Review

Current Developments in Lignocellulosic Biomass Conversion into Biofuels Using Nanobiotechnology Approach

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Abstract: The conversion of lignocellulosic biomass (LB) to sugar is an intricate process which is the costliest part of the biomass conversion process. Even though acid/enzyme catalysts are usually being used for LB hydrolysis, enzyme immobilization has been recognized as a potential strategy nowadays. The use of nanobiocatalysts increases hydrolytic efficiency and enzyme stability. Furthermore, biocatalyst/enzyme immobilization on magnetic nanoparticles enables easy recovery and reuse of enzymes. Hence, the exploitation of nanobiocatalysts for LB to biofuel conversion will aid in developing a lucrative and sustainable approach. With this perspective, the effects of nanobiocatalysts on LB to biofuel production were reviewed here. Several traits, such as switching the chemical processes using nanomaterials, enzyme immobilization on nanoparticles for higher reaction rates, recycling ability and toxicity effects on microbial cells, were highlighted in this review. Current developments and viability of nanobiocatalysts as a promising option for enhanced LB conversion into the biofuel process were also emphasized. Mostly, this would help in emerging eco-friendly, proficient, and cost-effective biofuel technology.

Keywords: lignocellulosic biomass; pre-treatment; biofuels; nanoparticles; nanobiocatalyst

1. Introduction

The emerging energy requisite raises queries about the hindrances of fossil fuels usage, in particular, their shortage in the future and adverse effects on the environment. Considering the current state, the need to search for alternative energy sources and biofuels has been augmented [1]. The swift decrease in scanty fossil fuel sources is currently a challenge for modern society [2]. Renewable and sustainable biofuel technology has been recommended as a prospective green substitute which may accomplish the global energy mandate [3]. Biofuel production using lignocellulosic biomass (LB) suggests an exceptional approach to reducing environmental pollution. The process of LB conversion into soluble sugars and corresponding bioproducts consists of numerous steps and requires precise technologies to downstream the final product [4]. The whole process of LB conversion to value-added products primarily involves: (i) pre-treatment of LB for disrupting biomass structure which makes it amenable for further enzyme attack; (ii) enzymatic hydrolysis of biomass components which releases sugars for microbial fermentation; and (iii) microbial fermentation which converts monomeric sugars into various value-added products depending on the selected strain [5] (Figure 1).

Figure 1. Three step process of lignocellulosic biomass conversion into various value-added products.

First generation biofuels are typically produced from different food crops, such as sugarcane, corn, etc., via microbial fermentation [6], but there will be food insecurity issues due to the impact on the global food supply and sustainability of the land [7]. This forced us to think about the second generation energy sources such as non-food-based LB materials for production of biofuels and chemicals. A short document prepared by the BIO-TIC project provided a concise overview of hurdles and possible solutions on biomass processing (<http://www.industrialbiotech-europe.eu/wp-content/uploads/2015/10/Summary-of-Hurdles-and-Solutions-BIO-TIC.pdf>). Therefore, second generation biofuel production can be performed using feedstocks, which are inedible and abundantly available sources such as LB, agricultural waste, organic residues, etc. [8]. Second generation substrates for biofuel production generally involve the utilization of LB sources [9], which suggests an interesting solution to these problems, as they do not adversely affect the food chain.

LB is an abundantly available organic source and still remains unused in bulk amounts. The worldwide production of LB is around 1×10^{10} MT [10], which constitutes a huge resource for energy production. The global demand for LB-based products will increase day by day. LB is a complex 3-dimensional network structure mainly composed of cellulose (30–50%), hemicellulose (25–30%), and lignin (15–20%) [5].

The cellulose structure is made up of a linear unbranched polymer chain of 100–1000 glucose units linked through β -glycosidic linkages. Cellulose is bundled into a structure called microfibrils which are surrounded by lignin and linked to each other by hemicellulose. The hydroxyl groups on the glucose units are linked through inter- and intra-molecular hydrogen bonds between polysaccharide chains forming microfibrils which makes the cellulose structure crystalline. In addition, the amorphous

region of cellulose plays an important role in enzyme attack [11]. Hemicellulose is another abundantly available biopolymer which is a short and highly branched heteropolymer composed of xylose, mannose, galactose, and arabinose units. Depending on the major sugar type, they are named xyloglucans, xylans, mannans, or galactans [11].

Typically, hemicellulose represents the main xylan content in hardwoods and glucomannan in softwoods. Lignin is known as the second largely available polymer after cellulose which makes plants recalcitrant towards biological/physical attacks by providing strength [12]. Lignin is made up of monomeric phenylpropanoid units, i.e., hydroxyphenyl, guaiacyl, and syringyl units [13]. Lignin is one of the hurdles to biomass processing on the economic front, due to the presence of phenolic compounds, which impart deactivation of cellulolytic enzymes and are toxic to microbial growth. Several studies have been reported for decades regarding the conversion of lignin to monomeric and oligomeric units with further upgrading to fuels and chemicals [14].

Lignin is a well-known section of LB restricting the hydrolysis of polymeric components (i.e., cellulose and hemicellulose) using biocatalysts/enzymes. Hence, for the complete utilization of polysaccharide components, LB pre-treatment is the ultimate requirement for lignin removal [15]. After pre-treatment, the isolated cellulose/hemicellulose-rich fractions can be exploited for enzyme hydrolysis followed by fermentation. Various LB sources such as sugarcane bagasse, corn cob, corn stover, wheat straw, etc. can be utilized for biofuel fermentation. To date, numerous pre-treatment methods have been used, such as chemical, physical, biological, and physicochemical methods. However, most of the mentioned pre-treatment processes are costly and generate toxic compounds during the reaction [16]. These complications can be overcome by the emergence of a sustainable and economically viable process, such as nanobiotechnology, which provides a key to the currently available pre-treatment processes. This new nanobiotechnology method is broadly used in different fields for the production of value-added products, i.e., bioproducts and biofuels [17]. The nanobiotechnology processes comprise the use of nanoparticles (NPs). Due to the small size of NPs, they easily penetrate the cell wall of LB and intermingle with biomass constituents to release sugars [18]. NPs can be utilized in both pre-treatment and enzyme/biocatalyst hydrolysis during LB conversion process. LB pre-treatment using NPs improves the molecular and physical properties of LB components. Nowadays, magnetic NPs in fermentation broth can be easily recovered and reused for several cycles, which would make the process viable [19]. For LB hydrolysis, several enzymes, such as cellulases, hemicellulases, cellobiases, etc., have been immobilized using nanomaterials, which would serve as a cutting edge substitute to the reported methods [20]. Moreover, enzymes immobilized on NPs/nanomaterials would increase the hydrolysis rate and release larger amounts of simple sugars due to their high surface to volume ratio [21].

Despite several promising prospects, very limited studies using NPs in LB pre-treatment have been reported so far as the nanotechnology application of biorefinery is still in its nascent phase [22]. Nanomaterials can be used for LB hydrolysis using different ways, such as enzyme immobilization on nanomaterials and acid-functionalized nanomaterials. During LB hydrolysis, enzyme immobilization can prove to be an economically viable option which will make the overall process cost-effective [23]. The nanotechnology arena has recently attracted great attention as it has been applied in various fields such as biosensors, catalytic reactions, and biomedical fields [24,25]. Nanomaterials include NPs, nanocomposites (NCs), nanofibers, and nanosheets, which possess excellent properties, namely a large surface area and quantum size [26]. These fundamental characteristics of nanomaterials provide many advantages, such as higher catalytic efficiency and greater adsorption capacity [27]. There are very limited studies reported with respect to nanomaterial-associated biomass conversion into fermentable sugars [25]. In this review, we evaluate nanobiotechnology applications in the cellulosic biofuel production using microbial fermentation. The probability to decrease the overall cost of fermentative biofuel technology using nanomaterials from LB waste materials is reviewed. We also highlight the emergent role of nano- and biotechnology-based methods in LB conversion, current developments in the field, and the use of nanobiocatalysts in LB hydrolysis. In addition, pre-treatment and hydrolysis

of LB materials using nanobiocatalysts into sugars and further biofuel fermentation using released sugars are reviewed. Furthermore, the adverse/toxic effects of nanomaterials are discussed.

2. Lignocellulosic Biomass Pre-Treatment

The first step in LB conversion is a pre-treatment which disrupts the complex structure of LB to release polymers. A number of pre-treatment methods, such as physical (e.g., steam explosion), chemical (e.g., acid, alkaline), physicochemical, biological, or combinations thereof [28], have been broadly studied and regularly used for isolating carbohydrates (cellulose and hemicellulose) from LB materials [29]. Still, the application of pre-treatment approaches on a commercial scale is a challenging task. Pre-treatment is an insistent obstacle for LB-based refineries, primarily due to the recalcitrant nature of LB materials, total process cost, etc. [30].

LB pre-treatment is necessary to make it more amenable towards enzyme attack. LB deconstruction is usually implemented under extremely severe operation conditions to enhance enzyme hydrolysis, making the process more expensive [31]. Chemical and biological methods have been usually employed for biomass pre-treatment. However, both methods have pros and cons. Biological methods are slower with less production capacity and chemical methods are costly due to extra steps such as neutralization, detoxification, etc. [32]. The use of costly chemicals for pre-treatment is not commercially viable. Hence, efforts have been made to explore an economical and sustainable process to convert LB to cellulose and hemicellulose for further enzymatic attack [33].

Pre-Treatment of Lignocellulosic Biomass Using Nanobiotechnology Approach

It has been studied that the use of NPs or their dispersions can exhibit hydrolytic action that is nearly similar to that demonstrated using chemical pre-treatment for LB processing [34]. Treatment of wheat straw with perfluoroalkylsulfonic and alkylsulfonic acid-functionalized silica–cobalt ferrite NPs generated higher amounts of sugar (~46%) compared to the control (~35%). In addition, NPs are required in very small amounts and can be recovered from the fermentation broth and reused in the next process cycle [34]. The major benefit of employing NPs is their noteworthy role in pre-treating LB substrates using minimal amounts and their recyclability for further use. Khalid et al. [35] studied the effect of magnetic iron oxide (Fe_3O_4) NPs on LB pre-treatment, demonstrating a significant improvement in biogas production after treatment with Fe_3O_4 magnetic NPs (MNPs). Nonetheless, so far, very little information is available on LB pre-treatment using NPs. Therefore, more efforts are required to discover probable ways of making the process economically viable on a commercial scale.

LB conversion to biofuels or other bioproducts is mainly based on pre-treatment and the enzymatic hydrolysis process. In the biomass conversion process, enzyme production is one of the costliest steps. If enzymes can be recycled using NPs, it would help improve the economics of the overall process. Recently, researchers have used nanotechnology methods to pre-treat and hydrolyze LB materials for improved biomass conversion [36]. MNPs have also been used for LB pre-treatment/enzymatic hydrolysis to easily recover enzyme/nanobiocatalyst, making the overall process cost-effective [37]. NCs containing silica functionalized with sulfonates were altered with ferrous oxide (FeO) MNPs and used as a catalyst for cellobiose hydrolysis [38]. Using alkyl sulfonate treated NPs, 78% cellobiose hydrolysis could be achieved and the NPs-associated catalyst was recovered and further reused for biomass hydrolysis. These MNPs showed significant progress in hemicellulose hydrolysis at 80 °C. The silica coated MNPs could be easily isolated from the reaction mixture using a magnet [39].

LB sources, such as corncobs pre-treated with a carbon-based solid acid catalyst, generated xylose with higher yields (78%) using microcrystalline cellulose with H_2SO_4 [40]. A pre-treated biomass sample with protease-associated magnesium NPs (MgNPs) showed an enhanced level of amino acid generation at high temperature (95 °C) with increased lignin removal (18-fold) compared to cellulase pre-treated samples. MgNPs pre-treated samples that were further hydrolyzed with xylanase exhibited a 1.82-fold increase in reducing sugar production compared to untreated samples [41]. Consequently,

nanotechnology-associated biotechnology contributes considerably to various areas such as biofuels and bioenergy.

3. Lignocellulosic Biomass Hydrolysis

LB hydrolysis is known to be one of the most significant steps in the biofuel fermentation process which is usually employed for degrading biomass-derived carbohydrates (cellulose and hemicellulose) into simple sugars (glucose and xylose). After hydrolysis, sugars obtained can be converted into various value-added products such as biohydrogen, bioethanol, butanol, succinic acid, lactic acid, etc. [42]. (Figure 1). LB hydrolysis determines the overall proficiency of the process which has been attained by enzymatic/biocatalytic hydrolysis and chemical hydrolysis methods. Chemical degradation processes use numerous acid catalysts such as HCl, H₂SO₄, etc. for hydrolysis of different types of LB materials, resulting in lower efficiency toward sugar formation [43]. The sugar decomposition into unwanted compounds during acid hydrolysis includes furfural and hydroxymethylfurfural (HMF), which is one of the reasons for the lower efficacy of the acid hydrolysis method [44]. Limitations associated with acid hydrolysis, such as extreme operation conditions, low specificity, and generation of inhibitor toxic compounds, necessitated the development of a novel, green, and sustainable approach to LB disruption [45]. Enzymes possess better catalytic activity, selectivity, and specificity than other catalysis processes. Hence, enzymes were found to be promising biocatalysts for effective LB degradation into simple sugars [46]. The use of biocatalyst provides a green approach as no chemicals are used and thus no toxic compounds are generated. The reusability of biocatalysts/enzymes is a foremost concern in utilizing enzymes in LB hydrolysis [47]. Enzyme immobilization using various materials through crosslinking, adsorption, and covalent bonding improves enzyme stability and catalytic efficiency [48].

Enzymatic hydrolysis of LB material is recognized as a more proficient strategy than chemical hydrolysis methods. In general, specific enzymes such as cellulases, hemicellulases, and laccases are used for LB hydrolysis. Cellulases are a combination of enzymes containing endocellulase, exocellulase, and β -glucosidase which act serially/synergistically to degrade cellulosic materials. Endocellulase enzymes cleave randomly at β -1,4 internal linkages, creating free chain ends. Further, exocellulases act on 2–4 units at the ends of the exposed chains to generate disaccharides such as cellobiose. Lastly, β -glucosidase hydrolyzes cellobiose units, releasing glucose molecules [49]. There are also several other enzymes required for hemicellulose degradation, such as xylanase, β -xylosidase, glucomannase, etc. This set of enzymes can be applied to the hydrolysis of both cellulose and hemicellulose components [50]. The costly enzyme production step makes the overall process expensive, even though enzyme-mediated LB hydrolysis is an ideal method for biomass hydrolysis.

Mostly, bacterial and fungal strains are studied to produce cellulases and hemicellulases. Many bacterial species belonging to *Bacillus*, *Cellulomonas*, *Thermomonospora*, *Caldicellulosiruptor*, *Erwinia*, *Clostridium*, etc. and fungal strains including *Trichoderma*, *Penicillium*, *Aspergillus*, etc. are able to produce cellulases. Some strains have already been used on a commercial scale. Fungal strains usually yield higher levels of cellulases compared to bacteria. Therefore, commercially available cellulases are mainly expressed in fungi [51]. Because bacterial species lack the ability to degrade lignin, LB hydrolysis is constrained to the lignin content in LB materials [51]. Fungal strains, i.e., soft-rot, white-rot, and brown-rot fungi, are mostly used for LB hydrolysis and their action depends on the type of LB materials. These fungal species can disrupt lignin because they can secrete several lignin-degrading enzymes, i.e., laccases, lignin peroxidases (LiP), manganese peroxidases (MnP), etc. Several studies have been performed on LB hydrolysis using standard commercially available cellulases [52].

Enzymatic hydrolysis is preferred over acid hydrolysis because the process is performed at ambient conditions, has a higher specificity, and does not generate inhibitor compounds. However, the cost of enzyme production primarily contributes to the increase in the overall process cost. To make the LB hydrolysis process economical, a new approach of fabricating biocatalyst on NPs/nanomaterials has recently been introduced.

Lignocellulosic Biomass Hydrolysis Using Nanobiotechnology Approach

Recently, there has been an ingenious improvement in nanotechnology, which can be used for enzyme/biocatalyst immobilization. Immobilizing enzymes on nanomaterials is a new way to increase the catalytic efficiency of enzymes [53]. Enzymes immobilized on NPs are referred to as ‘nanobiocatalyst’, which is an illustration of growing and pioneering developments of nanobiotechnology [54]. Enzyme immobilization on nanomaterials through crosslinking makes it selective for substrates and more flexible due to the spacer [55].

There are numerous physical and chemical methods recommended for the immobilization of enzymes on NPs/nanomaterials. Even though a variety of methods have been studied for enzyme immobilization, comprehensive information on the association of enzyme active sites through immobilization using nanomaterials is not yet known. It has been exhibited that the active site of the enzyme plays a significant role in obtaining the resultant activity of the immobilized biocatalyst. However, it relies on the kind of process used for immobilization, the type of enzyme/biocatalyst used, and the nature of solid support [56]. It has been evident that the increased surface area of nanomaterials permits the loading of higher amounts of enzyme and decrease substrate mass transfer resistance, which is a substantial prerequisite for nanobiocatalyst development [57]. In addition, the synthesis of magnetic nanobiocatalysts by immobilizing enzymes on MNPs is preferred because these nanobiocatalysts can be easily recovered and reused in large-scale continuous processes (Figure 2). It significantly increases the life of the biocatalyst, reducing the cost of the biocatalytic process [55,58].

Figure 2. Schematic representation of LB-derived cellulose hydrolysis using immobilized enzymes on magnetic nanoparticles and their recovery after hydrolysis into simple sugars.

A number of nanomaterials, including MNPs, nickel nanoparticles (NiNPs), iron nanoparticles (FeNPs), and other metal oxide NPs, have been used as carriers for enzyme immobilization. Nanomaterials can potentially increase the efficiency of immobilized enzymes because they provide

a large surface area for enzyme attachment, which helps in higher enzyme loading per unit mass of particles [59]. Enzymes immobilized on MNPs permit the reuse of enzymes for several cycles of hydrolysis due to their magnetic nature. Various studies have demonstrated that biocatalysts immobilized on MNPs can be reused for 10–15 cycles of LB hydrolysis, eliminating the need to add new enzymes every cycle. Thus, the overall process cost is reduced due to the recycling of enzymes, which can help in making the LB hydrolysis process commercially viable.

For LB hydrolysis using enzymes/biocatalysts, an enhancement in the hydrolysis rate is a key aspect of the bioconversion process [60]. Cellulases immobilized on MNPs exhibited ~94% adsorption on nanomaterial support. The immobilized enzymes recovered after biomass hydrolysis still retained around 50% enzyme activity after being reused for several cycles. The pre-treated hemp hurd biomass with immobilized enzyme and free enzyme showed 93% and 89% hydrolysis, respectively, 48 h after enzyme pre-treatment. Sulfonated MNPs were used for the hydrolysis of numerous LB materials such as sugarcane bagasse, jatropha, rice straws, etc. and exhibited higher LB conversion efficiency [61]. For rice straw hydrolysis, *A. niger* cellulases immobilized on cyclodextrin-based MNPs produced higher concentrations of simple sugars compared to free enzymes, with recovery of 85% immobilized enzymes for the further hydrolysis process [62]. Acid functionalized silica coated Fe-MNPs were used for LB hydrolysis and biofuel production and exhibited improved catalytic activity and stability [60].

NP-mediated enzyme catalysis (by immobilizing cellulases/hemicellulases on MNPs) could effectively produce simple sugars and biofuels from LB conversion, and immobilized enzymes could be magnetically recovered and reused [63]. Recently, enzymes immobilized on MNPs are under potential consideration as they provide numerous benefits such as biocompatibility, higher stability, larger surface area, and improved mass transfer [64]. Compared to free enzymes, enzymes immobilized on carriers such as MNPs exhibited higher thermal and pH stability [63]. Immobilized β -glucosidases on MNPs were used as a nanobiocatalyst for cellobiose hydrolysis, achieving a binding efficacy of 93%. They were reused for about 15 cycles, possessing about 50% of hydrolytic activity until the last cycle [65].

For efficient hydrolysis of Avicel, cellulase was immobilized on Fe_3O_4 MNPs using carbodiimide as a networking polymer and reused for 6 cycles [66]. *Trichoderma reesei* cellulase immobilized on chitosan-associated MNPs was used for carboxymethylcellulose hydrolysis, which retained around 80% hydrolytic activity even after reuse for 15 cycles of hydrolysis [64]. Fe_3O_4 -chitosan mediated MNPs used for cellulase immobilization provided maximum LB hydrolysis at pH 5 and 50 °C [67]. Cellulases immobilized on ferrite NPs through the glutaraldehyde crosslinking agent exhibited 53% higher LB hydrolysis at 60 °C, while remaining active until reused for the next 3 cycles [68]. Most studies demonstrated that LB hydrolysis using immobilized cellulase showed better catalysis than free enzymes [16]. Few studies exhibited that LB hydrolysis using free enzymes showed a higher hydrolytic efficiency in converting cellulose to glucose (78%) than cellulases immobilized on MNPs (72%) [69]. Immobilized enzymes could be reused in the next cycles with an efficiency of 60–70%. Apart from MNPs, Si, Ni, and AgNPs have been used for cellulase immobilization in simultaneous saccharification and fermentation (SSAF) for bioethanol production from LB materials [70]. Cellulases aggregated on crosslinked magnet used for LB conversion demonstrated better hydrolysis while retaining 74% enzyme activity compared to free enzymes [21]. Chang et al. studied the effect of two different pore sized mesoporous SiNPs on biomass hydrolysis [70]. Cellulases immobilized on SiNPs with large pore size exhibited higher cellulose conversion with better stability and yield. Nickel cobaltite (NiCo_2O_4) NPs were evaluated for their influence on cellulase production, and this study showed maximum cellulase production using NPs at a concentration of 1 mM [71]. In addition, cellulase immobilized on NiCo_2O_4 NPs improved thermostability at 80 °C for 8 h compared to free enzymes, which can be one of the viable options for cellulase production from cheap sources such as LB materials [29]. Various enzymes immobilized using different types of NPs/nanomaterials are mentioned in Table 1. These immobilized biocatalysts have been further used for biomass hydrolysis.

Table 1. Various nanomaterials used for immobilization of different enzymes.

Nanomaterials Used on Support	Enzymes Used	References
Silica based NPs with carbon-derived mesospores	Lipase	[36]
MgNPs	Xylanase	[41]
Sulfonated magnetic carbonaceous acid NPs	Cellulase	[61]
β -cyclodextrin conjugated MNPs	Cellulase (<i>Aspergillus niger</i>)	[62]
Fe ₂ O ₃ MNPs	β -glucosidase	[65]
Fe ₃ O ₄ MNPs	Cellulase	[66]
ZnFe ₂ O ₄	Cellulase	[68]
Sulfonated mesoporous silica modified with Fe ₃ O ₄ NPs	Cellobiase	[72]
Zn MNPs	Cellulase	[73]
Chitosan-based magnetic microspheres	β -glucosidase	[74]
Superparamagnetic NPs	Cellulases (β -glucosidase A and cellobiohydrolase D)	[75]
ZnO functionalized NPs	Cellulase (<i>Aspergillus fumigatus</i> AA001)	[76]
MnO ₂ NPs	Cellulase	[77]
Multiwall carbon nanotubes functionalized with N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride	Cellulase (<i>Aspergillus niger</i>)	[78]
Magneto-responsive graphene nanosupports	Cellulase	[79]

4. Effect of Nanomaterials on Enzyme Properties

Improvements in enzyme stability may facilitate biocatalysts/enzymes to operate under adverse conditions such as higher temperature and pH, which will ultimately lead to the enhanced production of the final desired product [80]. An application of NPs/nanomaterials as a new approach involves the immobilization of enzyme on the surface and surrounding the protein molecules in NPs networking. Several magnetic NPs/nanomaterials were used to immobilize enzymes such as cellulase and hemicellulose, resulting in better stability than free enzymes [81–83]. Several efforts have been made to discover NPs-associated saccharification of LB materials for biofuel production [80,84].

Immobilized enzymes such as cellulases and hemicellulases exhibited higher catalytic activity and could be reused for several cycles. However, recovering the complete amount of immobilized enzyme is still impossible, which is a major obstacle to reuse [85]. In addition, enzyme immobilization improves pH and temperature stability, which can be beneficial in SSAF conditions where enzymatic saccharification and fermentation need to be performed using similar physical parameters [86,87]. Furthermore, enzyme immobilization provides a higher affinity towards LB substrates, leading to higher hydrolysis of biomass [88]. Due to the interaction of NPs and enzymes, a structure is formed in which the protein stability is altered due to changes in surface properties. Structure formation depends on the concentration and type of NPs used for enzyme immobilization [89,90]. Cellulases immobilized on glutaraldehyde-based iron oxide (Fe₃O₄) NPs showed a wide range of pH and temperature stability compared to free enzymes [88].

Several studies demonstrated similar profiles of improved cellulase stability using various NPs/nanomaterials such as iron oxide, nickel oxide, etc. [71,91]. Cellulases immobilized using NiCo₂O₄ exhibited increased temperature stability at 80 °C for 7 h compared to the control which retained stability for only 4 h. Enzyme immobilized on Fe₃O₄ NCs demonstrated improved thermal stability. From the above studies, it can be concluded that enzymes immobilized on NPs can improve the catalytic efficiency towards LB substrate hydrolysis to produce simple sugars. Table 2 describes several studies demonstrating the improvement of pH and temperature stability of immobilized cellulases using various nanomaterials.

Table 2. Improvement of pH and temperature stability using immobilized cellulases on different types of nanomaterials.

Nanomaterials Used	Improved Physical Properties	Immobilization Methods	References
MnO ₂ NPs	Temperature stability at 70 °C and pH stability at 5.0	Covalent binding through surface modification using glutaraldehyde	[77]
Fe ₃ O ₄ /Chitosan NPs	Temperature stability at 60 °C and pH stability at 5.0	Covalent binding through surface modification using glutaraldehyde	[67]
CoFe ₂ O ₄ NPs	Temperature stability at 50 °C and pH stability at 5.0	Covalent binding through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride & N-hydroxysuccinimide chemistry	[92]
Fe ₃ O ₄ NPs	Temperature stability at 60 °C and pH stability at 4.5	Covalent binding through surface modification using glutaraldehyde	[88]
SiO ₂ NPs	pH stability at 4.8	Physical adsorption through vinyl group	[93]
Fe ₃ O ₄ /Chitosan NPs	Temperature stability at 60 °C and pH stability at 5.5	Covalent binding through surface modification using glutaraldehyde	[64]
Attapulgit@chitosan NCs	Temperature stability at 60 °C and pH stability at 4.0	Covalent binding through surface modification using glutaraldehyde	[94]
Fe ₃ O ₄ @SiO ₂ /graphene oxide NCs	Temperature stability at 50 °C and pH stability at 4.0	Covalent binding through surface modification using (3-aminopropyl)triethoxysilane chemistry	[95]
Fe ₃ O ₄ /polymer NCs	Temperature stability in the range of 10–70 °C and pH stability in the range of 2.0–8.0	Covalent binding through surface modification using glutaraldehyde	[96]
Fe ₃ O ₄ @SiO ₂ NCs	Temperature stability at 60 °C and pH stability at 4.5	Covalent binding through surface functionalization using glycidyl methacrylate	[97]

5. Enzyme Immobilization

In LB bioprocessing, enzymes play a key role in enzyme substrate reactions which lead to the generation of products depending on enzyme activity and other process conditions [98]. The use of enzymes, during the LB pre-treatment process, for generation of simple sugars, contributes to higher costs in the overall LB to biofuel conversion process. Hence, enzymes can be immobilized on NPs or any suitable support materials for improvising its properties, so as to make the enzyme application in LB pre-treatment processes economically feasible. Various groups of hydrolytic enzymes such as cellulases, xylanase, pectinase, etc. act synergistically in LB hydrolysis for biofuel production [98].

The process of enzyme immobilization refers to the confinement of enzyme in a definite region, retaining its activity in order to reuse the immobilized enzyme repeatedly without losing activity [99]. The application of enzymes when immobilized onto a support such as MNPs imparts higher stability, easy product purification, and enzyme/biocatalyst reusability [48]. In general, immobilization has been carried out using chemical bonding and physical retention methods. Physical retention includes attachment through membranes, and chemical bonding involves crosslinking and binding on supports. Enzyme binding on support material/NPs can be performed by adsorption entrapment, ionic binding, and covalent binding. From the industrial perspective, enzyme immobilization on support material through covalent bonding is the utmost method chiefly based on the interaction between the chemical groups present on the support material and enzymes [100].

Various types of support materials, inorganic and organic substrates, have been used for the immobilization of many enzymes of varying porosity, size, shape, etc. The natural inorganic supports such as clays, pumice, silica, zeolite, and manufactured materials (e.g., glass, metal oxides, alumina, ceramics, silica gel, magnetic materials) are usually used for immobilizing enzymes. In addition, some other organic carriers can be classified as natural polymers (e.g.,

cellulose, starch, dextran, agar, alginate, chitosan) for enzyme immobilization on various support materials. [99]. Support nanomaterials are linked with enzymes through crosslinking methods widely used in enzyme stabilization using dialdehydes, diiminoesters, diisocyanates, bisdiazonio salts, and carbodiimide-activated diamines to cause intermolecular bonds between the support and the enzyme/biocatalyst [101].

6. Microbial Fermentation for Biofuel Production Using Nanobiocatalyst

Although various R & D efforts have been directed towards biomass valorization over several decades, the commercial production of biofuels such as bioethanol and biodiesel still needs optimization from both technical and economical perspectives. None of the current available methods are up to the mark since their outcomes depend on the type of feedstock, downstream process configuration, and many other factors. An efficient microbial system with new metabolic routes can be used for sustainable biofuel production along with nanobiotechnology approach. At present, biohydrogen, bioethanol, biomethane, biodiesel, etc. are being studied as proficient biofuels [102]. There has been a continuous increase in energy consumption worldwide every year till date which is going to rise in the future. It is receiving great attention to improve enzyme activity and desired products during fermentation using NPs/nanomaterials [103]. However, the adverse effects of NPs/nanomaterials on LB to biofuel production have not yet been studied in detail. Only few studies have been conducted using NPs for the conversion of LB to sugar [104]. Usually, NPs/nanomaterials can have toxic effects on microbial cells when used in high concentrations. In this section, we assessed the application of nanomaterials for improved production of bioethanol and biohydrogen from LB substrates. By employing various nanomaterials during LB to biofuel fermentation, the reduction in overall cost of LB to biofuel may be possible.

6.1. Bioethanol Production Using Various Nanomaterials

A number of approaches have been used to make bioethanol production economical, which comprises numerous pre-treatment methods, enzymatic hydrolysis, microbial fermentation, enzyme immobilization, and genetically modified/recombinant yeast strains [105]. For bioethanol fermentation process, cellulases, cellobiase, and β -glucosidase enzymes have been immobilized on different NPs and further used for the pre-treated LB hydrolysis and showed improved properties compared to free enzymes. Numerous studies have been reported on enzyme immobilization using diverse nanomaterials such as metal NPs, metal, oxides, polymeric nanomaterials, magnetic materials, etc. [48,103]. For bioethanol production, β -glucosidase immobilized on metal NPs has been reported by several researchers [106–109]. Cellulase immobilized on magnetic NPs was used for *Sesbania aculeate* biomass hydrolysis to produce 5.31 g/L of bioethanol and the nanobiocatalyst was reused for several recycles [110]. Verma et al. reported an immobilized thermostable β -glucosidase derived from *Aspergillus niger* on MNPs through covalent linking, which exhibited an immobilization efficiency of 93% and retained around 50% of the enzyme after several recycling [107]. In addition, the efficiency of β -glucosidase immobilized on chitosan-based magnetic microspheres was studied, releasing 60 g/L of sugars during corn straw hydrolysis and maintaining conversion efficiency even after 8 rounds of biocatalyst recycling [106]. *Trichoderma reesei* cellulases immobilized on MNPs crosslinked through glutaraldehyde were used for hemp hurd hydrolysis to release simple sugars. The immobilized biocatalyst improved the saccharification rate and thermostability compared to free enzyme [73]. Cellulases derived from *Aspergillus fumigatus* were immobilized on manganese oxide NPs for bioethanol production, resulting in a binding efficiency of 75%. These immobilized cellulases showed improved stability over a wide range of temperature and pH and were reused for 5 cycles. Finally, bioethanol production from sugarcane leaves using free and immobilized cellulase showed 18 and 22 g/L of bioethanol, respectively [77].

Alternaria alternata cellulases immobilized on ferric oxide (Fe_2O_3) NPs were used to hydrolyze sugarcane bagasse biomass, which converted 78% cellulose to glucose at 40 °C. This nanobiocatalyst

was reused three more times, showing 52% biomass conversion in the third cycle. [55]. All of the studies mentioned above demonstrated that immobilized biocatalysts improve pH and thermal stability compared to free enzymes and thus improve bioethanol production.

6.2. Biohydrogen Production Using Nanomaterials

Nowadays, the implementation of the nanobiotechnology approach is attracting consideration to increase the biological activity of biohydrogen production and bioproduct recovery [99]. However, the impacts of different nanomaterials on LB-derived biohydrogen production under dark fermentation conditions are still unknown. The main problems with biohydrogen production are slow production rate and low yields [111]. It has been observed that only 30–35% of the substrate is converted into biohydrogen and the residual 60–65% of the substrate leads to the production of other metabolites such as ethanol, acetic acid, butyric acid, butanol, etc. [112]. Therefore, it is important to improve the conversion efficiency from LB to biohydrogen through strain improvement using various approaches.

Hydrogenase is one of the important enzymes in biohydrogen production using dark fermentation by proton reduction. The presence of two cofactors, Fe and Ni, in the active site of hydrogenase exhibited their effect in the biohydrogen production pathway during dark fermentation [113]. Hence, the productivity of hydrogen fermentation is influenced meritoriously by the presence or absence of these metal cofactors [112]. To maintain the normal functioning of microbial cells, the use of precise quantities of metal cofactors is crucial. Higher amounts of metal cofactors inhibited microbial metabolism and ultimately affected biohydrogen productivity [113]. The application of nanomaterials is a new approach to increasing metabolic activities during the process of biohydrogen production. NPs/nanomaterials revealed the prospective in improving ferredoxin-oxidoreductase activities, which improved the electron transfer rate corresponding to better catalytic efficiencies [114,115].

Malik et al. reported an improvement in the H₂ production rate to 80.7 mL/h using Fe₂O₃ NPs in the acidic pH range [116]. Kinetic studies also demonstrated that the level of biohydrogen production decreases with increasing substrate concentration. The effects of various NPs/nanomaterials for improving biohydrogen production are reported and summarized in Table 3.

The use of hematite (α -Fe₂O₃) and nickel oxide (NiO) NPs enhanced hydrogenase activity with a 4.5-fold increase in hydrogen production [83]. Han et al. reported a higher yield of H₂ in sucrose medium in the presence of Fe₂O₃ NPs (0.2 g/L), which was 33% higher compared to the control medium [81]. Sugarcane bagasse biomass was hydrolyzed in a combination of diluted H₂SO₄ and titanium dioxide (TiO₂) NPs in UV light [121]. This new approach released 260% more sugar with a 127% increase in H₂ production compared to the control. In addition, the influence of NiNPs on H₂ production was studied, resulting in a 22.71% higher yield at pH 5.6 and 30–35 °C. In the presence of NiNPs (0.0567 wt %), 2.54 mol of H₂/mol of glucose was obtained under dark fermentation conditions [132].

The application of various NPs such as Cu, Pd, Ag, Fe₂O₃ produced improved levels of H₂ production from glucose by *Clostridium butyricum* under dark fermentation conditions. Of these NPs, Fe₂O₃ produced around 40% higher hydrogen compared to the control one [126]. The outcome of all the studies discussed here depends on the kind of interaction between enzymes and NPs/nanomaterials to improve the electron transfer rate, which ultimately enhances H₂ yield and production levels. It has been demonstrated that the compact (nanometer) size of Fe₂O₃ NPs can increase the reaction rate tremendously and thus can improve hydrogenase activity by removing unnecessary oxygen from the fermentation broth [133]. Mohanraj et al. used green synthesized Fe₂O₃ NPs for biohydrogen production and enhanced H₂ production levels from 23.0 to 25.3 mL/h with the yield of 2.33 mol H₂ per mol of substrate [125]. Recently, it was reported that H₂ production was improved using FeO NPs with a 73% increase in H₂ yield due to the enhancement of microbial activity associated with electron transfer between ferredoxin and hydrogenase [118].

Fe-based NPs are primarily used for biohydrogen production as Fe has been proven to be the major cofactor for hydrogenase. Besides Fe and Ni, several other NPs such as Ag, Cu, Au, Pd, etc. have been

used for improved H₂ production rate and yield under dark fermentation conditions [2,118,125,126,132]. Although the effects of different NPs on H₂ production have been well studied, it is still necessary to develop strains at the molecular level to reduce NP concentrations.

Table 3. Biohydrogen production from different substrates using various nanoparticles/nanomaterials.

Microbial Strains Used	Substrates Used	NPs/ Nanomaterials Used	% Increase in H ₂ Yield	References
<i>Bacillus anthracis</i>	Palm oil mill effluent	NiO	151	[117]
<i>Enterobacter</i> sp. and <i>Clostridium</i> sp.	Grass	CoO	167	
Anaerobic sludge	Sugarcane bagasse	FeO	73.1	[118]
Mesophilic culture	Starch	Fe ₃ O ₄	69	
<i>Enterobacter aerogenes</i>	Cassava starch	FeNPs	200	[82]
Anaerobic sludge containing H ₂ producing bacteria	Molasses waste	Fe ₂ O ₃	92	
Anaerobic sludge	Sugarcane bagasse	NiO	24	[83]
Thermophilic anaerobic mixed culture	Glucose	Fe ₂ O ₃	43	
Anaerobic mixed bacteria	Glucose	TiO ₂	127	[121]
<i>Enterobacter cloacae</i>	Glucose	Fe ₂ O ₃	53.6	
Mixed culture	Glucose	Fe ₂ O ₃ -Fe ₃ O ₄ /carbon nanocomposite	33.7	[123]
<i>Clostridium butyricum</i>	Glucose	FeNPs	130	
<i>Clostridium butyricum</i>	Sucrose	Pd(II) NPs	9	[125]
Anaerobic sludge	Glucose	Pd, Ag, Cu, and Fe encapsulated	38	
Anaerobic sludge	Sucrose	SiO ₂ NPs	32.64	[81]
Anaerobic sludge	Glucose	α-Fe ₂ O ₃	~50	
Anaerobic sludge	Sucrose	Carbon nanotubes	62.5	[98]
Anaerobic sludge	Sucrose	Activated carbon	70	
<i>Clostridium butyricum</i>	Glucose	Nano activated carbon	67.5	[127]
Anaerobic sludge	Acetate	AgNPs	-	
<i>Rhodobacter sphaeroides</i>	Malate	AuNPs	19.4	[129]
		Fe		

7. Future Perspectives

In this review, we discussed the role of nanobiotechnology for LB conversion into simple sugars through pre-treatment and the hydrolysis process. The nanobiotechnology strategy used NPs/nanomaterials for biocatalyst immobilization which can easily penetrate through the LB cell wall. Thus, after interaction with nanobiocatalyst, the LB material can be more amenable towards enzyme attack for carbohydrate (cellulose and hemicellulose) conversion into sugars and further microbial fermentation to produce biofuels and other bioproducts. Most recent studies reported the use of MNPs that can recover and reuse enzymes/biocatalysts immobilized on magnetic nanomaterials by applying a magnetic field. While many studies have demonstrated the positive effects of nanomaterials on LB conversion, the thorough mechanism of NPs degrading LB structures has yet to be elucidated. The use of nanobiocatalyst for generation of sugars from LB conversion is still in its infancy. Thus, several aspects need to be studied in more detail, including the effect of NPs on enzyme functionalities, toxicity of nanomaterials in microbes during the SSAF processes, etc. Further studies are needed to evaluate the efficacy of NPs and other nanomaterials. From all of the studies discussed in this review, it can be concluded that nanomaterials are essential components for the pre-treatment and hydrolysis of LB materials for viable biofuel production. The application of nanobiotechnology would play a remarkable role in the pre-treatment and hydrolysis of LB materials.

Though NPs in powdered form have been used to produce biofuels, the use of nanosheets, nanotubes, etc. can show better binding capacity and enzyme activity. In addition, the application of biologically-derived NPs can reduce the risk of toxicity to microbial cells during fermentation. Biologically-derived NPs consist of bioactive compounds on surface matrix which might protect

microbial cells from death/lysis. These possibilities can be implemented on a commercial scale to increase the yield and productivity of biofuels such as bioethanol and biohydrogen.

8. Conclusions

The search for developing efficient and sustainable pre-treatment processes is constantly an issue of huge concern for producing fermentable sugars. To date, no ideal pre-treatment process has been developed. The use of a nanobiotechnology approach provides a potential pre-treatment for LB conversion. LB hydrolysis is a fundamental stage in the biofuel production process. Acid hydrolysis methods usually generate several toxic inhibitor compounds which make the downstream process even more challenging. On the other hand, LB hydrolysis using enzymes/biocatalysts is highly specific towards substrate and more sustainable, but the enzyme production makes the overall process costlier. Immobilization of various enzymes on different metal oxides/NPs proliferates the stability and proficiency of enzymes. Additionally, immobilization of enzymes on MNPs creates nanobiocatalysts with high potential and widely recognized for LB hydrolysis. Such nanobiocatalysts possessing magnetic properties can be recovered simply by applying a magnetic field and can be reused for further processes which would make the whole process cost-effective. Nevertheless, the treatment, recycling, and preservation of NPs in pre-treatment processes need to be investigated in detail for future applications on a commercial scale.

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Abbreviations

LB	Lignocellulosic biomass
NPs	Nanoparticles
MNPs	Magnetic nanoparticles
NCs	Nanocomposites
Fe ₃ O ₄	Iron (III) oxide
Fe ₂ O ₃	Ferric oxide
FeO	Ferrous oxide
FeNPs	Iron nanoparticles
SiNPs	Silica nanoparticles
NiNPs	Nickel nanoparticles
MgNPs	Magnesium nanoparticles
ZnO	Zinc oxide
NiCo ₂ O ₄	Nickel cobaltite
TiO ₂	Titanium dioxide
LiP	Lignin peroxidase
MnP	Manganese peroxidase
SSAF	Simultaneous saccharification and fermentation

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