Nanotechnology Applied for Cellulase Improvements

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Abstract The biotechnological potential of cellulases has been demonstrated in various industrial segments including food, animal feed, pulp and paper, and several others. Among them, one of the most important applications of cellulases is the bioethanol production from lignocellulosic biomass. Despite the great potential of this enzyme in cellulosic biofuel production and also the interest in such products, expansion has been limited by relatively high production costs and other drawbacks. In this sense, several strategies have been proposed to overcome these obstacles and major challenges, such as the utilization of nanotechnology. This technique has raised the interest of research and can be considered a potential candidate to boost the biofuel refineries aimed at new developments in the area. In this approach, the main goal of this chapter is to conduct a broad and recent review of the potential of nanotechnology to improvements in cellulase production and hence to drive advances in the production of second-generation ethanol. The material will cover the main microbial sources used for the production of cellulases and their applications in different industrial segments. Finally, the applications of nanotechnology for cellulase improvements in bioprocesses will be addressed.

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1 Introduction

Human and industrial activities have been considerably intense over the years. Problems associated with the large-scale use of fossil fuels and oil reserves are in the fact that these resources are depleting very fastly, implying in serious future limitations. Besides that, they can also be considered as the major contributors for emission of harmful gases, with negative impacts in loss of biodiversity, climate change, rise in sea level, among others (Agarwal 2007).

In this perspective, considering the increased depletion of nonrenewable resources and also the greenhouse effect, bioconversion of renewable lignocellulosic materials into biofuel, biochemicals, and other value-added products is of great significance in replacing traditional fossil fuel (Sánchez and Cardona 2008; Han et al. 2017; Bischof et al. 2016; Kumar et al. 2016).

Lignocelluloses, composed of cellulose (ranging from 35 to 50%), hemicelluloses (25–30%) and lignin (from 25 to 30%) (Behera and Ray 2016), are one of the most worldwide available and renewable biomass resources, reaching the production rate of 200 billion tons biomass per year (El-Bakry et al. 2015). Among them, cellulose is a key structural component of plant cell walls and is the most abundant source of renewable carbon on Earth (Greene et al. 2015).

Several cellulosic biomass materials such as agricultural and forestry residues, agricultural by-products, and woody biomass are produced abundantly worldwide (Zhang et al. 2017) and arouse much interest for the future of the bioprocess industry. These materials are essentially potential raw materials for the production of fermentable sugars, which are fundamental for various industrial products such as biofuels, biodegradable plastics, biosurfactants, enzymes, etc. (Pandey et al. 2000).

Biofuels technology presents several advantages over the conventional petroleum fuels, such as a much more sustainable process chain, their biodegradable property besides being more environmental friendly (Gaurav et al. 2017). Thus, one important example is that the biological conversion of the stored potential energy in cellulose to biologically derived biofuels has gained much attention over the past few decades as the drive to shift human energy dependence from fossil fuels to renewable sources continues (Greene et al. 2015).

Ethanol production from lignocellulosic biomass is a complex process, however it can be briefly summarized in the main steps: pretreatment of lignocelluloses, hydrolysis of cellulose and hemicellulose, sugar fermentation, and distillation of ethanol (Sánchez and Cardona 2008). The economic feasibility of second-generation bioethanol relies mainly on the substrate and the enzymes which are the two major cost factors. In this way, it becomes essential to select a cheaper, abundant, and easily hydrolyzable material to be used as substrate, playing a critical role for an economical production of fermentable sugars (Gomes et al. 2016).

Similarly, the selection of an enzyme complex that displays satisfactory performance, capable of withstanding the process conditions, and presenting broad spectrum of activity and efficiency in the presence of lignocellulosic materials

substrates is essential. In this context, the biological depolymerization of cellulose found in lignocellulosic biomass is primarily achieved from the action of synergistic cellulases (Greene et al. 2015), increasing considerably the potential of these enzymes.

Cellulase is a general term for cellulolytic enzymes (Kuhad et al. 2016) of which three classes are recognized on the basis of the mode of enzymatic actions and the substrate specificities: endoglucanases (EC3.2.1.4), exoglucanases (EC3.2.1.74 and EC3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Molina et al. 2016). They are members of the glycoside hydrolase families of enzymes, according to the CAZy database (www.cazy.org), with the capability of hydrolyzing oligosaccharides and/or polysaccharides (Teeri 1997). Cellulases are capable of breaking down insoluble crystalline cellulose into soluble sugars that can then be fed to ethanologens to produce bioethanol or other engineered microorganisms to produce other fuel precursors (Wen et al. 2013).

Cellulases have been commercially available for more than 30 years for both research and industrial applications, and have demonstrated their biotechnological potential in various industries including food, animal feed, pulp and paper, brewing and winemaking industries, and also in agriculture, biomass refining, textile (Cherry and Fidantsef 2003; Ferreira et al. 2014), wastewater treatment (Fitzpatrick et al. 2010), and most importantly for bioethanol production from lignocellulosic biomass (Chapple et al. 2007).

Various methods have been reported for the cellulase-catalyzed hydrolysis of cellulose-producing biofuels (Singhvi et al. 2014). However, it is well known that to produce biofuels effectively, the key step is improving the efficiency of converting lignocellulose to fermentable sugars using cellulases (Sun and Cheng 2002). The performance of cellulase mixtures in biomass conversion processes depends on several of their properties including stability, product inhibition, specificity, synergism between different enzymes, productive binding to the cellulose, physical characteristics as well as the composition of cellulosic biomass (Heinzelman et al. 2009).

Despite the great potential of this enzyme group in cellulosic biofuel production and also the interest in such products, expansion has been limited by relatively high production costs (Cherry and Fidantsef 2003). This is linked to a series of process drawbacks, such as low enzymatic hydrolysis efficiency that is one of the main factors that restrict the industrialization of the second-generation bioethanol (Saini et al. 2016). Low rates of enzymatic hydrolysis efficiency were reported as one of the most critical issues due to the nonproductive adsorption of cellulase on the lignin in substrates (Lin et al. 2016; Saini et al. 2016), which decreased the effective concentration of cellulase in enzymatic hydrolysates (Cai et al. 2017).

Other important factors of bioalcohol production are related to the rate-limiting enzymatic saccharification step due to the challenges of degrading complex mixtures present in plant cell walls (Greene et al. 2015) and limited solubility of lignocellulose in traditional aqueous phase (Bose et al. 2010). Currently, the cellulose saccharification by cellulases remains costly, thus hindering the commercial bioethanol production process (Banerjee et al. 2010). Hence, bringing many

challenges to understanding the overall process involving the use of this enzyme system (Kuhad et al. 2016).

Therefore, the enzymatic hydrolysis of the cellulose component has been acknowledged as one of the bottlenecks for the biorefinery of lignocellulosic biomass (Liu et al. 2013). This fact drives efforts in the research and development of numerous techniques to make improvements in the process.

In this sense, several strategies have been proposed to overcome these obstacles and major challenges, such as the enhancement of cellulase productivity that has been studied through strain modification and bioprocess improvement strategies (Kuhad et al. 2016). Aiming to reduce enzyme costs, cellulase recycling seemed as a promising strategy (Wang et al. 2016) and the use of low-cost substrates, such as sugar mixture (Li et al. 2016) and cornstarch hydrolysate (Zhang et al. 2017) were proposed. Several approaches were conducted for improvements in bioethanol production using cellulases, such as genetic engineering tools (Greene et al. 2015), new immobilization systems (Salem et al. 2016), application of ionic liquids (Xu et al. 2016b; Mihono et al. 2016) and modulation of cellulase activity by charged lipid bilayers (Mihono et al. 2016), and purification techniques (Yang et al. 2017). Process developments were studied by using solid-state fermentation (Ray and Behera 2017), use of nutrient limitations (Callow et al. 2016), repeated fed-batch fermentation (Han et al. 2017), and residues and by-products as alternative substrates (Gomes et al. 2016), among several others.

More recently, nanotechnology has been raised the interest of research and can be considered a potential candidate to boost the biofuel refineries aimed at new developments in the area. The use of nanotechnology has increased broadly over the last years in several areas of knowledge, including medicine, robotics, chemical engineering, biology, and advanced materials. In this context, some efforts have been made to combine nanotechnology with biomass degradation and second-generation ethanol (Chandel et al. 2015).

In this approach, the main goal of this chapter is to conduct a broad and recent review of the potential of nanotechnology for cellulase improvements and hence to drive advances in the production of second-generation ethanol. The material will cover the main microbial sources used for the production of cellulases and their applications in different industrial segments. Finally, the applications of nanotechnology for cellulases improvements in bioprocesses will be addressed.

2 Cellulase-Producing Microorganisms

Lignocellulosic material is the main component of plant cell walls and is found worldwide, mainly in the composition of several agro-industrial residues. Lignocellulose is generally composed of cellulose, hemicellulose, and lignin. Cellulose is the most abundant organic polymer on earth (Klemm et al. 2005) and is composed of linear chains of β (1 → 4) linked D-glucose units. These linear chains are compacted among each other through hydrogen bonds what results in the

intense recalcitrance of this polymer in plant cell wall. In addition, cellulose is the most abundant component of lignocellulosic biomass and is considered the most interesting candidate for the substitution of either fossil fuels or oil refineries through the utilization biorefineries, due to its high abundance in agro-industrial residues.

Cellulose degradation is very interesting to produce high quantities of glucose that can be used for the production of second-generation ethanol through fermentation by yeasts, among other applications. One of the most efficient strategies for the hydrolysis of cellulose is by the action of enzymes produced by microorganisms known as degraders of lignocellulosic material. Cellulose is hydrolyzed by cellulases that correspond to a group of enzymes that act in different parts of cellulose cleaving β (1 → 4) bonds among glucoses. Cellulases are classified as endoglucanases (EC 3.2.1.4) that cleave random β (1 → 4) bonds of the amorphous region of cellulose, exoglucanases, or cellobiohydrolases (EC 3.2.1.91) that hydrolase (1 → 4)-beta-D-glucosidic linkages in cellulose, releasing cellobiose from the free chain ends. The cellobiose released is degraded into glucoses by the action of β-glucosidases (EC 3.2.1.21) (Segato et al. 2014).

Cellulolytic microorganisms share their ecological niche with other cellulolytic and non-cellulolytic fungi and bacteria. Interestingly, the degradation of lignocellulose is performed by different sets of microorganisms and enzymes, since cellulose is commonly coated by other polymers, such as lignin and hemicellulose, resulting in a great variation of structural and chemical arrangement and therefore recalcitrance. In nature, the degradation of cellulose results in the formation of cellobiose that is inhibitory to the cellulase system of fungi and bacteria. However, due to the presence of several other saccharolytic microorganisms, the excess of cellobiose is utilized, allowing the continuity of the degradation of cellulose. In return, these saccharolytic strains help to neutralize toxic effects of lignin-degrading fungi, as well as providing vitamins and other nutrients assimilated by cellulolytic strains. Therefore, the complex machinery of cellulase system and nature of enzymes have been coordinated by the evolution of these organisms (Bayer et al. 1994).

2.1 Bacteria as Producers of Cellulases

Both fungi and bacteria have been studied for cellulase production. Although, the former have been more studied, lately bacteria have received more attention due to their higher growth rate than fungi and the fact that they produce more complex and multi-enzymatic complexes (Sadhu and Maiti 2013). Several bacteria produce cellulases, including species from *Cellulomonas*, *Clostridium*, *Bacillus*, *Erwinia*, *Ruminococcus*, *Thermomonospora*, *Bacteriodes*, *Microbispora*, *Streptomyces*, and *Acetovibrio* (Bisaria 1991).

There are works focused on the optimization of the production of cellulases by bacteria. (Manfredi et al. 2016) studied the production of endoglucanases by the strain *Bacillus* sp. AR03 in the peptone-based broth supplemented with 10 g/L

CMC (carboxymethyl cellulose) and 10 g/L sucrose after 48 h cultivation at 30 °C reaching 3.12 ± 0.02 IU/mL of enzyme activity. In a study carried out by Sethi et al. (2013), three bacteria capable of producing cellulase were isolated from soil and identified as *Escherichia coli, Pseudomonas fluorescens*, *Serratia marcescens*, and *Bacillus subtilis*. The optimal conditions for cellulase production were found at 40 °C, pH 10, using glucose and ammonium sulfate as the carbon and nitrogen source, respectively. In addition, coconut cake induced cellulase production. The author observed that *Pseudomonas fluorescensis* was the best producer of cellulase among the other four strains.

In the early 1980s, a multifunctional, multienzyme complex, capable of solubilizing cellulose, called cellulosome, was discovered in the cellulolytic thermophilic anaerobe *Clostridium thermocellum* (Lamed et al. 1983; Bayer et al. 1983). This complex is produced by some anaerobic cellulolytic bacteria, since the energy level generated by anaerobic bacteria limits the production of enzymes. Cellulosomes are remarkably efficient, organized, cell surface enzymatic system (Bayer et al. 2004) that result in enzyme recycling and direct assimilation products. Moreover, this complex can have a better access to cellulose surface because it physically separates cellulose microfibrils (Resch et al. 2013; Ding et al. 2012).

Cellulosomes are composed of dockerin-containing enzymes or different types of ancillary protein, and cohesin-containing structural proteins, termed scaffoldins. These two main blocks are bound to each other since they are complementary modules. Interestingly, these multienzyme complexes can be released as cell-free cellulosomes or attached to the bacterial cell surface (Hamberg et al. 2014; Xu et al. 2016b).

Cellulosomes are produced by different anaerobic bacteria such as different species of *Clostridium* and *Ruminococcus* and *Acetivibrio cellulolyticus.* In addition, there are different types of enzymes in cellulosomes system depending on the bacterial producer, including cellulases, hemicellulases, and pectinases (Artzi et al. 2016).

2.2 Fungi as Producers of Cellulases

In nature, there are innumerous fungi capable of producing different lignocellulolytic enzymes, including species from ascomycetes (i.e., *Trichoderma reesei* and *Aspergillus niger*), basidiomycetes including white-rot fungi (i.e., *Phanerochaete chrysosporium*), brown-rot fungi (i.e., *Fomitopsis palustris*), and a few anaerobic species (i.e., *Orpinomyces* sp.) The last group corresponds to fungi found in gastrointestinal tracts of ruminant animals (Ljungdahl 2008; Kim et al. 2007).

Filamentous fungi are well-recognized decomposers in nature and present important role in the decomposition of lignocellulosic material. Among the most interesting fungi cellulase producers, one can cite species from *Aspergillus, Trichoderma, Penicillium* and *Schizophyllum* (Sternberg 1976; Duff and Murrayh 1996).

Taking into consideration the high costs of enzyme obtainment, different strategies have been applied to optimize cellulase production, including

fermentation optimization and the use of molecular biological tools. The first focuses on the optimization of bioprocesses and includes the selection of the best media that can be used for submerged fermentation (SmF), or solid-state fermentation. In this context, the use of agro-industrial residues can be of great relevance, since they are low-cost and have high nutritional value. The composition of the cultivation medium, as well the fermentation condition, including temperature, pH and agitation must be optimized for a maximum cellulase production, using different techniques, for instance, response surface methodology. In the work of Pirota et al. (2016), the best conditions for the production of different cellulases, including endoglucanases and β-glucosidases were 28 °C, with an initial substrate moisture content of 70%, 80% of inlet air humidity and 20 $mL.min^{-1}$ of airflow rate. Matkar et al. (2013) isolated and identified a strain of *Aspergillus sydowii*. The cellulase production was optimized showing that endoglucanase (1.32 IU/ml), exoglucanase (3.99 IU/ml), and β-glucosidase (cellobiase 9.24 IU/ml) were optimal on the 6th day under SmF using 10% (v/v) inoculum with 0.1% Tween-20 at 40 °C, pH 5.5, and 120 rpm. In addition, the best carbon source was lactose.

Besides the optimization of fermentation for protease production, there are several techniques of molecular biology applied to increase production of specific cellulases or cocktails. Ascomicetes are not only recognized as excellent cellulase producers but also efficient hosts for the secretion of heterologous proteins that can be native or engineered. They include *Aspergillus* species, such as *A. nidulans*, *A. oryzae*, and *A. niger*, and *T. reesei* (Zoglowek et al. 2015). These techniques include the use of constitutive promoters to improve enzyme secretion (Bando et al. 2011) and deletion of genes involved in different pathways (Schuster et al. 2012). Different strategies are also used like the work of Patyshakuliyeva et al. (2016) that applied adaptive evolution in *A. niger* generating a mutant that showed a five times higher production when compared to the parental strain. The authors observed that the expression of *noxR* gene was reduced in the mutant strain, what was proved after the obtainment of *noxR* knockout strains.

Secretomic analysis of both *T. reesei* and *A. niger* cultivated in sugarcane bagasse showed that, since 6 h of cultivation, these ascomicetes were capable of secreting enzymes involved with deconstruction of polysaccharides from sugarcane cell walls. Although *A. niger* produced more enzymes, quantitatively and qualitatively, both fungi secreted important cellulases, including cellobiohydrolases, endoglucanases, and β-glucosidases, as well as some other hemicellulases. In addition, the authors concluded that a combination of enzymes from both fungi could be interesting to increase saccharification processes (Borin et al. 2015).

Trichoderma reesei is probably the most important producer of cellulases being used for industrial production of cellulolytic cocktail. The strain *T. reesei* QM6a that has been engineered through classical mutagenesis for the last three decades resulted in the industrial strain *T. reesei* RUTC30 that has a massive capability in producing a combination of cellulases. This engineered strain showed a surprisingly high number of mutagenic events, leading to the loss of more than 100 kb of genomic DNA that was related to 43 genes that are involved in nuclear transport, secretion/vacuolar targeting, mRNA stability, metabolism, and transcription (Le Crom et al. 2009).

3 General Applications of Microbial Cellulasesin Industrial Sectors

Due to the enzymatic complexity of cellulases, these microbial enzymes can have a wide spectrum of application and have shown their biotechnological potential in several industrial processes (Kuhad et al. 2011; Cherry and Fidantsef 2003; Ferreira et al. 2014). Among them, the main industries that seek the use of cellulase enzyme complex are the food, detergents, and the pulp and paper industries. In order to extend the understanding of the potential of application of these enzymes, with exception of lignocellulose conversion and bioethanol, this chapter briefly presents more information on these processes.

3.1 Application of Microbial Cellulases in the Food Industry

In the food industry, clarification of fruit juices is one of the main focuses of enzyme application. The process of juice production results in the disruption of cell wall and the generation of insoluble particles that interfere with the final appearance of the juice. In this context, cellulases have been applied in combination with other enzymes, mainly pectinases and hemicellulases. It improves filtration, clarification, and stabilization of the juice (Kuhad et al. 2011). A study characterized and applied immobilized pectinases–cellulases for grape juice clarification. Enzymatic preparations were tested for turbidity reduction in grape juice, resulting in a decrease of 50% in 1 h (Magro et al. 2016). In a different work, the combination of xylanase, pectinase, and cellulase was studied for the clarification of pineapple juice, resulting in a 90.2% yield and 80.9% clarity (Pal and Khanum 2011).

Cellulases have also been studied for the extraction of different industrially relevant compounds. The extraction of water-soluble polysaccharides from pumpkin (*Cucurbita moschata*) has been investigated. The authors observed that optimal conditions of extraction were determined as 40 min, 55 °C, pH 4.5, and 4000 U/g of cellulose. After polysaccharide purification and pulverization, it had high antibacterial activity against *Escherichia coli, Bacillus subtilis,* and *Staphylococcus aureus* at the concentration of 100 mg/mL (Qian 2014). Cellulases have also been applied in the extraction of water-soluble fiber diary from apple pomace that presents functional properties. In comparison with other methods, cellulase presented the best results (Li et al. 2014).

Secoisolariciresinol, the main flax seed lignin, is converted into enterodiol by human intestinal microbiota. This compound is related with the reduction of mammary and prostatic tumors. The extraction of secoisolariciresinol from seed hulls and whole seeds of flax has been studied using an enzymatic step with cellulase R10 from *Trichoderma reesei*. The best conditions were found using 1 unit ml^{-1} of cellulase R10 in 0.1 M citrate–phosphate buffer pH 2.8 at 40 °C for 6 h (Renouard

et al. 2010). On the other hand, pigments are important compounds that can also be extracted using cellulases. In the study of Zuorro et al. (2011), tomato skins were pretreated by a food-grade enzyme preparation with cellulolytic activities and pectinolytic and subjected to hexane extraction. An 8- to 18-fold increase in lycopene recovery was obtained when compared to the untreated plant material.

In wine and beer industry, cellulases are applied mainly in the maceration process for the extraction of relevant compounds that provide sensorial or functional properties for the product. In the work of Bautista-ortín and Jiménez-pascual (2013), the combination of cellulases and polygalacturonase was important to the degradation of seed cell walls, resulting in the diffusion of proanthocyanidins located in the peels and seeds of grapes. These compounds interfere with the astringency, bitterness and color stabilization of red wines. Regarding brewing, Sensidoni et al. (2011) investigated the addition of cellulases from *Aspergillus* spp. in beer production and observed that after the enzymatic treatment the levels of β-glucan, maltotriose and maltose were reduced, while the levels of glucose and fructose increased. Consequently, the flow rate during filtration increased to 21.97 L/h/m^2 compared to the non-treated beer (8.1 L/h/m^2) and resulted in better filtration.

3.2 Application of Microbial Cellulases in Detergent Industry

Besides, the industrial applications already mentioned of cellulases, these enzymes are also used in detergent formulations along with protease, lipase, and amylase enzymes. The most used cellulases for this application are cellobiohydrolase CBH1 and endoglucanase EGIII, being the filamentous fungi, *T. reesei, T. viride, T. harzianum,* and *A.niger* being some microbial sources of these enzymes. Cellulases from *Humicola insolens* and *H. grisea* var. *thermoidea* are also employed in washing powders and detergents because of their particular properties of being enzymes active under mild alkaline condition and at high temperatures. These cellulases are reported to improve the color brightness, dirt removal, and protuberances in cotton industries, making a step named biopolishing. Alkaline cellulase along with lipase and protease are able to remove the oil from interfiber space of clothes and enhance the cleaning potential of a detergent (Kuhad et al. 2011; Sukumaran et al. 2005; Kottwitz and Schambil 2005; Karmakar and Ray 2011; Gaubert et al. 2016). Nowadays, little is known about this subject and further studies are necessary aiming to investigate cellulases and their role on boosting the activity of detergents.

3.3 Application of Microbial Cellulases in the Pulp and Paper Industry

The interest in the application of cellulases in the pulp and paper industry has increased considerably during the last years (Kuhad et al. 2011), along with

xylanase, laccase, and lipase that display important applications in this area (Demuner et al. 2011). In the process chain of the pulp and paper industry, cellulases can be employed in different segments including enhancement of drainage, deinking, and mainly in fiber modification (Tolan 2010; Kirk and Jeffries 1996).

Enzyme treatments improve drainage by removing the fines or peel off fibrils on the fiber surface and dissolved and colloidal substances, which often cause these problems in paper mills and impact on the production rate (Bhat 2000). Cellulases have also been reported to enhance the bleachability of softwood kraft pulp resulting in a final brightness grade comparable to that of xylanase treatment (Singh et al. 2007). It was shown that cellulases used alone or combined with xylanases are beneficial for deinking of different types of paper wastes (Singh et al. 2007). Mechanical pulping process can also be improved with cellulases and other enzymes providing energy savings which may vary from 20 to 40%, due to the lower energy input of these enzymes (Karmakar and Ray 2011).

Cost-effectiveness is essential for enzymatic treatment of dissolving pulp toward industrial application. In this perspective, the strategy of cellulase recycling with fresh cellulase addition was demonstrated. This technique resulted in decreasing the viscosity (470 mL/g) and increasing the Fock reactivity (80%) of the dissolving pulp. Thus, cellulase recycling should be considered as a promising strategy to reduce enzyme cost (Wang et al. 2016).

Besides that, cellulases can have several other applications in the pulp and paper industry, such as to reduce chlorine requirement, to improve fiber brightness and strength properties, and also can be used during the production of biodegradable cardboard, paper towels, and sanitary paper (Kuhad et al. 2011). Some other specific applications of cellulose in this industry were well reviewed in the literature conducted by Kirk and Jeffries (1996) and Bhat (2000), among others.

4 Nanotechnology and Cellulases for Bioethanol Production

The use of nanotechnology has increased broadly over the last years in several areas of knowledge, including medicine, robotics, chemical engineering, biology, and advanced materials. The miniaturization trend and the development of more precise equipment have allowed a great range of different applications for nanotechnology and attracted increasing attention due to the possibility of optimizing and improving the productivity of processes, such as nanoimmobilization of enzymes and development of nanomaterials of industrial interest (Mamalis 2007; Verma et al. 2016; Cipolatti et al. 2016). In this context, some efforts have been made to combine nanotechnology with biomass degradation and second-generation ethanol. Therefore, nanotechnology has been elected as a potential candidate to boost the biofuel refineries (Chandel et al. 2015).

Over the past years, the increasing global demand and dependence for fossil fuels has emerged as one of the main environmental concerns due to the emission of greenhouse\gases and the climate change associated and depletion of fossil fuels reserves (Goldemberg 2007; Banerjee et al. 2010). Thus, it is of paramount importance to replace the petroleum and other fossil fuels by biofuels produced from sustainable energy sources, such as the lignocellulosic feedstocks (agricultural residues and industrial wastes, for instance). Bioethanol is one of the promising alternatives among the biofuels to replace fossil fuels and it might be obtained through the fermentation of sugars released from vegetal cell wall degradation, conversely to the sugarcane sucrose and cornstarch used by the major producers United States and Brazil, respectively, for the production of first-generation ethanol (Jørgensen et al. 2007; Agbor et al. 2011; Kubicek and Kubicek 2016).

Lignocellulose is basically composed of three elements: cellulose, hemicellulose, and lignin. All these components are arranged in a robust and recalcitrant network that must be deconstructed in order to make sugar monomers available from lignocellulose to microbial fermentation and bioethanol production. Thus, briefly the lignocellulose requires physical/chemical pretreatments to have its backbone more accessible to the enzymes of the hydrolysis step. The list of enzymes used for lignocellulose deconstruction is vast, but the main ones are the cellulases cellobiohydrolases, endoglucanases, β-glucosidases and the hemicellulases xylanase, β-xylosidase, β-arabinofuranosidase, xyloglucanase, and esterases. Cellulases sources have been already discussed previously, and it is well known that filamentous fungi are superb producers and secretors of this type of enzyme in nature and their cellulases mixtures are employed in diverse commercial cocktails (Cannella and Jørgensen 2014; Van Den Brink and De Vries 2011; Kim et al. 2015). However, one of the major drawbacks of the bioethanol production still is the economic viability due to the high cost associated with enzyme production, purification, and concentration (Jönsson et al. 2013; Chandel and Singh 2011; Cannella and Jørgensen 2014). Therefore, one alternative to overcome this challenge is the reuse of the enzymes to hydrolyze the lignocellulose components by immobilization in nanomaterials.

Enzyme immobilization is an interesting method used to optimize industrial processes fixing a biocatalyst (an enzyme, for example) in a biocompatible and inert support (Romo-Sánchez et al. 2014). It offers several advantages compared to free biocatalyst, such as improvement of enzyme loading and activity, better thermal and pH stability, recovery of desired products with high purity degree, and biocatalyst reusability (Eş et al. 2015; Ansari and Husain 2012; Abraham et al. 2014a). Enzyme nanoimmobilization is a particular immobilization method that uses materials at nanoscale having higher surface area and superior physical properties (like strength, chemical reactivity, and conductivity) than conventional materials. These nanomaterials reduce the diffusion limitations, maximize the functional surface area to improve the enzyme loading, and provide a strong cross-linking immobilization through covalent bonds (Abraham et al. 2014a; Chandel et al. 2015). An improvement on the stability of proteins adsorbed onto nanomaterials in denaturing conditions was also observed (Dordick et al. 2012).

Several different nanostructures for immobilization, such as nanoparticles, nanofibres, nanopores, nanocomposites, nanotubes, nanorods, and nanosheet (Verma et al. 2013a, b, 2016) have been reported, however, the most used nanostructures related to lignocellulose hydrolysis and bioethanol production are magnetic (MNPs), gold, and silica nanoparticles (Table 1) (Dwevedi 2016; Verma et al. 2016). Adsorption and covalent binding were the attachment approach of most of them to immobilize cellulases and each method has cons and pros based on the enzymatic biocatalyst, nanomaterial, and substrate. Adsorption of cellulase is supported by van der Waals forces, hydrogen bonding, and hydrophobic interactions between the enzyme and the nanostructures. It presents lower costs and is relatively a nontoxic method. For covalent binding immobilization, the surface nanomaterials have to be modified, however, this attachment is the safest method to reduce protein desorption (Gokhale and Lee 2012).

Enzyme reusability is one of the key factors for industrial processes, because it has impact directly on the cost production of a desired product, making the production economically viable or not. Thus, it is necessary that the nanoimmobilized enzyme has an efficient recovery, does not suffer much leaching, and still retains high residual activities after several recycles and purification steps (Miletić et al. 2012). Among all the studies presented, it is quite difficult to compare the best results and nanoimmobilization methods because of the differences between the growth conditions, strains, and substrates used, and also due to different methodologies applied. However, it is worth to mention the interesting results obtained by Verma et al. (2013a, b). In this study, the authors immobilized a β-glicosidase from *A. niger* in iron oxide MNPs by covalent binding method. Using the synthetic substrate pNPG and a temperature of 60 °C, the nanoimmobilized enzyme retained more than 80% of its residual activity after eight recycles of 10 min each. Furthermore, immobilized β-glicosidase was able to hydrolyze more than 90% of cellobiose within 5 h incubation, while the free enzyme reached the same cellobiose conversion only after 16 h (Table 1) (Verma et al. 2013a, b).

Besides MNPs, silica nanoparticles have also been used for cellulase immobilization and ethanol production. Lupoi and collaborators adsorbed a cellulase from *T. viride* in 40 nm silica nanoparticles and observed a greater ethanol production (>10 mg ethanol) on simultaneous saccharification and fermentation (SSF) compared to free enzyme (4 mg ethanol) using a temperature of 35 °C, cellulose as substrate and a time incubation of 96 h. In addition, the cellulose conversion yielded 1.6 times more glucose by nanoimmobilized cellulase than free enzyme at pH 4.8 and 35 °C (Table 1) (Lupoi and Smith 2011).

Although there are various types of characterized nanoparticles and different attachment methods for nanoimmobilization, further efforts are needed to optimize the conditions of enzymatic hydrolysis and ethanol production yield in order to overcome the lignocellulose hydrolysis bottleneck using this approach.

Nanotechnology has also progressed on the discovery of new materials and methods. Qi and collaborators, for example, reported high glucose concentration from permeate after an enzymatic hydrolysate being filtrated on polyamide membranes (NF90 and NF270 from Dow Filmtec™). Steam exploded wheat straw

Table 1 Cellulase nanoimmobilization studies using different nanoparticles and substrates

Nanoparticles	Nanoparticles size	Cellulase and microbial source	Substrate	Optimum pH and temperature	Km, Vmax	Reusability (percentage relative to the original activity)	Reference
SiO2-coated Fe3O4	–	Commercial cellulase (Runyang Ltd Co., China)	CMC	–	–	77% after 7 cycles	Tao et al. (2016)
MNP	40 nm	β-glucosidase from *A. niger* (Sigma)	pNPG	6.0, 60 °C	4.3 mM, 0.89 U/mg	>80% after 8 cycles	Verma et al. (2013)
β-cyclodextrin-Fe3O4	6.2 and 4.5 nm	Cellulase from *A. niger* (Sigma)	Rice straw	–	–	44.15% after 16 cycles	Huang et al. (2015)
PMMA[a]-Fe3O4	150 nm	Endoglucanase from Thielavia terrestris (Cellusoft CR, Novozymes, Brazil)	CMC	5.0–6.0, 55–65 °C	–	69% after 8 cycles	Lima et al. (2016)
Silica modified gold	6.3–9.6 nm	Cellulase complex from T. reesei (Sigma, USA)	Waste bamboo chopsticks powder	8.0, 50 °C	–	>99% after 6 cycles	Cheng and Chang (2013)
Nanogold-coated PU[b]spheres	35 ± 7 Å[e]	Endoglucanase from *Fusarium* sp.	CMC	5.0, 70 °C	–	43% after 5 cycles	Phadtare et al. (2004)
MNP-PMAA[f]shellparticles	100 nm (MNP), 30 nm (PMAA[f])	Cellulase mixture from *T. reesei* (Celluclast 1.5 L, Novozymes)	Avicel	–	–	–	Kamat et al. (2016)

(continued)

Table 1 (continued)

Nanoparticles	Nanoparticles size	Cellulase and microbial source	Substrate	Optimum pH and temperature	Km, Vmax	Reusability (percentage relative to the original activity)	Reference
Sílica	40 nm	Cellulase from T. viride (Sigma)	Avicel	–	–	–	Lupoi and Smith (2011)
PMMA[a] core-shell	80–120 nm	Cellulase from *Aspergillus* sp. (Sigma)	CMC	7.0, 50 °C	–	–	Ho et al. (2008)
TiO2	<25 nm	Commercial cellulase (Sisco Research Labs)	CMC	–	3.35 mg, 4.02 µmol/min (physically immobilized); 0.67 mg, 2.68 µmol/min (covalently immobilized)	<10% after 5 cycles (physically immobilized); 60% after 5 cycles (covalently immobilized)	Ahmad and Sardar (2014)
PAA[c] polymer-silica	25 nm (PAA), 100 nm (silica)	Cellulase from *T. reesei,* β-glucosidase from *A. niger* (Novozymes)	Filter paper, solka-floc cellulose, cellobiose	–	–	–	Samaratunga et al. (2015)
PAA[c] polymer-silica	25 nm (PAA), 100 nm (silica)	Cellulase from *T. reesei,* β-glucosidase from *A. niger* (Novozymes)	Solka-floc cellulose, cellobiose	4.4, 50 °C	–	–	Samaratunga et al. (2015)

(continued)

Table 1 (continued)

Nanoparticles	Nanoparticles size	Cellulase and microbial source	Substrate	Optimum pH and temperature	Km, Vmax	Reusability (percentage relative to the original activity)	Reference
MAPS [d]covered enzyme particle	3 nm (polymer layer); 5–30 nm (polymer layer + enzyme)	Celluclast BG enzyme from *T. reesei* (Novozymes)	Filter paper	7.0–12.0, 70 °C	–	–	Hegedüs et al. (2012)
MNP-magnetosome system	75 nm	Endoglucanase and β-glucosidase from *C. thermocellum*	CMC	–	–	>70% after 5 cycles	Honda et al. (2015)
MNP	40 nm	Cellulase from *T. reesei* (Sigma)	CMC	4.0, 60 °C	2.6 mg/mL, 2.0 mg/mL/min	70% after 3 cycles	Abraham et al. (2014b)

[a]PMMA: poly(methyl methacrylate); [b]PU: polyurethane; [c]PAA: poly(acrylic acid); [d]MAPS: 3-(trimethoxysilyl) propyl methacrylate; [e]Gold nanoparticle diameter; [f]PMAA: Poly(methacrylic acid)

(SWES) was hydrolyzed by a commercial cellulase (Genencor Bio-Products) at 50 °C and then a nanofiltration step was used to recover the soluble sugars from the hydrolysis and the water for recycling. Thus, a glucose concentration of 110.2 and 70.6 g/L using the NF270 and NF90 was found, respectively, operated at 13.3 L/m^2h, versus 30.2 g/L of glucose from the non-nanofiltrated hydrolysate. Along with this strategy, a previous filtration step was able to recover the cellulases from the hydrolysate to recycling, showing the feasibility of using this combination of filtration methods to improve the bioethanol production process (Qi et al. 2012).

Finally, Zhao and collaborators introduced a real-time assessment of morphological changes of cellulose promoted by a sodium chloride (NaCl) treatment using atomic force microscopy (AFM) imaging. Shortly, they fixed microcrystalline cellulose powder on a nanomechanical sensor named microcantilever, previously rinsed in a polyvinylamine (PVAM) solution and were monitoring the differences of cellulose roughness using an increasing NaCl concentration (0.1, 0.5 and 1 M). PVAM layer was used to improve the adhesion of the 10–20 nm cellulose layer and the microcantilever surface. The authors observed that 1 M of NaCl was enough to cellulose having 43% increase in roughness, what suggests the correlation between the bending of microcantilever and the morphological changes on the cellulose (Zhao et al. 2010). A few years later, the same leading researcher published a work showing that the addition of 0.15 μM of cellobiohydrolase CBH1 from *T. reesei* induced microcantilever bending and this change was related to cellulose deconstruction (Xi et al. 2013). Despite some efforts have been made, this field of nanotechnology has to be more explored in order to better understand the breakdown of cellulose at molecular level. This real-time technique could be improved to use other enzymes and substrates, broadening its applications. The combination of structural analysis of lignocellulose and the assessment of molecular changes is also a valuable tool to get insights of the cell wall recalcitrance and its deconstruction (Chandel et al. 2015).

5 Concluding Remarks

The wide complexity of cellulase system boosts their research and industrial potential. In this way, the understanding of its activity and its application in a suitable industrial process are essential for bioethanol production from lignocellulosic biomass. Despite the significance in substituting traditional fossil fuel and usual technologies, the ethanol production from lignocellulosic biomass is a complex process. This technology presents a massive potential for the future of the industry but has still faced several technological challenges that directly impact the adoption of an industrial scale.

Therefore, nanotechnology can be considered as a potential candidate to boost the biofuel refineries aimed at new developments in the area, offering new improvement with cellulases as a new technological tool. Some of the advances include efficient recovery of cellulase enzymatic complexes through nanoimmobilization and the cost reduction associated with their reuse. Nanotechnology has also progressed on the

discovery of new materials and methods, opening up many future prospects in this area.

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