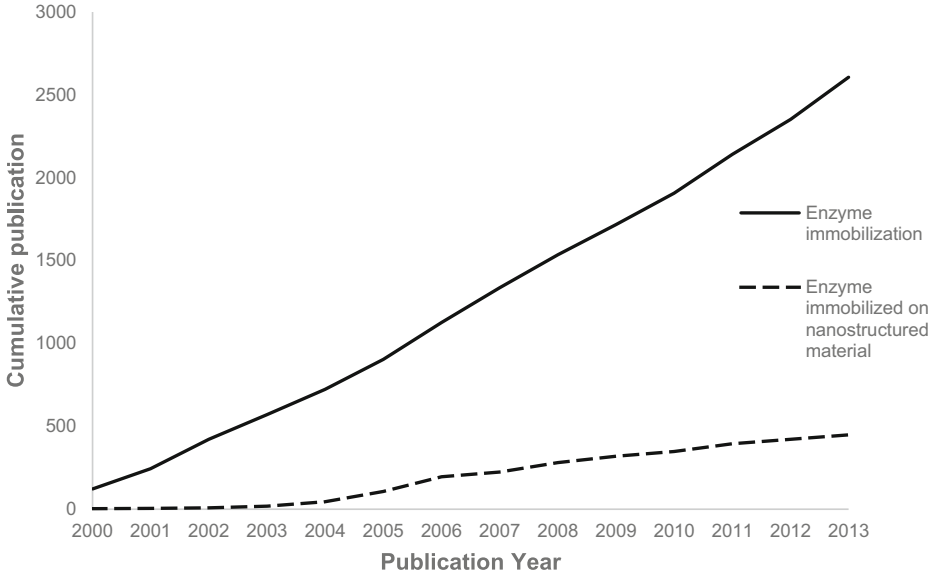

Fig. 1 Cumulative annual number of English scientific publication-based articles, reviews, and book chapters since 2000 according to the keywords used to describe "enzyme immobilization," "immobilized enzyme," and "nanostructured for enzyme immobilization." Data analysis by Scopus scholar search system (December 2013)

traditional immobilized systems. Development of nanoscale biocatalyst system would show a benefit for both enzyme and nanosized support, since the size of enzyme molecule is already in the nanoorder.

Nanoporous silica, nanotubes, nanoparticles, nanofibers, nanocomposite, and nanosheets are types of nanostructured supports. Nanosized support provides opportunities for great efficiency in manipulation of the nanoscale environment, making possible new innovations, and offering promise for various advanced technologies in the enzyme immobilization field [28]. However, some nanostructured materials have their own limitations. For example, nanoporous silica usually confines enzyme molecules on its inner porous surfaces, and this inhibits mass diffusion of substrate or product and results in lower enzymatic production [29, 30]. Non-porous nanoparticles and nanotubes are remarkable with respect of providing a minimum mass transfer limitation, but their recycling and dispersion are more difficult [12]. Nanocomposites could damage or block the reactive functional groups and possibly affect the stability of multipoint interaction of enzymes [31]. On the contrary, nanofibers have a great potential as a solution for those problems that can be prepared as non-porous supports and remain easy to be recycled or reused. Nanofiber support normally has sufficient dimension (diameter and length) in certain range of distribution, which provides a wide area for interaction of immobilized enzyme, compared with other types of nanostructured supports. The use of a nanofiber support is a promising approach for enzyme immobilization, and it offers unique capabilities, as described previously. Nanofibers has attracted continuous operation of enzymatic process especially in membrane application and other potential applications due to high enzyme loading and its efficiency [29, 32].

Natural resources can be generally described as rigid and partially crystalline objects. They are composed of cellulosic micro-fibrils reinforced with amorphous lignin, hemicellulose, waxes, and several water-soluble compounds. Cellulose is the main constituent in the most abundant organic compound on earth, especially within wood and natural fibers (kenaf, palm,

cotton, hemp, flax, etc.). Almost 65–70 % of cellulose compound is contained in plant fibers and comprised C, H, and O elements [33]. Cellulose is insoluble in water. The poor solubility can attribute to strong intra- and inter-molecular hydrogen bonds within and among individual chains [34]. As a result, the cellulose fibers present a moderately hydrophobic surface at its exterior, and this feature renders it as a promising material for enzyme immobilization. In addition to the hydroxyl group on the cellulose surface, it provides an ideal site for participating in covalent bonding of enzyme immobilization [35].

The basic chemical structures of cellulose play a central role in determining the nature of cellulosic nanomaterials. Cellulose consists of linear chains of homopolysaccharide composed of β-D-glucopyranose units linked together by β-1-4-linkages [35–37], with a degree of polymerization (DP) of approximately 10,000 for cellulose chain in nature and 15,000 for native cellulose cotton [38], as illustrated in Fig. 2. Each monomer unit has three hydroxyl groups, which make possible the formation of a dense system of well-ordered hydrogen bonds, which is especially evident in the crystalline packing of cellulose [39, 41]. These hydrogen bonds give cellulose a stable structure, with no melting point and lack solubility in typical aqueous solution. Krässig [42] reported that the presence of hydroxyl groups located in the amorphous regions were found to be highly reactive and accessible, while for those present in compact crystalline regions showed much lower accessibility. Thus, it provides a cellulose chains at high axial stiffness [43]. Stiffness allows an enzyme to separate itself from the matrix surface effectively by minimizing any steric hindrance, such that the enzyme can retain essentially its full activity while in the immobilized state. The approaches for the enzyme and support ratio depend on the experimental work and enzyme loading onto the surface support, which can be expected in 100 U/g support [44].

The production of cellulose nanofibers (CNF) and their application in enzyme immobilization have gained increase attention due to their green support, high strength and stiffness, low weight, low cost, biocompatibility, biodegradability, and renewability [45]. Furthermore, several researchers have reported that CNF consist of high cellulose composition, high crystallization, superior directional strength, and low coefficient of thermal expansion, which exhibit good mechanical properties of support [46–50]. High mechanical properties could make CNF a preferable support for this application, to increase enzymatic performance because of its immobilized enzyme stability. Distribution of similar size of CNF support gives numerous advantages in enzyme immobilization, which are listed in Table 1.

CNF can be isolated either by using top-down or bottom-up approach method. The top-down approach is defined as a preparation method in which nanosized fibers can be generated by stripping down a complex multicellular living plant organism [57]. This term is used especially when the support is produced from natural resources. The bottom-up approach

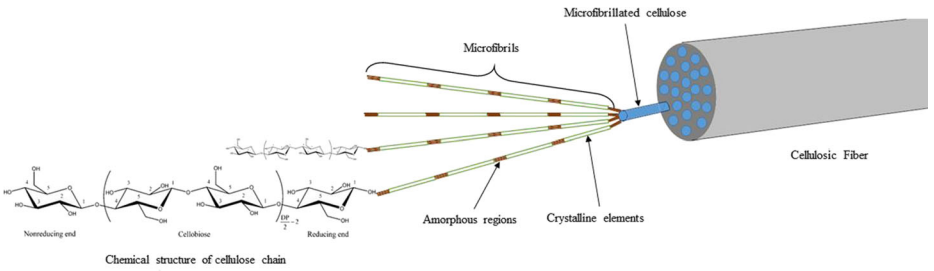

Fig. 2 Chemical structure of cellulose [39, 40]

Table 1 Advantages of CNF properties in enzyme immobilization

Factors	Advantages	References
Surface area to volume ratio	It provides high surface area to volume ratio for enzyme interaction. High surface area would increase the number of functional groups on the surface CNF support, which improves the covalent binding efficiency.	[4, 23, 51]
Enzyme activity	Surface modification of CNF support benefits the stability of enzymatic system, which potentially increases the enzyme activity.	[52]
Enzyme loading	It offers high enzyme loading due to their high relative surface area, perhaps giving better results in biocatalytic activity and stability.	[28]
Functional groups	CNF possesses a large number of specific surface functional groups compared with micro-sized fibers. Therefore, more enzymes will interact to achieve higher enzyme loading in immobilization.	[53]
Flow rate	It behaves as a stable, monodispersed in aqueous suspension eventhough exhibiting the Brownian motion. Based on Stokes–Einstein theory, diffusivity, and mobility, the nanostructured materials should be smaller than those native enzymes to achieve higher enzymatic activity.	[28, 29]
Mass transfer effects	High surface area and interconnectivity of CNF support provide low mass transfer limitation, which results in high activity and stability of enzyme performance.	[12, 20, 28, 54]
Microporous membrane-like structure	Non-woven of CNF support distribution forms a microporous membrane-like structure which improves the mass transfer rate of substrate.	[52]
Easily recovery	The enzyme is easily recovery from reaction media compared to freely suspended enzymes.	[11]
Design of enzymatic bioreactor	Immobilization is a fundamental platform for development of bioreactors and biosensors. CNF have high flexibility; they can be easily prepared as membrane or fixed-bed system. Some factors especially high flow rate and small pressure drop in CNF membrane represent the most promising advantages in enzyme immobilization compared with traditional immobilized enzymes bioreactor and fixed-bed bioreactors.	[32, 55, 56]

refers to the assembly process of material from a small molecule into a more complex structure or the production of a new nanofiber structure [58]. For example, electrospinning method can be used to electrospun nanofibers from cellulose solutions, which are initially in a solid nanoparticle form [2, 59–61]. This term merely involves the use of polymer material as a support in enzyme immobilization.

Top-down approach technique mainly involves the use of mechanical treatments [48, 50, 62–64], chemical treatments [65–69], biological treatments [70–72], or a combination of two or more of these methods, to remove the plant cell constituents other than cellulose, such as hemicellulose, lignin, pectin, minerals, oils, and other extractive compounds. However, isolated CNF also participate in aggregation of nanofibers with a random distribution due to complicated multi-layer nanofiber structure and interfibrillar hydrogen bonds [73]. The structural images of isolated CNF using TEM are shown in Fig. 3, based on different natural resources.

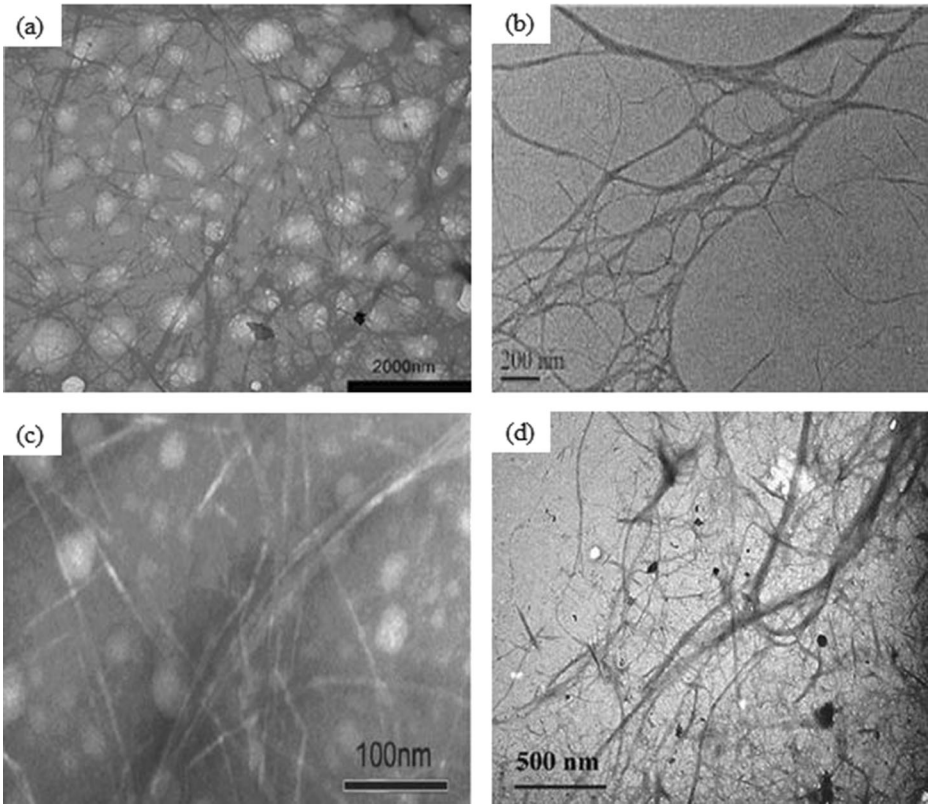

Fig. 3 The transmission electron microscope (TEM) images of isolated CNF with different natural sources: **a** kenaf [66], **b** bamboo [48], **c** wood [74], and **d** hemp [75]

CNF have been categorized into three main types, as follows: microfibrillated cellulose (MFC), nanocrystals cellulose (NCC), and bacterial nanocellulose (BNC). Fibrils, which can be described as relatively long and very thin pieces of cellulosic material, have been used in various applications [76–78]. In nature, cellulose has been found as assemblies of individual cellulose chain-forming fibers and does not occur as an isolated individual molecule. Microfibrils or several elementary fibrils are defined as larger units of cellulose molecules. MFC consists of an assemblage of fibrils that are partly separated from each other [35, 36]. There are several terms in nomenclatures that are synonymous to common types of cellulose nanofibers, as described in Table 2.

Surface Modification of CNF Support

The support to be used for enzyme immobilization must present a high external surface area to achieve a good geometrical conjugation with the enzyme [87]. CNF support is attractive to the enzyme because of its support characteristic under inert physiological conditions and its ability to adapt into hydrophilic or hydrophobic conditions, depending on the applications needed [39, 88]. Non-porosity of CNF support allows high accessibility onto the active site, low

Table 2 The different terminologies of CNF family

Types of cellulose	Synonym nomenclature	Description	Average size	References
Microfibrillated cellulose (MFC)	Cellulose microfibril, microfibrils, nanofibrils, and nanofibrillated cellulose (NFC)	Produced through delamination of fibers pulp by mechanical treatment either before or after chemical or enzymatic treatment.	Diameter, 5–60 nm; length, several micrometer (μm)	[63, 79–82]
Nanocrystalline cellulose (NCC)	Cellulose nanocrystals, crystallites, whiskers, and rodlike cellulose microcrystals	Formed by acid hydrolysis and often followed by ultrasonic treatment, in order to remove an amorphous sections of cellulose source. Known as whiskers, consist of rodlike cellulose crystals. Similar to MFC size, but limited in flexibility, as well as do not contain amorphous regions but instead exhibit elongated crystalline rodlike shapes.	Diameter, 5–70 nm; length, 100–600 nm (from natural plants), 100 nm to several micrometers (from tunicates, algae, and bacteria)	[2, 38, 41, 83–85]
Bacterial nanocellulose (BNC)	Bacterial cellulose, microbial cellulose, and biocellulose	Synthesized by aerobic bacteria. Formed a polymer and nanomaterials by biotechnological process from low-molecular-weight sugar (D-glucose) and alcohols.	Diameter: 20–100 nm	[86]

diffusion resistance, and easy recoverability as well as potential applicability for continuous operations [23, 32, 59, 89, 90].

Many factors such as moisture absorption, quality variations, poor compatibility, surface hydrophilicity/hydrophobicity, rigidity, and durability may affect the behavior of mechanical properties of CNF support, which make a less attractive toward covalent interaction. By manipulating the CNF properties and characteristics using surface modification treatment, it would maintain or enhance the immobilized enzyme conformation and its apparent activity. Moreover, the appropriate surface modification treatment of CNF can increase the availability of hydroxyl groups, change the degree of crystallinity, and break hydrogen bonds, which increase the reactivity of cellulose [91].

Chemical treatment method is often used to perform surface modification of CNF support, compared with UV oxidation and plasma method [92]. For example, chemical treatment (mercerization) is a well-known treatment for surface modification of CNF. Chemical reagents such as acidified sodium chlorite ($NaClO_2$) and sodium hydroxide (NaOH) are often used to treat the structure of CNF to increase the surface roughness [2], providing a platform with reactive functional group for higher interaction in this application. In fact, the treatment will remove other matrix substances covering the surface fibers (e.g., lignin, hemicellulose, pectin, wax, and oil) to obtain high purity in morphological size and composition of CNF [49].

Surface hydrophilicity or hydrophobicity of the CNF support is one of the most important criteria in covalent interaction. Hydrophilicity or hydrophobicity characteristic of CNF may change the conformation structural in enzyme interaction due to reduction of free energy in an enzymatic process. In a few cases, a hydrophobic surface of support also shows an untightened bond onto accessible hydrophobic group of amino acids due to complimentary unfolding of the hydrophobic core toward the surface. However, lipase is known as an exception enzyme; lipase activity increases when it is immobilized on hydrophobic supports [93]. This phenomenon is because of hydrophobic "lid" on the surface enzyme in open position state, which ables contact with a hydrophobic surface support. Open position of the lid may lead toward the increase of enzyme activity compared with the free enzyme [94]. Meanwhile, a hydrophilic surface may result in loss of enzyme conformation because of competitive hydrogen bonding between hydrophilic amino group and aqueous solvent [95]. Some researchers have reported that hydrophilic supports (e.g., cellulose, agarose, chitosan, dextran, alginate, gelatin, and collagen) have high retention activity, but they can be degraded under harsh chemical reaction conditions especially during chemical coupling preparation [96]. However, these supports can be manipulated to be more suitable in retaining immobilized enzyme activity, depending on their interface characteristic; they can be rendered as either hydrophobic or hydrophilic surface [97–99]. Unfortunately, hydrophobic interactions between amino acid group and support surface may cause dehydration of the enzyme. This may cause a shift in the enzyme structure, leading to loss of enzymatic activity [100].

Surface modification of CNF support is also able to create the surface interfacial that consists of hydrophilic enzyme molecules onto hydrophobic support [101]. This technique will reduce the complimentary unfolding toward hydrophobic-side group in amino acids, as well as it will provide a suitable interface for retaining enzyme activity [97]. Moskovitz and Srebnik [186] reported that by using computational modeling, they suggested that a hydrophilic chain on a hydrophobic surface can be used to preserve the activity of an immobilized enzyme. An early patent related to the invention technique for enzyme immobilization through covalent bond on a cellulose support was claimed by Monsan [187].

Numerous studies have been published related to functional groups on the surface of lignocellulosic materials. The types of the functional groups can be identified by using Fourier transform infrared (FTIR) spectroscopy analysis. On the CNF surface, there is a high surface density of –OH functional groups, which are evident in the region of absorbance around 3400

to 3300 cm^{-1}, corresponding to intra- and inter-molecular hydrogen bonding [65, 66]. The –OH group can be a potential target functional group for the interaction of covalent binding. The rest of the functional groups in CNF support are listed in Table 3.

Chemical Modification of Covalent Immobilization of Enzyme onto CNF Support Surface

Basically, covalent binding is a well-established mechanism, based on the chemical reaction of side chain attachment between active amino acid group on the enzyme surface and active functionality that attached onto the CNF support surface. Interaction of covalent binding can be achieved either by the activation of surface functional group on CNF support and/or enzyme molecule before binding preparation. Often, the activated of surface functional group on CNF support provides more efficient interaction with enzyme during immobilization [7]. Only in a few cases is the control mode of binding or the number of bonds onto reactive group of enzyme molecules is necessary, as well as the surface modification of enzyme molecules should be considered first before binding to the support, which represents a complex and reverse covalent interaction method [110]. This present study only focuses on the activation of surface functional group of CNF support, as discussed previously in surface modification section and followed by the detail on chemical modification by using chemical coupling agent.

Covalent immobilization of enzyme onto CNF support belongs to one of the fundamental natures which does not only reflect on the inactivation of enzyme active site but also possibly on the irreversible binding reaction, alteration of the reactive group of enzyme (because of chemical modification), and misdirection of enzyme orientation [7, 111]. However, these drawbacks can be avoided if a proper handling in chemical modification pretreatment is considered. Well-defined covalent immobilization will provide an oriented and reproducible immobilization, as well as avoid enzyme denaturation [112]. Understanding the fundamental mechanism based on covalent interaction provides a key to maintain and enhance the enzymatic performance in the covalent immobilization of enzyme.

There are two main requirements that should be met in order to achieve a good covalent interaction; (1) choice of biocompatible structure that possesses a necessary functional group

Table 3 Details of peak assignment for surface functional groups of CNF that have a potential for covalent binding

Surface functional groups	Component	FTIR wavelength (cm^{-1})	References
–OH	Cellulose	3400–3300	[65, 66]
–CH stretching in aromatic ring and alkanes	Lignin, cellulose	2900–2800	[102, 103]
–C=O stretching from acetyl group	Hemicellulose and lignin	1800–1700	[104, 105]
–OH	Adsorbed by water	1650–1600	[106]
$–CH_2$ symmetric bending	Pectin, lignin, and hemicellulose	1435–1425	[66, 107]
–C=C, aromatic groups	Pectin, lignin, and hemicellulose	1435–1425	[108]
–CH, −CO, aromatic ring	Polysaccharide and cellulose	1380–1320	[106]
–CO stretching of acetyl groups	Lignin	1240–1230	[107]
–COC, anti-symmetric bridge	Cellulose and hemicellulose	1161–1159	[109]
–CH, symmetric to polysaccharides	Polysaccharide and lignin	899–895	[46]

and able to recognize the target group that present on the external enzyme surface, and (2) availability of the specific functional group of the enzyme [14]. Choosing a biocompatible supporting material is essential. In most cases, a native material does not meet all the needs of enzyme immobilization and therefore it requires further functionalization steps. In other words, it is necessary to identify and/or to introduce any different functional group onto the support surface (e.g., −NH2, −COOH, −OH, and etc.).

The functionalization step (chemical modification) can be achieved by using chemical coupling agents with different lengths and types to form spacer arm or ligand or both. This technique required two mutual reactive functional groups on CNF support surface and enzyme side, to form linkage either essential or non-essential in covalent immobilization of enzyme [113]. Chemical coupling is defined as a "smart" linker that represents a high affinity for the specific group of enzyme [14, 114]. It was designed as a sandwich-like structure (support-specific linker enzyme), which forms a thin layer on CNF support surface in successive independent steps separated by washing of excess activation agents. Thus, it will permit a very strong binding between the target enzyme molecules and CNF support at a specific point of reactive group. The reactive group acts as a platform for the interaction between two end-terminals of the chemical coupling, which shows a more robust way to create bio-functionalized linkage with the amino group of enzyme [115]. Most chemical coupling agents contain at least one different functional group that could co-react with the organic phase [114]. The covalent interaction to construct ligand and spacer arm–ligand is illustrated in Fig. 4.

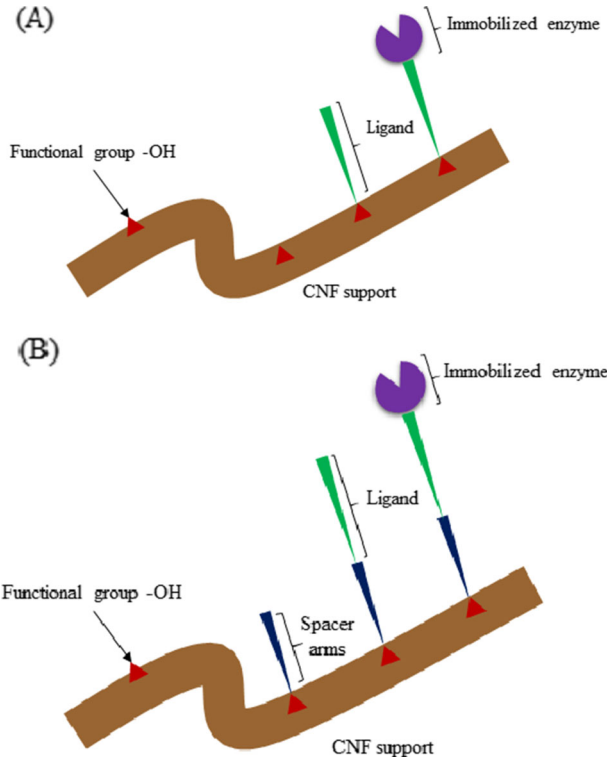

Fig. 4 Illustration of the interaction of chemical coupling agents in covalent immobilization: **a** immobilized enzyme through ligand and **b** immobilized enzyme through both spacer arm and ligand

A ligand can be described as an interaction between surface chemistry of enzyme and support or spacer arm via covalent binding [116]. The ligand provides a promising site to bait the target enzyme molecule [117]. The ligand can be arranged to construct a direct linkage between reactive group of enzyme molecule and reactive group of support, as illustrated in Fig. 4a. However, there are some cases in which ligand weakly binds with the functional group on the surface support. For example, the best ligand may be selected due to its high suitability for enzyme immobilization, but the ligand is not suitable for the support (e.g., ligand does not bind or weakly binds on the surface of CNF). Due to this, enzyme will potentially leach out, which results in low enzyme loading activity and poor reusability. Therefore, to improve this covalent binding, the selected suitable spacer arm is required to construct the linkage between support and ligand (as shown in Fig. 4b).

Spacer arms act as indirect linkages, and they involve different types of chemical couplings that do not react to the target reactive groups of the enzyme. The spacer arm functions as an aliphatic linear chain structure that consists of bi-functional amine groups. The amine group is one of the functional group of terminals that interact with the surface functional group located on the support, whereas another terminal would bind to the functional group of the ligand [115]. It acts as an anchor with one other side of functional group to guarantee flexibility to the target ligand and facilitate the accessibility to the target enzyme molecules onto the functional group of ligand [18]. The role of the spacer arm is not only to present a function to dissociate the ligand from the surface functional group of the support but also to reduce the steric hindrance when the one side terminal group of spacer arm is bound to the ligand [12]. In fact, a spacer arm provides a greater degree of mobility to the coupled biocatalysts so the activity could be higher than that directly bound to the nanofibers surface, under certain circumstances [118].

Chemical coupling via spacer arm and/or ligand could form either a multipoint or single-point interaction through the functional groups of enzyme. Single-point interaction offers high ligand flexibility and easy to access into the enzyme functional group. In fact, single-point coupling interaction provides a better site recognition, but generally they are not strong enough, compared with multipoint interactions [119]. A multipoint covalent interaction promotes a rigidification of the immobilized enzyme structure by attachment on highly activated CNF support through a spacer arm and ligand, as illustrated in Fig. 5 [31]. Multipoint attachment also results in a strong and secure interaction, but it can impede the accessibility between ligand-spacer arm and CNF support [120, 121]. It can also prevent various residual interactions such as protein extraction, aggregation, and any undesirable contact with a hydrophobic interface. For example, a hydrophobic interface of gas bubble might act as an enzyme inhibitor, but it cannot

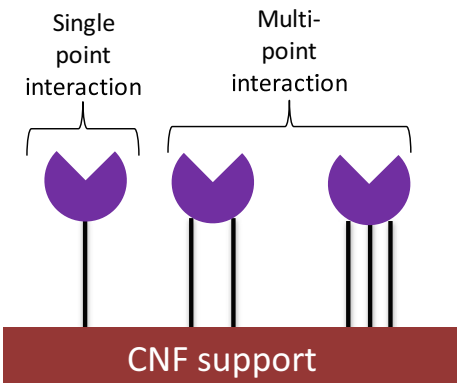

Fig. 5 Illustration of the multipoint interaction of chemical coupling of covalent immobilization of enzyme

inactivate the enzymatic process [92]. The relative distances among all residues involved in these covalent bindings should be close to each other in order to achieve multipoint interaction. Thus, there are a possible number of reactive amino acid sites that can react onto the surface functional groups of CNF, resulting in a strong interaction among them. Multipoint interaction could reduce any conformation changes in enzyme inactivation such as heat, organic solvents, and extreme pH values, thus greatly enhancing enzyme stability [10]. However, the formation of multipoint covalent bond also resulted in inactivation of the enzyme due to the conformation change from native to distortion state [122].

This covalent interaction should be carried out under a compatible micro-environment medium (e.g., buffer solution, pH, and temp) to perform an optimum reaction of chemical coupling process. Chemical coupling process depends on the presence of reactive group within the enzyme molecule. This approach requires the addition of a single chemical coupling agent, acts as ligand (e.g. cyanogen bromide, carbodiimide, aminoalkylethoxysilane, isothiocyanate, epichlorohydrin, etc.) into the reactive group of the support or modification of support backbone using multi-chemical coupling agents, acting as spacer arm ligands to produce an activated group [21]. These cases are desirable to generate reactive group on the support that will react on the amino group on the surface of enzyme molecule. In other words, this process will generate strong electrophilic group on the support that will react with nucleophilic group on the protein molecule, which depends on the nature of these groups [18, 123].

Chemical coupling through covalent immobilization of enzyme involves many types of reaction procedures such as diazotation, Schiff's base formation, thiol-disulfide, peptide bond, amidation reaction, amino bond, and alkylation [124]. For example, Schiff's base formation is a reversible reaction, and immobilization could not happen at a neutral pH value [125] because the amine groups of lysine residue on the enzyme surface at that pH are protonated and consequently non-reactive. However, at alkaline pH (pK around 10), most of them are unprotonated and reactive enough to undergo immobilization reaction [122, 126].

A primary functional group of amino acid is located at the outer layer of the enzyme structure, as an interaction point for immobilization. The functional groups that are most often involved in covalent binding are amine group ($-NH_2$) of lysine, carboxyl group (−COOH) of aspartic acid or glutamic acid, and thiol group (−SH) of cysteine [127]. Both amine and thiol are good nucleophilic groups, while carboxylic acid groups can be activated to be reactive toward nucleophiles [128, 129].

The amine group ($-NH_2$) is the most used moiety for covalent immobilization of enzymes. Amine group of lysine is present in most proteins and frequently is located on the surface of enzyme molecules [130]. Lysine is a reactive nucleophilic group, which reacts toward electrophilic agents (e.g., aldehydes, epoxy, cyanogens bromide, tosyl, etc.), and it provides a good stability for the coupling interaction [126, 131]. For example, the *N*-hydroxysuccinimide (NHS) ester is one of the chemical coupling agents that are often used to react with amine group by forming a stable peptide bond between lysine and a carboxylic acid group of the support [132]. Ester is also a good electrophilic group, which can react with amine to form amide [133]. Other examples of chemical coupling agent to react with amine groups include aldehyde [134], cyanogen bromide [111], epoxide [135], sulfonyl chloride [136], and isothiocynate [137].

The thiol group (−SH) is more nucleophilic (pK_a ~8 for cysteine) compared with the amine group (pK_a ~10.5 for side chain of lysine) [138]. Generally, cysteine often reacts faster than lysine due to selective modification of cysteine over lysine residue. Cysteine is relatively rare in enzyme and mostly appears in oxidized bridges. Reducing agents such as dithiothreitol and tris(2-carboxyethyl)phosphine are required to expose the enzyme to thiol group [138]. Cysteine is an interesting tag in immobilization reaction because the reaction occurs in random orientation.

Covalent immobilization through carboxylic group (−COOH) may be an alternative to amine-based immobilization procedure, because aspartic and glutamic acid constitute major

potential sites of reaction on the surface activation groups of protein [111]. Carboxylic group reacts slowly with amine group using chemical coupling agent such as carbodiimide or carbonyldiimidazole by producing an ester bond, to activate the protein binding reaction [131]. However, this coupling strategy has the disadvantage of causing rapid cross-linking of enzyme molecules [135].

There are also other functional groups in amino acids such as aldehyde and tyrosine. For example, an aldehyde group is good if it is reacted with guanidino functional group located in arginine and imidazole functional group located in histidine [115]. However, hydroxyl group (−OH) of serine or threonine in amino acid on enzyme molecule is not reactive enough. The only reactive hydroxyl group is from tyrosine, and it is located near to active sites of enzyme [139]. Tyrosine is relatively rare on enzyme surface and can be genetically introduced without changing the overall charge state or redox sensitivity [140]. The phenolic ring of tyrosine was reported as being extremely reactive in a diazo-coupling reaction, and the hydroxyl group in amino acid reacts as an excellent nucleophile at alkaline pH [141]. A medium with an optimum alkaline pH would increase the reaction rate of chemical coupling toward nucleophilicity group [16]. Targeting tyrosine for modification or immobilization therefore needs careful consideration.

The basic principle in controlling the covalent interaction of chemical coupling onto a support is analogous to that applied in the chemical modification of enzyme molecules [115]. Previous researchers have listed numerous types of chemical coupling agents that possibly react to the reactive functional groups of enzyme and support, as described in Table 4.

Table 4 Reactive functional groups in enzyme and possible relationship to the activation support groups

Reactive groups on solid supports	Types of chemical coupling	Reactive groups of enzymes	References
$–NH_2$	Carbodiimide	–COOH	[142, 143]
	Glutaraldehyde	$−NH_2$	[134, 144, 145]
–COOH	Carbodiimide		[146]
	Carbonyldiimidazole		[147, 148]
–SH	2-iminothiolane		[118]
	Cysteine/cystamine	–SH	[149]
	Thiol sepharose		[150]
	2,2-dipyridyldisulfur		[151]
−OH	Silane epoxide	$−NH_2$	[152]
	Cyanogen bromide		[153, 154]
	Epichlorohydrin		[155, 156]
	Cyanuric chloride		[157]
	Tosyl chloride		[158]
	Thiol silane		[114]
	Triethoxysilane		[159]
	Trichlorotriazine		[160]
	Succinimide ester		[161]
	Glyoxly-agarose		[162]
	Cyanogen bromide	–OCN	[118]

Natural types of amino acid in enzyme: $−NH_2$ (N-terminus, ε-amino group of lysine), −OCN (serine, threonine, tyrosine), −COOH (C-terminus, glutamic acid, aspartic acid), −SH (cysteine).

Chemical Coupling of Reactive Hydroxyl Group (−OH) on CNF Support

CNF mainly have hydroxyl group (−OH) on the surface of the support. Since the hydroxyl group is known as a weak electrophilic group, the functionalization step is required. For example, cyanogen bromide is often used as a chemical coupling agent for the activation of –OH support group. When the –OH group on CNF support is activated, it is able to interact covalently to the reactive group from enzyme molecule through N-terminus or amine amino group of lysine [141]. In fact, the interaction between enzyme and CNF support through chemical coupling is potentially susceptible to hydrolytic cleavage, which would potentially cause enzyme leaching, unless by performing multipoint covalent interaction.

The interaction through chemical coupling agent is extremely popular in covalent immobilization especially in the lab scale. Most of the use of chemical coupling agents such as cyanogen bromide, cyanuric chloride, carbodiimide, and others are extremely toxic. Some other factors such as degradation rate and microbial contamination need also to be considered, since the CNF support is based on natural polysaccharides. The mechanism of the hydroxyl group on the support with coupling agent (interaction via ligand and spacer arm-ligand) and subsequent enzyme immobilization is shown in Fig. 6.

Potential Applications of Immobilized Enzyme on Nanofibers

Progress in various studies of enzyme immobilization has led to the increase of potential applications in the field of enzyme biotechnology. Recently, more attention has been paid to the regulation of the microenvironment for the enzyme on a support in order to obtain significant stabilization of the immobilized enzyme and development of new supports [163]. New developments of nanobiocatalysis have revived the enzyme immobilization field by successfully stabilizing the activities of various enzymes and demonstrating their potential applications in various fields such as biosensors, medical and clinical antibiotic production, protein digestion in proteomic analysis, antifouling, food industry, biofuel cells, and bioremediation. Since the CNF support is a natural-based cellulose biomaterial, types of enzyme used to immobilize toward CNF support must not include those involved in degradation of cellulose elements, especially “cellulase” enzyme. In addition, CNF possess almost similar properties and benefits with other nanofibers, as a support for enzyme immobilization application. Table 5 shows the summary of the potential applications in covalent immobilization enzyme onto nanofibers support, which also can be applied using CNF support.

Conclusions and Future Perspectives

CNF have a huge potential to be applied in nanobiotechnology especially in enzyme immobilization applications. Enzyme immobilization onto a natural-based support, especially CNF is has the potential for improving enzymatic performance and production yield, as well as contributing toward green technology and sustainable sources. The CNF surface consists mainly of an –OH functional group that can be directly interacted weakly with enzyme, and its binding can be improved by surface modification and interaction of chemical coupling that forms a strong and stable covalent immobilization of enzyme. Application of CNF in nanobiocatalyst has also the potential to be applied in wide applications, including biosensors, medical diagnostics, pharmaceuticals, food, and agriculture industries.

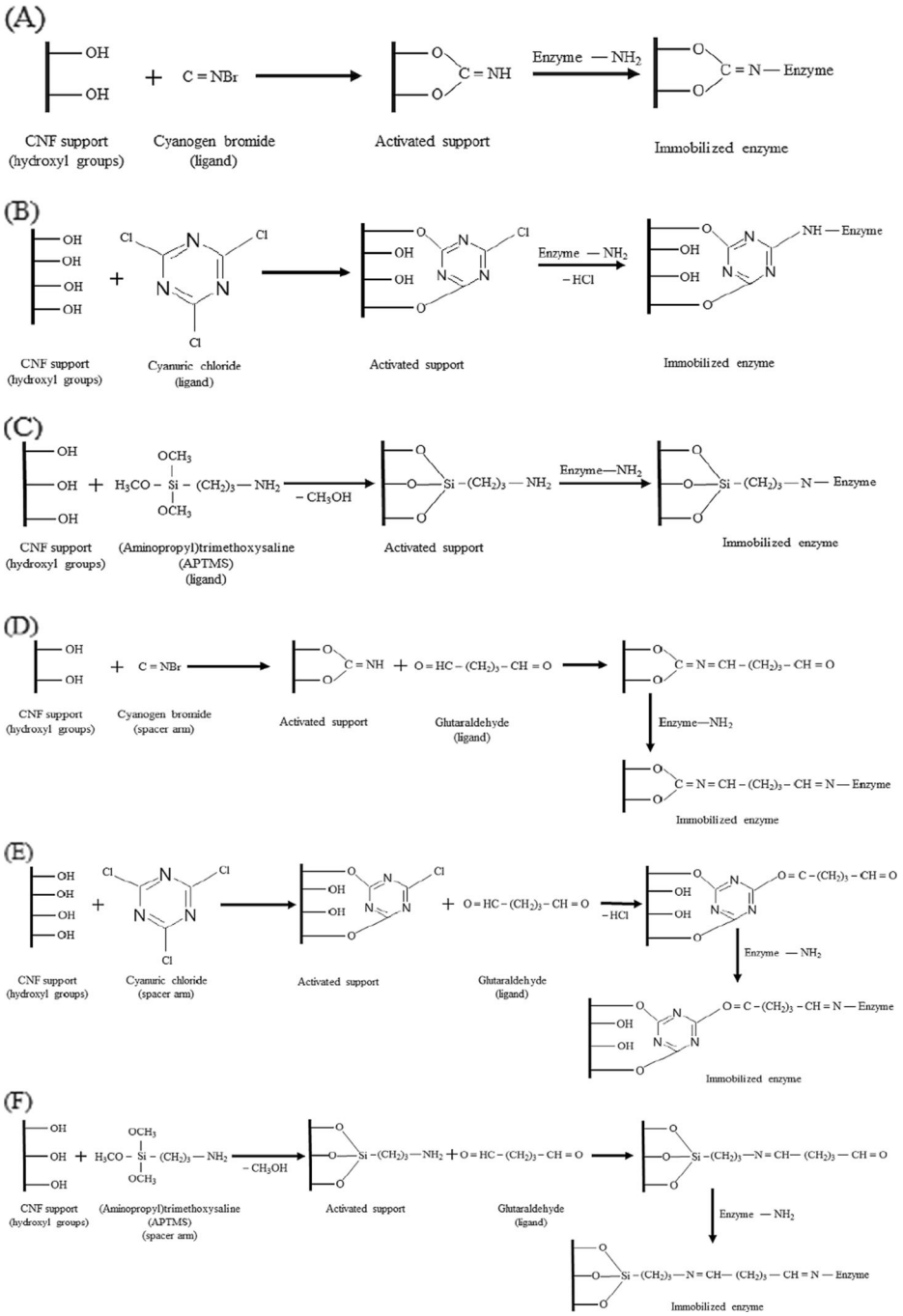

Fig. 6 Multipoint reaction mechanisms of the interaction chemical coupling agents in immobilized enzyme onto CNF support. Interaction activation through ligand with **a** cynogen bromide, **b** cyanuric chloride, and **c** aminopropyl)trimethoxysaline. Interaction mechanism by using both chemical coupling agents (spacer arms: **d** cyanogen bromide, **e** cyanuric chloride, and **f** (aminopropyl)trimethoxysaline) with involved glutaraldehyde as a ligand

Table 5 Potential applications of immobilized enzymes onto nanofibers supports

Applications	Descriptions	References
Biosensor	Device that has capability to detect biological species selectivity.	[164]
	Sensor that responds to low concentrations of analytes and is able to discriminate among species according to the immobilized molecules on surface.	[164]
	Its performances and capabilities in high specificity and sensitivity, low cost, rapid response, compact size and user friendliness, make biosensors an important tool in chemical and biological detection.	[165]
	Biosensors based on immobilized enzymes have good operational and storage stability, high sensitivity, high selectivity, short response time, and high reproducibility.	[113]
	Functions of nanofibers for immobilized enzyme in biosensor are to maintain its structure, retain its biological activity after immobilization, and to remain tightly bound to surface.	[164]
	Widely used in major applications in pharmaceutical, medical treatment, food, and agricultural industries.	[166]
	For example, covalent interaction of l-cysteine (l-cys) onto modified poly(diallydimethylammonium) chloride (PDDA)/gold nanoparticles (PDDA-Au) has been used for immobilization of glucose oxidase (GOD), represented as third-generation of glucose biosensor.	[167]
Medical and clinical	Immobilized enzymes have been applied to specific target drug delivery, disease diagnosis, and molecular imaging.	[168]
	Advantages in development in diagnosis, treatment, and prevention of disease for the future in terms of time, accuracy and reliability, sensitivity, ease to handling, and low cost compared with conventional detection methods.	[169]
	Major application in medical due to immobilized enzyme is the enzymatic electrode, which allows the detection for a complex sample matrix such as blood, serum, urine, and food with minimum of sample pretreatment.	[170, 171]
	Polymerase chain reaction (PCR) and surface plasmon resonance (SPR) were reported as recent technology in clinical application, which allows the qualitative and quantitative measurement of biomolecular interaction without required labeling procedures.	[172]
	Piliarik et al. introduced an efficient SPR technique based on a single-mode polarization-maintaining optical fiber for biosensors. Polarization-maintaining fiber has been found to give more stable and accurate measurements compared with micro-prism fiber-SPR and polished-end fiber SPR	[173]
	For example, immobilizing a monoclonal antimyoglobin antibody toward chemical coupling of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/ N-Hydroxysuccinimide (NHS) showed an effective result at pH 4 compared with direct immobilization technique.	[133]
Antibiotic production	Implementations of biological enzymes are important in antibiotics compared to fine chemical processes.	[164]
	For example, immobilized of penicillin G acylase to cephalexin by conversion of 7-amino-3-deacetoxy-cephalosporanic acid (7-ADCA) had been reported with ~85 % conversion of 7-ADCA to cephalexin and with reuse around 10 cycles under optimum conditions.	[174]
Protein digestion in proteomic analysis	Currently used because of its ability to expound the protein molecules and to regulate pathways of cells in the development of novel drug.	[175]
	All proteins including protease are digested by trypsin before their analysis, and trypsin is crucial for the protein identification in the sample.	[176]
	Optimize the protein identification, but also can reduce the time of protein digestion	[177]
		[178]

Table 5 (continued)

Applications	Descriptions	References
	Immobilized trypsin onto hydrolysis of beta-lactoglobulin (BLG) through the presence of a spacer arm to degrade the allergenic protein in bovine milk and to create safe milk products, especially in food applications.	
Antifouling	Defined as an adsorption of proteins on surfaces, which represents a significant problem for a bio-device system and medical purpose.	[4]
	Several types of antifouling agents such as proteases, subtilisin, chymotrypsin, pronase, trypsin, pepsin, and papain were considered.	[179]
	Approach in nanobiocatalyst which effectively reduces the protein binding onto the surface, perhaps in increasing enzyme stability.	[4]
	Contribute to long-lasting applications which prevent the attachment of barnacles and microorganisms on the surface of medical implants, biosensors, and membranes.	[4]
	For example, proteases have been immobilized onto antifouling molecule structured to reduce the protein binding on the surface and prevent any contamination.	[179]
Food industry	Immobilized enzymes provide a great value in the food processing especially in production of high-fructose corn syrup and trans-free oils.	[180]
	High-fructose corn syrup (HFCS) can be produced by using immobilized D-glucose/xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5). Production of non-metabolizable sugars, colored products and reduced sweetness occur based on the reaction of conversion starch to HFCS, allowed the reaction process under ambient temperature and pH, formed a high concentration of fructose.	[181]
	The reaction of immobilized lipase (EC 3.1.1.3) could enzymatically change triacylglycerols in a low aqueous environment with soy oil and free fatty acid (oleic acid), resulted in the production of soy oil with a higher oleic acid content with improved oxidative stability. Water level is a main factors to control the reaction process. Synthesis of triacylglycerols with new fatty acid profiles happen after hydrolysis reaction process of fatty acids from the triacylglycerol molecules and finally, oil need to be deodorized to remove the residual free fatty acids. This immobilization of lipase is very convenient and simple technique to produce a trans-free fatty acid compared to partial hydrogenation and inter-esterification techniques.	[180]
Enzymatic bio-fuel cells	Enzyme-based biofuel cells generate the converted chemical energy into electrical energy, which has been used as a power source for low-powered sensors, medical implants, and communication devices.	[182]
	Work by oxidizing the enzyme producing electrons, protons, and other byproducts at the anode electrode cell via a concentration gradient.	[183]
	Nanofibers fabricate the enzyme electrodes that meet both high power density and long lasting in stability requirements.	[183]
	Improving enzyme geometrical surface area of electrodes for enzymatic bio-fuel cells with increase of the enzyme-loading rate in active sites.	[4]
	Mano et al. reported that immobilized glucose oxidase and bilirubin oxidase onto two 7 μm electrode could operate to generate electricity without using proton exchange membrane as in a conventional fuel cell.	[184]
Bioremediation	Biodegradation employing nanobiocatalyst technology has promised great effectiveness against contaminated soil or water and also toxic pollutants compared with conventional methods.	[164]
	For example, immobilized peroxidases from *M. charantia* onto nanostructured supports had been found to be effective in decolorizing reactive textile dyes with 50 % in retaining the activity of an enzyme and reusability up to more than 10 cycles.	[185]

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