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Chapter

Microbial Cellulases: An Overview and Applications

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Abstract

Cellulases are a complex group of enzymes which are secreted by a broad range of microorganisms including fungi, bacteria, and actinomycetes. In the natural environment, synergistic interactions among cellulolytic microorganisms play an important role in the hydrolysis of lignocellulosic polymer materials. In fact, it is the combined action of three major enzymes which determines the efficiency of this process. They are exoglucanases, endoglucanases, and β-glucosidase. Microorganisms produce these enzymes in a diverse nature which determines their efficiency in cellulose hydrolysis. During the cellulose degradation reaction, the enzyme targets the β-1,4-linkages in its polymeric structure. This is an essential ecological process as it recycles cellulose in the biosphere. The application of this same scenario for industrial purposes is identified as an emerging area of research. Biofuel production, textile polishing and finishing, paper and pulp industry, and lifestyle agriculture are among the key areas where cellulase enzyme shows a broader potential. The objective of this chapter is to discuss the structure, function, possible applications, as well as novel biotechnological trends of cellulase enzymes. Furthermore, possible low-cost, enzymatic pretreatment methods of lignocellulosic material in order to use it as an efficient raw material for biofuel production will be discussed.

Keywords: applications of cellulases, cellulase activity, cellulose, cellulolytic microorganisms, industrial applications of cellulase, pretreatment of cellulose

1. Introduction

Biomolecules derived from natural resources are playing a major role in manufacturing products needed for daily use. Enzymes are one of those molecules that are globally recognized for their multifarious applications in industries. For instance, their utility in brewing, dairy products, detergents, food and feed, pharmaceutical production, and paper and pulp industry is huge. One of those most widely used enzymes is cellulase. According to recent global cellulase market analysis reports, the demand for this enzyme is exponentially increasing.

Cellulose, the substrate of cellulase, is the most abundant polysaccharide present on earth. It is the main substance in plant materials. Anselme Payne was the very first person to discover and isolate this amazing compound from green plants [1]. It happened more than two centuries ago. From the past, cellulosic materials have played a crucial role in daily human life. They used it to fertilize their soil for crop cultivation. It was also fodder for their cattle. It was firewood for cooking, and they were igniting cellulosic material to generate heat whenever they needed to produce energy.

Currently, the role played by cellulose is not that simple. Especially, as it is recognized as a cost-effective raw material, the useful applications of cellulose in the industrial sector have become much more complex. This has laid a huge platform for scientists to do cellulose-based research in multidisciplinary approaches. One such area is hydrolysis of cellulose. In nature, this is usually accomplished by cellulases. Cellulase is catalyzing hydrolysis of cellulose.

However, cellulase is not a single enzyme. It is a group of enzymes which is mainly composed of endoglucanase and exoglucanases including cellobiohydrolases and β-glucosidase. Fungi, bacteria, and actinomycetes are recorded to be efficient cellulase enzyme producers in the natural environment. These microorganisms must secrete cellulases that are either free or cell surface bound. Their enzyme production efficiency and the enzyme complex composition are always diverse from each other. Although both aerobic and anaerobic microorganisms produce these enzymes, aerobic cellulolytic fungi, viz., *Trichoderma viride* and *T. reesei*, are excessively studied. The enzyme breaks β-1,4-linkages in cellulose polymer to release sugar subunits such as glucose. This notion is applied in industries either cellulose is utilized as a raw material or cellulose degradation is a must.

According to recent enzyme market reports, the key areas of the industry where cellulase enzyme is increasingly being applied are healthcare, textile, pulp and paper, detergent, food, and beverages. Its wide application in coffee processing, wine making, and fruit juice production is related to food and beverage segment. In other industrial applications, it is broadly used to produce laundry detergents and cleaning and washing agents. Cellulase is also being highly recognized as an effective alternative to available antibiotics for treatment of biofilms produced by *Pseudomonas*. Therefore, the potential of cellulases to fight against antibiotic-resistant bacteria is an amazing trend which will overcome problems in the healthcare sector [2].

Application of microorganisms or microbial enzymes for pretreatment of lignocellulosic material is currently earning a huge attention of the industry. This is a result of growing interest about depletion of fossil fuel resources in the world which have inspired the production of bioethanol from lignocellulosic biomass through enzymatic hydrolysis [3]. Lignocellulosic biomass is one of the best options as a low-cost, readily available, eco-friendly raw material. However, it is not found alone. Cellulose is forming lignocellulose in combination with hemicellulose and lignin which finally becomes a compact network structure [4]. Moreover, it has a crystalline structure which is hard to break down. Therefore, cellulose is insoluble in water and causes limitations in hydrolysis. That is why it is essential to pretreat lignocellulosic material in industries like bioethanol production. During pretreatment, it will loosen up the crystalline structure and facilitate the degradability to release fermentable sugar forms. There are several methods available for pretreatment of lignocellulose, viz., physical, chemical, and biological methods. Biological pretreatment using cellulolytic microorganisms and their enzymes is found to be the best way of addressing this problem.

By all means, cellulase is an enzyme which can cause a huge economic impact. However, there are some considerable bottlenecks of utilizing this enzyme in the industry. For example, the higher cost of cellulase and less catalytic efficiency are especially understood. Another important point is less understanding of the relationship between hydrolysis mechanisms and molecular structure of the enzyme. This knowledge is important to carry out further improvements in the enzyme to enhance its catalytic activity. Therefore, this chapter is discussing about the structure and function of cellulase in order to understand its mechanisms of action. The details on current applications of the enzyme have also been summarized here. Furthermore, the efforts have been taken to bring together information on novel biotechnological trends of cellulase. Moreover, it is discussing about possible low-cost, enzymatic pretreatment methods that have been practiced for lignocellulosic materials in order to use it as an efficient raw material to produce bioethanol.

2. Cellulose

Before moving on to cellulase, it is essential to understand cellulose which is the substrate of cellulase enzyme. This section will provide a short description about cellulose.

2.1 Cellulose is a polysaccharide

Cellulose is a linear polysaccharide. In this polymer, D-glucose subunits are attached together by formation of β-1,4-glycosidic linkages between individual glucose molecules. The molecular formula of cellulose is $(C_6H_{12}O_6)_n$. The "n" indicates the degree of polymerization (DP). It symbolizes the number of glucose subunits connected with each other. This number is varying from hundreds to thousands. Two glucose repeating units together are called cellobiose. In other words, this polymer is made by β-(1 → 4)-D-glucopyranose units in $^4C_1$ conformation. It consists of long chains of anhydro-D-glucopyranose units (AGU) with each cellulose molecule having three hydroxyl groups per AGU with the exception of the terminal ends. Cellulose has both crystalline and amorphous regions in its structure in various proportions [5]. Those regions are intertwined to form the structure of cellulose. There are four major crystalline forms, for instance, Iα, Iβ, II, and III. This crystalline structure is a result of intramolecular and intermolecular hydrogen bonding between glucose monomers in cellulose. These hydrogen bonds construct a huge network that directly contributes to the compact crystal structure of cellulose polymer. On the other hand, this strong intramolecular and intermolecular hydrogen bond formation leads to poor solubility of cellulose.

2.2 Organization of cellulose

In plant cell walls, cellulose exists as different levels of structures, i.e., single cellulose chains, elementary fibrils (consisting of tens of single cellulose chains), and microfibrils (bundles of elementary fibrils). It is proposed that the macrofibril is composed by the attachment of several newly synthesized elementary fibrils. With the cellular growth, the macrofibrils divide to form individual microfibrils. Microfibril is consisting of a single elementary fibril. Although elementary fibrils and macrofibrils are composed of mere cellulose, microfibril has noncellulosic polymers like hemicelluloses along with cellulose. It is noted that others consider a microfibril as consisting of a number of elementary fibrils. Microfibril is an elementary fibril associated with noncellulosic polymers. Each microfibril might contain up to 40 cellulose chains and is about ~10 to 20 nm in diameter. Many such cellulose chains aggregate into bundles called micelles and micelles into microfibrils. Micelles are interconnected with few cellulose fibers. The plant cell wall structure is stabilized by the macrofibrils. The cross-links between hemicellulose and pectin matrices also support this stabilization process. Lignin is a complex polymer which usually fills the spaces between cellulose and pectin matrices. It forms covalent bonds with hemicellulose. This provides more mechanical strength to the plant cell wall. This structure which is present in plants is collectively called lignocellulose. Other components known as extractives including fats, phenolic, resins, and minerals are also present in lignocellulosic biomass.

3. Cellulase

3.1 Global demands for enzymes

Enzymes are known to be very useful in many industrial processes. Their broad applicability has created a significant market demand in the recent years. According to market reports on world enzyme demand (2017), they have recognized several key factors which lead to huge consumer demand for enzymes. Some of them are completely bound with economical advances. For example, increased per capita income in developing countries causes huge growth in consumer-related industrial applications [6]. A recent industry study done by Freedonia in January 2018 on "Global Industrial Enzymes" reveals that global demand for industrial enzymes is projected to grow 4.0% per year to $5.0 billion in 2021. This report also emphasizes the gains in personal incomes in developing countries as the key factor which is supporting growth in demand for enzymes. The development of scientific research on enzymes is mainly based on disciplines such as biotechnology,molecular biology and genetics. Continued advances in these areas of research, particularly related to DNA manipulation and sequencing, result in extensive increases in enzyme demand worldwide. Cellulase is one such enzyme which earns consecutively increasing demand. Therefore, collection of knowledge about this enzyme is essential for further development of fundamental and applied research on cellulase and for consequent application in human life.

3.2 Molecular structure and function of cellulase

3.2.1 Molecular structure of cellulase

It is produced by fungi, bacteria, actinomycetes, protozoans, plants, and animals. According to Carbohydrate-Active Enzymes database, there is information of the glycoside hydrolase families. Glycoside hydrolases, including cellulase, have been classified into 115 families based on amino acid sequence similarities and crystal structures. A large number of cellulase genes have now been cloned and characterized. They are found in 13 different families. Furthermore, there are 3D structures of more than 50 cellulases. All of cellulases cleave β-1,4-glucosidic bonds. However, they display a variety of topologies ranging from all β-sheet proteins to β/α-barrels to all α-helical protein.

In the structure of cellulase, there are catalytic modules and non-catalytic modules. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures. The non-catalytic carbohydrate-binding modules (CBMs) and/or other functionally known or unknown modules may be located at the N- or C-terminus of a catalytic module. Usually, fungal and bacterial cellulase mainly has two or more structural and functional domains. Both aerobic and anaerobic microorganisms are producing this enzyme. Therefore, there are two types of cellulase systems: noncomplex and complex. A noncomplex cellulase system is produced by aerobic cellulolytic microorganisms, and it is a mixture of extracellular cooperative enzymes. In a noncomplex cellulase system, the common arrangement is joining of a catalytic domain with a cellulose-binding domain (CBD). A complex cellulase system is produced by anaerobic microorganisms and it is called "cellulosome." Cellulosome is assembled by joining a catalytic domain with a dockerin domain. The enzyme is a multiprotein complex anchored on the surface of the bacterium by non-catalytic proteins that serves to function like the individual noncomplex cellulases but is in one unit.

In addition to these two major domains in the cellulase structure, there are some other domains that are present in many cellulases, for instance, S-layer homologous (SLH) domain, fibronectin-type 111 domains, and NodB-like domain, and there are also other regions of unknown function. These domains are often connected by Pro and hydroxy amino acid (threonine and serine) enriched linker sequences. Among all these domains, catalytic and cellulose-binding domains are the most important because they are the domains which are considered participating in hydrolytic mechanisms of the enzyme.

3.2.2 Catalytic function of cellulase enzyme

Cellulase catalyzes the decomposition of cellulose polysaccharide by simply breaking down β-1,4-glycosidic bonds. Three major types of enzymes are generally involved in hydrolyzing cellulose microfibrils in the plant cell wall: endoglucanase, exoglucanase, and β-glucosidase. Complete cellulose hydrolysis is mediated by the combination of these three main types of enzymes. Endoglucanase usually attacks amorphous areas of cellulose. The random attack of this enzyme on internal bonds of loosely bound, amorphous areas of cellulose creates new chain ends. These new chain ends are then easily attacked by other types of enzymes. The highest activity of this enzyme usually occurs against soluble cellulose forms or acid-treated amorphous cellulose. The function of exoglucanase is to produce glucose or cellobiose units by attacking the reducing or nonreducing end of cellulose chains. Endoglucanase is different from exoglucanase because it is usually very active against crystalline cellulose substrates such as Avicel or cellooligosaccharides. Finally, β-glucosidase can hydrolyze cellobiose to glucose from the nonreducing ends, and it is inactive against amorphous or crystalline cellulose. Although an exact mechanism is not yet finalized, fragmentation of cellulose aggregations into short fibers has been observed and reported during the beginning of cellulose hydrolysis prior to releasing any detectable amount of reducing sugars. This is known as amorphogenesis.

There are two catalytic mechanisms of cellulases. They are simply introduced as retaining mechanisms and inverting mechanisms. Cellulases cleave glucosidic bonds by using acid-based catalysis. The hydrolysis is performed by two catalytic residues of the enzyme: a general acid (proton donor) and a nucleophile/base. The catalytic mechanism which occurs depends on the spatial position of the catalytic residues. The retention and inversion of the anomeric configuration of cellulose are the two mechanisms which hydrolyze cellulose. The "retaining" cellulases retain the same configuration of anomeric C bearing the target glucosidic bond even after a double-displacement hydrolysis with two key glycosylation or deglycosylation steps. "Inverting" cellulases inverts the configuration of the anomeric C configuration after a single nucleophilic displacement hydrolysis [7].

4. Applications of microbial cellulases

For many decades, cellulases have played a crucial role as biocatalysts. They have shown their potential application in a large number of industries. Textile, paper and pulp, laundry and detergent, agriculture, medicine, and food and feed industries are some of the major industries which employ microbial cellulases. According to Coherent Market Insights, the textile industry is the dominant market for cellulases in 2017. According to most of the enzyme market research reports published in 2018, food and beverages, textile industry, animal feed, and biofuels have been reported to be the major areas of applications.

According to another Global Cellulase (CAS 9012-54-8) Market Research Report published in 2018, Asia-Pacific is the largest consumer of cellulase, with a revenue market share nearly 32.84% by 2016. Furthermore, the reported data showed 29.71% of the cellulase market demand in animal feed, 26.37% in food and beverages, and 13.77% in the textile industry in 2016. This same report forecasts that the applications of cellulases will reach 2300 million USD by the end of 2025, growing at a compound annual growth rate (CAGR) of 5.5% during the 2018–2025 period. These data suggest that the application of cellulases in industries is drastically rising annually. Novozymes and DuPont from Denmark are key cellulase enzyme producers supplying these enzymes to the global market for industrial applications. From this point forward, in this chapter, our major effort was to discuss about the current applications of cellulases in major fields that have been listed above. The novel biotechnological trends emerging in those fields while understanding the key areas of research where further studies required also surfaced to an extent.

4.1 Textile industry

The textile industry is one of the largest industries in the world. The customer demand for fashion is increasing as they want uniqueness in styles, colors, and the clothes they wear. There was a significant growth in this industry during the last few decades as a result of this increasing customer demand. This enzyme has now become the third largest group of enzymes used in these applications [8]. This creates a very competitive market platform for manufacturers that are always looking for environmentally friendly approaches of giving their products a unique look. Cellulase is used for many purposes in the industrial sector.

Especially for textile wet processing, biostoning of denim fabric, biopolishing of textile fibers, softening of garments, and removal of excess dye from the fabrics are some of the major applications of this enzyme in the industry. Fungal cellulases from *Trichoderma reesei* are the mostly applied enzyme in the textile industry. Apart from that, actinomycetes from the genera *Streptomyces* and *Thermobifida* and other genera of bacteria, such as *Pseudomonas* and *Sphingomonas*, are some of the sources of enzymes to be used for decolorization and degradation of textile dyes [9].

Biostoning and biopolishing are well known for the best applications of cellulases in the current textile industry.

4.1.1 Biostone washing

The conventional washing process of denim usually has three steps. The denim fabric is first treated with amylase enzyme to remove the starch coating of the fabric. This process is called desizing. During this process, starch is broken down into maltose which is a water-soluble disaccharide composed of two glucose molecules. Then, the fabric is given treatment by providing abrasion to the material in pumice stones added to the washing machine. This wash was completely achieved by adding chemicals like sodium hypochlorite or potassium permanganate. This traditional process has several disadvantages. The addition of pumice stones must be done in larger quantities. This was affecting the machine's productivity in an adverse way causing tear effects. After the wash is completed, the manual removal of stones is needed. This is causing further reduction of the process efficiency. The excessive back-staining was another disadvantage of the traditional process. Backstaining is the reaction by which the removed dye molecules are deposited on the denim fabric again.

Application of microbial cellulases was found to be an efficient alternative for pumice stone washing. It was first staged in the 1980s. The use of stones is currently replaced by cellulases in a successful way. During this process, cellulases act on the denim fabric which is made of tough cotton. The indigo dye which is used to color the fabric is trapped inside the cellulose fiber in this cotton material. Usually, the indigo dye is mostly attached to the surface of the yarn and to the most exterior short cotton fibers. When the fabric is treated with the enzyme, it hydrolyzes and breaks small fibers coming out of the fabric which loosens the dye. For this purpose, the β-1,4-linkages of cellulose chains will be broken down, and simple water-soluble sugars will be formed. This will remove the fibers which traps indigo dye. Then, the dye is easily removed from the fabric giving a faded look.

Trichoderma reesei acidic endoglucanase II has been found to be a very efficient candidate for biostoning [10]. The neutral cellulase enzyme extracted from *Humicola insolens* is also reported to be commonly applied in this process [11]. The use of cellulases has several advantages over stone washing with pumice stones including high productivity; less work-intensive, safer environment; short treatment times; and less wear and tear of machines. Currently, denim with a worn-out look has a huge demand in the textile market.

The major disadvantage associated with the application of microbial cellulases is again backstaining. The redisposition of dye on the fabric covers up the shaded look given by the treatment. In order to overcome this problem, several biotechnological approaches have been already experimented by researchers. Immobilization of cellulases on pumice stones is one such cost-effective way of doing this. It has also been observed that acidic endoglucanase causes a better abrasion and less backstaining compared to neutral endoglucanase. For example, the cellulase given by *Trichoderma reesei* is more efficient in preventing backstaining as compared to neutral endoglucanase of *H. insolens*.

The latest trend of biostone washing is to utilize an enzyme mixture composed of amylase, cellulase, and laccase [12]. The sizing is the process by which denim material surface is covered by a compound like starch to provide rigidity and stiffness to raw denim and provide strength and friction resistance during handling. During washing, this surface layer of starch must be removed first to facilitate interaction between cellulases and cotton fibers. The amylase hydrolyzes starch from the fabric and causes desizing. Cellulase hydrolyzes small cellulose fibers, and laccase usually causes bleaching of the fabric. Laccases (EC 1.10.3.2) with intrinsic electron-donating tendency can decompose indigo in the solution as well as on the fabric creating bleaching effect on denim garments. The indigo dye is converted into isatin and anthranilic acid like chemical forms. This prevents backstaining of dye on the fabric surface. Eventually, this will give a complete faded look to the denim fabric. The purpose of using a mixture is to improve the efficiency of biostone washing process by allowing those three enzymes to work together in a sequential manner.

In a recent study, it is reported that an alkali-stable cellulase in combination with xylanase from *Thermomonospora* sp. has a reduced tendency of backstaining [13]. However, the effluents generated during biostone washing must be pretreated to remove dye material and the intermediate chemical compounds present after the reaction. Otherwise, these dyes might pollute natural waterways and soil. Most of these dye residues are toxic and carcinogenic that would cause adverse health effects in humans and animals. Although biodegradability of enzymes is a positive advantage here, chemical by-products formed during dye removal must be neutralized.

4.1.2 Biopolishing and biofinishing

These two processes are simply similar to each other. Cellulosic fibrous materials like cotton and linen are always loosing appearance because of fuzz formation on the fabric surface. Fuzz occurred due to short fibers protruding out from the surface of the fabric. Fuzz is sometimes loosely attached to the fabric forming a ball-like appearance which gives an unattractive look to the fabric. This is called pilling. The biopolishing process basically aims on removing microfibrils of cotton. It enhances fabric look, hand feel, and color by giving a smooth and a glossy appearance. This is also leading to improvement of color brightness, hydrophilicity, and moisture absorbance by the fabric [13]. The acidic cellulases produced by *T. reesei* and *Aspergillus niger* are found to be enormously effective in this process. Biopolishing is eco-friendly because the enzymes used in this process are readily biodegradable and nontoxic.

The repeated washing of a cotton garment makes it fluffy and dull. This is due to partially removed microfibrils on the fabric surface. Biofinishing by cellulases can remove these fibrils and give back the smooth surface and original color to the fabric. This will also give a soft hand feel to the material, and also this is a good way of removing stains and dirt spots that are trapped within the cotton fiber network [14, 15].

4.1.3 Bioscouring

This is the process that removes noncellulosic material from the surface of the cotton. This is usually done with cellulase alone or in combination with other enzymes such as pectinase. Pectinase digests the pectin substance present among cellulose fibers. This helps to remove the intact connection between the cuticle and the main body of the cellulose fiber. This helps to degrade the primary cellulosic wall of the fiber. The ultimate result is the destruction of the cuticle [16]. This reaction increases the softness of the fabric.

4.1.4 Biocarbonization and wool scouring

This is a kind of a biological mode of cleaning the fabric from the cellulosic or vegetative impurities with the help of enzymes. When a pure cotton or cotton blend fabric is prepared, some traces of unwanted cellulosic material still may remain in the fabric. They may result in imperfect finishing and lower quality of the fabric. The earliest methods of carbonization involved application of sulfuric acid. It was not only expensive but also corrosive, unsafe, and hazardous. Being a nonhazardous, non-corrosive, and eco-friendly method, enzymatic carbonization was a promising alternative. This method was perfect for removal of cellulosic impurities from the material because it was least affecting the color and the hand feel of the fabric. The removal of vegetative impurities from the surface of raw wool using cellulases is called wool scouring [17]. Cellulases can be used alone or in combination with other enzymes such as pectinases to increase the efficiency of this process. These methods are doing less damage to the fabric when compared to the treatment with sulfuric acid.

4.1.5 Defibrillation of lyocell

Lyocell is the generic name for a biodegradable fabric that is made out of treated wood pulp. This material is used in everything from clothing to cars. This is obtained from wood pulp using a solvent-spinning method. The solvent system which is usually applied is an organic compound called N-methylmorpholine N-oxide. Some main characteristics of lyocell fibers are that they are soft, absorbent, and very strong when wet or dry and resistant to wrinkles. One chief defect of this material is fibrillation. This is the formation of small tangled fibrils on the surface of the fabric. Cellulases can be efficiently applied to remove these fibrils and give the fabric an increased softness and an improved appearance. This is also good for preventing fuzz and pill formation.

Although the applied enzymes are nontoxic and biodegradable in the above processes, the final effluent produced will show increased biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), and total suspended solids (TSS) to a certain extent because the effluent may contain digested sugar and cellulosic forms. Direct release of this effluent to natural water bodies may cause water pollution. Alkalinity and pH fluctuations of the effluent will also result in polluted water which is not good for human and animal consumption. This may cause health issues like skin irritations in humans. On the other hand, the enzymatic treatments need an incubation period to facilitate the reaction between enzyme and fabric. As this is a fermentation reaction, this may release certain odors to the environment which may cause air pollution to a certain extent. Moreover, textile dyes are removed during textile processing. Most of the dyes are toxic and some are highly carcinogenic. Mixing this type of dyes with water is definitely causing adverse health effects in humans. Therefore, direct release of effluent without applying any pretreatments to neutralize these toxic compounds will breach the stability in ecosystems to which they are released. Therefore, establishment of pretreatment facilities and water quality testing procedures are essential for these enzymatic textile processing plants.

Lyocell production has a different impact on the environment compared to the other textile polishing and finishing processes. The solvent which is used to manufacture this textile is N-methylmorpholine N-oxide. This is usually causing acute toxicity (oral, dermal, inhalation), skin irritation, serious eye damage and irritation, skin sensitization, and specific target organ toxicity. These are also hazardous to aquatic environments. These possible environmental impacts must be always addressed although application of enzymes in textile processing is eco-friendly.

4.2 Paper and pulp industry

This is one of the largest industrial sectors in the world. According to the World Wildlife Fund (WWF), the pulp and paper industry, which includes products such as office and catalog paper, glossy paper, tissue, and paper-based packaging, uses over 40% of all industrial wood traded globally [18]. On the other hand, the latest paper industry statistics reveal China, the United States, and Japan as the three countries where the largest paper production occurs in the world. Half of the total paper manufacture of the world is done by these three countries. However, Germany and the United States are the world's leading paper importers and exporters [19]. Moreover, the United States is reported to be the largest consumer of papers.

Papers and pulp are renewable resources. Therefore, recycling and reusing are two popular concepts related to this industry. Application of microbial cellulases is usually utilized for this purpose. The application of cellulases in this industry is broader. Starting from the 1980s up to now the possible applications are branching toward many areas. For instance, deinking, pulping, bioremediation of industry wastes, bleaching, and fiber enhancement can be taken.

4.2.1 Pulping

The drawbacks in mechanical pulping processes of woody raw materials such as refining and grinding resulted in pulps with higher amounts of fines, bulk, and stiffness. On the other hand, the process was high energy consuming which was not a profitable option for an industry. Meanwhile, biopulping using enzymes such as cellulases is an energy-saving way, and also it is eco-friendly [20]. The substantial energy saving is reported around 20%–40%. During the refining process, it generates small particles of the pulps. These particles reduce the drainage rate during the paper-making process. These particles can be readily degraded by cellulases in order to increase the drainage ability of the pulp. Mixtures of cellulases (endoglucanases I and II) and hemicellulases have also been used for bio-modification of coarse pulp material to improve fiber properties. It is strengthening the hand sheets. On the other hand, biological pulping has the potential to improve the quality of pulp and properties of the paper while reducing energy costs and environmental impact [21].

4.2.2 Deinking

In traditional deinking, large quantities of chemicals are used which make the method expensive and environmentally damaging and increase the release of contaminants [22]. The main advantage of bio-deinking is the ability of avoiding the alkali use during the process. This prevents yellowing of the paper. Cellulases alone, or used in combination with xylanase, are beneficial for deinking of different types of paper wastes. In most of the applications, partial hydrolysis of carbohydrate molecules releases ink from the fiber surface. This is done by a mixture of cellulases alone or in combination of cellulases and hemicellulases. The advantages associated with enzymatic deinking are clean look of paper, enhanced brightness, as well as environmental pollution reduction..

4.2.3 Bio-modification and bio-characterization of fibers

Successful application of cellulase and hemicellulase mixtures has been reported to modify properties of fibers. Usually in the paper industry, the making of paper is made easier by improving the beatability, runnability, and drainage of paper pulp during the process. Modifications of fiber properties are also achieved through treatment of paper by cellulase enzyme [20]. Not only that but also the enzymatic hydrolysis helps in characterization of fiber using various techniques such as scanning electron microscopy (SEM) and HPLC [23].

4.3 Laundry and detergent industry

The application of enzymes in manufacturing enzymatic washing agents or biological detergents dates back to the 1960s. Using enzymes in detergent formulae is a common practice today. In fact, according to market reports, by 2014, the detergent industry was the largest single market for enzymes at about 25–30% of total sales [22]. Another market research report published in 2017 on laundry detergent market stated that its global market size valued at 133.3 billion USD in 2016. The latest trend in the industry is to use alkaline enzymes in large amounts. For instance, protease, cellulase, α-amylase, lipase, and mannanase are broadly applied in heavy-duty laundry and automatic dishwashing detergents..

The capability of enzymes to remove stains is the major focus of using them in manufacturing detergents. Cellulases are available in the market in different brands. For instance, Celluzyme® and Carezyme® are two main brands applied in detergent blends. These detergent blends are mainly applied in washing fabrics made of cotton and cotton blends. These detergents are making fiber modifications in the fabric in order to improve color brightness, softness, and particulate soil removal.

Cellulases extracted from fungi like *Trichoderma* sp. (*T. longibrachiatum*, *T. reesei*, *T. viride*, and *T. harzianum*), *Aspergillus niger*, *Humicola* (*H. insolens* and *H. griseathermoidea*), and *Bacillus* sp. have been excessively studied so far for application in detergents. Alkaline cellulases are the most suitable additives to conventional detergents. It is because of their ability to remove soil and dirt particles from the interfibrillar spaces of the fabric. The cellulases remove the rough projections of cellulose fibers or cellulose aggregates attached to the fabric. This gives an increased gloss and smoothness to the fabric [23].

The most recent innovation is to use combinations of enzymes in detergents. Cellulases are used in combination with other enzymes like proteases and lipases. The combination of enzymes is used to increase efficiency on stain cleaning and fabric care. For instance, SaniZyme® is a four-enzyme liquid detergent containing lipase, cellulase, amylase, and protease. This is a bacteriostatic enzymatic detergent for the removal of blood, protein, mucous, fats, lipids, and carbohydrates from all types of endoscopic equipment and surgical instruments. Another example is Getinge Clean MIS Detergent® which is also a formulation which includes protease, lipase, amylase, and cellulase enzymes, surfactants, sequestering agents, and corrosion inhibitors (typical pH in use dilution 8) which is specifically designed to clean complex, minimal invasive instrumentation.

4.4 Agriculture

The application of cellulases in agriculture is usually reported in enhancement of crop growth and a control agent of plant diseases. For this purpose, combinations of cellulases, hemicellulases, and pectinases are broadly applied. Certain fungal cellulases are with the ability to degrade cell wall of plant pathogens. There are lots of details about application of bacteria such as plant growth-promoting rhizobacteria (PGPR) to improve plant performance. It is reported that these bacteria play a major role in reducing application of chemical fertilizers increasing plant development and also controlling potential plant pathogens and protecting plants from diseases. Moreover, many fungi including *Trichoderma* sp., *Geocladium* sp., *Chaetomium* sp., and *Penicillium* sp. enhance seed germination, support rapid plant growth, accelerate flowering, improve the root system and increase the crop yield. However, exact mechanisms behind these reactions are not yet clearly understood. But all these organisms have the ability to produce cellulase and related enzymes which may have a direct participation in these reactions. Some reports are about possible synergisms between bacterial cellulase production and bacterial antibiotic production against plant pathogenic fungi.

According to available information, it is evident that cellulolytic microorganisms are participating in many processes, viz., rhizosphere soil decomposition, increasing the availability of nutrient for the plant, controlling plant pathogens, facilitating root colonization, and penetration of cereal crops improving yields and nutritional contents. However, as there are no solid evidence to prove the mechanisms behind these, this area needs further research. The studies should be performed in order to characterize and improve applications of microbial cellulases in this field.

During traditional agriculture practices, especially in countries like Sri Lanka, farmers used to add straw and *Gliricidia* leaves like cellulosic materials into their fields. They observed that the incorporation of these types of plant material not only improved the quality of the soil but also increased the yield due to added nutrients. Therefore, it is obvious that in this type of processes cellulolytic microorganisms must have a direct contribution.

4.5 Medical applications

Medical pharmacology is currently a very active field of research that novel discoveries are coming into action. One such area is cellulases for development of medicine. By the way, humans are not cellulase producers, but the recent research on health and medicine reveals the benefits of consuming blends of enzymes including cellulase. As a result of global demand for enzyme blends, cellulase produced by the natural fermentation process of *Trichoderma reesei* and *Bacillus licheniformis* has been included in commercially available enzyme blends. This type of enzyme blends target collective digestion of cellulose-rich fibrous substances such as fruits and vegetables, cereals, legumes, bran, nuts and seeds, soy, dairy, healthy greens, sprouts, and herbs along with fats (lipids), sugars, proteins, carbohydrates, and gluten. One such example is VeganZyme®. Apart from that digestive aids (e.g., Digestin, P-A-L Plus Enzymes, Polyenzyme Plus, etc.) to treat people suffering from metabolic disorders are evolving as a promising strategy in medicine.

In some records, the direct and indirect applications of cellulase in medicine have been mentioned apart from using it as consumable enzyme blends.

4.5.1 Indirect applications of cellulases for medical purposes

Cellulase of fungal origin in combination with chitinases and lysozymes has a reported use in chitosan degradation. To obtain chitosan, a partial degradation of chitin must take place. As cellulose, chitin is a structural polysaccharide present in animals such as marine animals like shrimp and insect exoskeleton as well as participates in the formation of some parts of fungal cell walls. Chitin is a poly-ß-1,4-N-acetyl-D-glucosamine, conforming crystalline microfibrils. This polysaccharide provides structural integrity, stability, and protection to animals. Chitosan is the most important semicrystalline derivative form of chitin. This is obtained by partial deacetylation of chitin (around 50%, soluble in aqueous solution) under alkaline conditions or enzyme hydrolysis. Chitosan and its derivatives have many medical applications, viz., surgical sutures, bone rebuilding, production of artificial skin, anticoagulant, antibacterial agent, hemostatic dressings, anticancer and antidiabetic agents (in combination with metals), hypocholesterolemic effectors, elaboration of cosmetics, production of biopharmaceutics, and encapsulation of diverse materials [24, 25].

Apart from these applications, a lot of reports have been published on several studies which discuss how cellulases hydrolyze chitosan and their potential biomedical effects. For instance, antitumor activity of cellulase-treated chitosan [26] and antimicrobial activity of low-molecular-weight chitosan obtained by *Trichoderma* commercial enzymes could be discussed. However, the lack of solid evidence is a common issue on this area.

4.5.2 Direct applications of cellulases for medical purposes

A bezoar is a mass found trapped in the gastrointestinal system. Phytobezoars, as its name suggests, are composed of indigestible plant material (e.g., cellulose). In other words, it is a gastric concretion formed by vegetable fibers, seeds and skins of fruits, and sometimes starch granules and fat globules trapped inside the gastrointestinal tract. This is a common problem frequently reported in patients with impaired digestion and decreased gastric motility. Although sometimes surgeries are needed to remove these stagnated substances, certain minor conditions can be treated by cellulases. The common application reported is fungal cellulases. As there are no much reports about application of bacterial cellulases, research can be conducted to find out the application of potential cellulolytic bacterial cellulases to treat this condition. However, what is most important is that any of these individual enzymes or enzyme cocktails should not adversely affect the healthy body cells.

Another possible direct application of cellulase in medicine is degradation of cell walls of pathogenic organisms. *Acanthamoeba* is a Protista which causes a very rare as well as serious corneal infection which leads to blindness. This is simply called keratitis. This amoebic keratitis is an acute sight-threatening corneal infection associated with contact lens misuse [27]. This organism has two stages in its life cycle: the cyst and trophozoite. Both these structures are available when the eye is infected by this organism. The cyst wall resembles the plant cell wall. It is plausible that amoebae secrete cellulose because its cyst wall is primarily composed of cellulose. There is evidence which has been found to prove this matter about the presence of cellulose in [28]. Therefore, it is possible to apply cellulases in controlling the pathogen which causes this disease. Cellulases can be used to break off the cyst wall and control the pathogen. However, it needs a lot of research before using cellulase as a treatment for the eye.

Pathogenic microorganisms usually form biofilms. A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and is enclosed in an extracellular polymeric substance (EPS) matrix. Usually, pathogenic microorganisms form this type of assemblages. They may be found on a wide range of surfaces including living tissues and indwelling medical devices. Artificial hip prosthesis, central venous catheter, prosthetic heart valve, intrauterine device, and urinary catheter are some examples for indwelling medical devices that are commonly associated with biofilms. Most of the microorganisms produce extracellular polymeric substances composed of backbone structures that contain 1,3- or 1,4-β-linked hexose residues and tend to be more rigid, less deformable, and in certain cases poorly soluble. This is the exact linkages present in cellulose polymer. Therefore, cellulases can be further studied for their efficient application in removing this type of biofilms from medical devices.

4.6 Food and feed industry

4.6.1 Food processing industry

Food is essential for all living organisms to obtain nutritional support for their growth and well-being. The huge demand for food has laid a path to a very complex, interconnected global business that supplies most of the food consumed by the world's population.

Food biotechnology nowadays considers cellulases as an invaluable resource due to their increased applicability in a broad range of processes. Fruit and vegetable juice clarification, reducing the viscosity of nectars, concentrating purees, alteration of fruit sensory properties, carotenoid extraction, olive oil extraction, and the quality improvement of bakery products are among the various processes in food biotechnology that cellulase is exploited worldwide.

The cloudiness which is usually present in fruit and vegetable juices is a result of floating polysaccharide materials such as cellulose, hemicellulose, lignin, pectin, starch, metals, proteins, and tannins. The presence of these materials in the juice makes it low quality and draws less consumer demand. "Rapidase pomaliq" is a commercially available enzyme preparation composed of cellulase, hemicellulases, and pectinases obtained from *Trichoderma reesei* and *Aspergillus niger*. The application of this product in fruit juice clarification was beneficial to a considerable level. It is also reported that cellulase produced by bacteria such as *Bacillus* and *Paenibacillus* in combination with other enzymes such as pectinases and hemicellulases carries out the fruit and vegetable juice clarification. Apart from that, treatment of nectars and purees also found to be efficiently carried out by this enzymatic process. Rheological parameters such as viscosity of these products are brought down to a commercially acceptable level.

Modification of sensory parameters of food is another important area where application of cellulases is highly recommended. The aroma properties, flavor, and texture of fruits are some sensory properties which play a crucial role in food biotechnology. The infusions of pectinases and cellulase enzymes have been found to be effective in altering the sensory properties of fruits and vegetables [29].

These enzymes are also applied in degradation of grape fruit peels to release sugars. These sugars will be used in many industries including food production. Another important application of cellulase is extraction of phenolic compounds from grape pomace.

During extraction of olive oil, malaxing (mixing) is an indispensable step. This period allows the tiny oil droplets to attach with bigger ones and increase the oil yield which is coming from the olive paste. The use of cellulases alone or in combination with other hydrolytic enzymes like pectinases in this step has been found to have an enhancing effect on the extraction as well as the quality of olive oil. The enzymatic treatment of olive oil at the extraction stage causes significant enhancements in phenolic content and antioxidant activity of olive oil, thereby ultimately improving its quality.

The enzyme cocktails of cellulases with other hydrolytic enzymes such as amylases, proteases, and xylanase result in increased loaf volume, improvement in bread quality, and production of softer crumb. Enzyme cocktail containing cellulases, hemicellulases, amylases, lipases, and phospholipases results in dough conditioning with improvement of flavor, prolonged shelf life, and increase in volume after baking [30].

Another important application of cellulases is in pigment extraction from plants and plant products. Natural pigments such as carotenoids are nowadays earning a huge consumer demand as food colorants because of their natural origin, less toxicity, and availability of a wide range of colors. Brightly colored fruit peels such as orange, sweet potatoes, tomatoes, and carrot cell walls are rich in carotenoids. These are applicable as natural food colorants. The treatment of fruit peels with enzyme cocktails including cellulase leads to carotenoid extraction.

4.6.2 Animal feed industry

In animal feed production, cellulases are applied to enhance digestibility of cereal-based food and to increase nutritive values for a higher quality of forages. There are reports about efficiently using *Trichoderma* cellulase as feed additives for significant improvements in feed conversion ratio as well as digestibility of the cereal-based food [31]. Forage feed of ruminants is quite complex in composition containing cellulose, hemicellulose, pectin, and lignin. There is a suggestion that it is possible to use cellulase preparations to enhance digestibility of forage [32, 33]. Usually, cellulase from bacteria such as *Bacillus subtilis* is used in this feed production and nutritional enhancement processes. Enzyme preparations containing cellulases and hemicellulases are utilized for numerous activities like milk yield, body weight gain, and feed. Another aspect of treating animal feed with these enzyme mixtures is to remove anti-nutritional factors present in grains and other cellulosic materials.

Furthermore, cellulases play an important part in increasing the rate and extent of fiber digestion. This can be used as a positive effect on natural gastrointestinal processes of the ruminants. The ultimate result will be the increased availability of absorbable nutrients by digestibility enhancement of fodder.

The partial hydrolysis of lignocellulose materials also leads to better emulsification of food in the animal digestive tract resulting in ultimate improvement of nutrients availability.

5. Pretreatment methods of lignocellulosic biomass for bioethanol production

Energy is the life blood of the modern world. Fossil fuel, among all energy sources, holds the highest consumer demand. Since the industrial revolution, there is a drastic increase in fuel consumption. As fossil fuels are not renewable resources, the depletion of its natural deposits is inevitable. Therefore, we are in an era of limited and expensive energy. With the recent rise in fossil fuel prices, along with growing concern about its adverse effects on environment such as global warming caused by carbon dioxide emissions and subsequent climate change problems, biofuels have been gaining popularity. In our study area, we are focusing on bioethanol production using cellulolytic microorganisms as well as fermentative yeast using cellulose as the substrate.

Bioethanol is a renewable form of energy. Especially, second-generation bioethanol production is an emerging trend because of the abundance of low-cost raw materials. The largest potential feedstock for this purpose is lignocellulosic biomass, which includes materials such as agricultural residues (corn stover, crop straws, and bagasse), herbaceous crops (alfalfa, switch grass), short-rotation woody crops, forestry residues, waste paper, and other wastes (municipal and industrial). Lignocellulose is the most abundant renewable biomass. The yield of lignocellulose can reach approximately 200 billon metric tons worldwide per year [34]. Bioethanol production from these feedstocks has certain advantages. It is an attractive method of disposing those lignocellulosic materials which is the nonedible part of plants. Less production of pollutants makes it environmentally friendly. Most importantly, bioethanol production using lignocellulosic biomass does not create any food insecurity because it does not utilize any food crops before harvesting. Finally, it is abundant all around the year as a raw material.

However, the major drawback of this production process is associated with the structure of lignocellulosic biomass. It mainly consists of lignin, cellulose, and hemicellulose which collectively form a very stable structure. In order to release fermentable sugars from this substrate, it needs to undergo a pretreatment process to break open the stable structure. One of the main focuses of this chapter is to discuss about the possible biological pretreatment of lignocellulosic biomass with special references to the current studies conducted by scientists. It also includes a description about our own attempts in this particular area of research.

5.1 What is biological pretreatment?

Biomass contains about 40–50% of cellulose, a glucose polymer; 25–35% of hemicellulose, a sugar heteropolymer; 15–20% of lignin, a non-fermentable phenyl-propane unit; and lesser amounts of minerals, oils, soluble sugars, and other components. Biological pretreatment of lignocellulosic biomass uses the ligninolytic potential of certain microorganisms (fungi and bacteria and actinomycetes) to reduce the recalcitrant nature which is mainly caused by lignin component of the feedstock and enhance its digestibility by hydrolytic enzymes [35]. The breakdown of lignin barrier changes the structure of lignocellulose and enhances the access to the cellulose and hemicellulose carbohydrate components present.

5.2 Advantages and drawbacks of biological pretreatment

Biological pretreatment seems to be a promising approach due to its low capital cost, low energy, and little dependence of chemicals, mild environmental conditions, eco-friendly nature, and the absence of inhibitor generation during the process which affects in bioethanol production. Moreover, this process does not release any toxic materials or any toxic effluents to the environment.

However, there are few limitations in this strategy. The main drawback against the industrial scale application is the prolonged incubation time consumed to achieve the efficient delignification [36]. This is because of low hydrolysis rate of the microorganisms. Another possible drawback that comes into mind is the possible consumption of carbohydrates as well as fermentable sugar formed by the same microorganisms used to pretreat the material. This is possible to take place because most of the lignolytic microorganisms are producing cellulolytic enzyme batteries as well. Then, the substrate left for fermentative organisms will be minimal which could consequently lead to lower bioethanol yields. Therefore, it is essential to have poor cellulolytic microorganisms for delignification process.

To minimize this type of drawbacks in a biological way, it is possible to use cocultures or biofilms of efficient ligninolytic microorganisms. Introduction of fermentative yeast isolates into the same microbial coculture would also be a perfect approach. However, developing the most efficient microbial consortium is not that simple. Excessive laboratory-scale studies are required to understand the optimum physiological as well as biochemical parameter setup.

5.3 Microorganisms employed in biological pretreatment

Fungi are found to be more efficient in degrading lignocellulosic biomass. For instance, white-rot fungi, brown-rot fungi, and soft-rot fungi can be taken. The first two are basidiomycetes and soft-rot fungi are classified in ascomycete group.

Among these efficient ligninolytic microorganisms that have been studied so far, white-rot basidiomycete fungi are found to be more versatile in the process. Most research has been concentrated on species such as *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*) which is considered as the model organisms for lignin degradation as it completely mineralizes lignin to CO_2 and water. *Ceriporiopsis subvermispora*, *Phlebia subseralis*, *Pleurotus ostreatus*, and *Lentinus edodes* are also some other white-rot fungi that have been studied. This is thanks to the ligninolytic enzyme systems produced by these fungi. Lignin peroxidase, manganese peroxidase, and laccase are the major enzymes involved in this process. Lignin peroxidase and manganese peroxidase enzymes catalyze H_2O_2-dependent oxidation of lignin, while laccase which is a copper-containing enzyme catalyzes demethylation of lignin components. These are a set of high-redox potential oxidoreductases.

Recently, some bacterial laccases have also been characterized from *Azospirillum lipoferum*, *Bacillus subtilis*, etc. Unlike fungi, the bacteria are considered as low potential for lignin degradation. However, the three groups of bacteria, namely, actinomycetes, α-proteobacteria, and γ-proteobacteria, are known to have ligninolytic systems. Some actinomycetes were studied for their role in lignin biodegradation [37]. These degraded lignin into low-molecular-weight fragments. Some studies have shown potential of *Penicillium camemberti* for lignin degradation.

The biological pretreatment can be performed by growing the microorganism directly on the feedstock or using the enzyme extracts. Solid-state fermentation is the method of choice for biological delignification. Thus, from the reports available, it is evident that white-rot fungi and actinomycetes can be used to remove lignin from lignocellulosic substrates. However, further studies are required to shorten the incubation time and to optimize the delignification process.

5.4 Enhancement of biological pretreatment efficiency

The importance of enhancing enzymatic hydrolysis has been increased because of the urgent need for efficient biological pretreatment processes. For this purpose it is essential to search for high enzyme-producing organisms from the natural environment. Selecting the most effective strain and its culture conditions can make the process more efficient. Another important aspect is to find unique microbial communities for biological pretreatment. These communities can be called consortia. The efficient biodegradation of lignocellulosic biomass could be achieved by the synergistic action of various bacteria and fungi in a microbial consortium. There are a number of advantages in using a microbial consortium for biological pretreatment. The increase of adaptability, improved productivity, improved efficiency of enzymatic saccharification, control of pH during sugar utilization, and increase in substrate utilization are some of them. With the development of biotechnology and molecular biology, the production of hyperlignolytic mutants by genetic modification of wild-type species is one approach that could be studied further. Furthermore, complete understanding of the theoretical basis behind the mechanisms of actions of these hydrolytic enzyme systems is very useful in the process of enhancing hydrolytic efficiency.

Various process parameters affecting biological pretreatment like incubation temperature, incubation time, inoculums concentration, moisture, aeration, and conditions of pH have to be optimized. This must be done with well-planned laboratory-scale experiments. It is essential to pay attention to the microorganism used as well as the type of lignocellulosic material utilized because these parameters obviously change based on these two factors. Accessory enzymes are those enzymes which act on less abundant linkages found in plant cell walls. These include arabinases, lyases, pectinases, galactanases, and several types of esterases. Some studies have reported that addition of these accessory enzymes will improve hydrolysis efficiency.

Recently, several studies have been conducted in Sri Lanka on efficient lignocellulose-degrading microorganisms isolated from the natural environment. The effect of coculturing these fungal isolates for degradation of lignocellulosic material has also been reported. Coculturing of *Trichoderma* spp. with other cellulolytic fungi has found to improve the activity of lignocellulose-degrading enzymes compared to its monocultures [36]. In a different study, 18 basidiomycete isolates from the natural environment of Sri Lanka has been evaluated for their lignocellulose-degrading enzyme production. An *Earliella scabrosa* species with higher laccase activity (79,600 U/l) when cultured in 50 g/l rice bran has been reported [37]. Thus, it can potentially be used for industrial production of laccase using rice bran as a cheap carbon source for high laccase production.

6. Conclusions

The current progress in applications of cellulases is truly remarkable and attracting worldwide attention. It has already conquered the global market in an unbeatable way. Microbes are an attractive topic of interest for the production of cellulases due to their immense potential for cellulase production. However, it is apparent that more efficient species are still out there in the environment unnoticed by researchers. Further exploration and understanding of hidden mechanisms behind the activity of these enzymes are much more important. Microbial cellulases are preferred for their potential applications in a broad range of industries. Their ventures are expanding day by day. More and more researches are required to produce scientific knowledge to meet the growing demands for microbial cellulase. The advances in the emerging fields such as biotechnology, microbiology, and molecular biology will open up novel strategies to magnify the still-unlocked potentials of these enzymes. Eventually, it will be able to fine-tune the areas which still are dragging on the way to their utmost success.

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Conflict of interest

No conflicts of interest.

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