PREFACE

Cellulase refers to a class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the cellulolysis (or hydrolysis) of cellulose. The enormous potential that cellulases have in biotechnology is the driving force for continuous basic and applied research on these biocatalysts from fungi and bacteria. Cellulases are found in many fields, such as animal feeding, brewing and wine, food, textile and laundry, pulp and paper products. The growing interest toward the conversion of lignocellulosic biomass into fermentable sugars has generated an additional request for cellulases and their related enzymes. This book presents research in the study of cellulase, including biotechnological applications of microbial cellulases; using agro-industrial by-products as raw material for cellulase production; and the enzyme saccharification of cereal crop residues using dilute alkali pretreatment.

Chapter 1 - Cellulases (EC 3.2.1.4) catalyze the hydrolysis of 1,4-β-D-glucosidic linkages in cellulose, and play a significant role in nature by recycling this polysaccharide which is the main component of plant cell wall. Cellulases work in synergy with other hydrolytic enzymes in order to obtain the full degradation of the polysaccharide to soluble sugars, namely cellobiose and glucose, which are then assimilated by the cell.

The enormous potential that cellulases have in biotechnology is the driving force for continuous basic and applied research on these biocatalysts from Fungi and Bacteria. Nowadays, cellulases found application in many fields, such as animal feeding, brewing and wine, food, textile and laundry, pulp and paper industries. Moreover, the growing interest toward the conversion of lignocellulosic biomass into fermentable sugars has generated an additional request for cellulases and their related enzymes. In fact, bioconversion of biomass has significant advantages over other alternative energy production strategies because lignocellulose is the most abundant and renewable biomaterial on our planet. Bioconversion of lignocellulose is initiated primarily by microorganisms which are capable of degrading lignocellulosic materials. Several Fungi produce large amounts of extracellular cellulolytic enzymes, whereas bacterial and few anaerobic fungal strains mostly produce cellulolytic enzymes in a complex associated with the cell wall which is called "cellulosome". However, the heterogeneous and recalcitrant nature of lignocellulosic waste represents an obstacle for an efficient saccharification process, and pretreatment techniques are required to make the polysaccharide more accessible to the enzymatic action.

Thermostable enzymes can offer potential benefits in the hydrolysis of pretreated lignocellulosic substrates because the harsh conditions often required by several pretreatments can be harmful for conventional biocatalysts. The enhanced stability of thermostable enzymes

to high temperature and extreme operative parameters allows improved hydrolysis performance and increased flexibility compared to process configurations, all leading to enhancement of the overall economy of the process.

The present review gives an outline of several mesophilic and thermophilic cellulases from Fungi and Bacteria that have been characterized in the last years. Moreover, applications of these enzymes in some biotechnological fields, with particular regard to lignocellulosic biomass bioconversion, will be illustrated.

Chapter 2 - Cellulases, responsible for the hydrolytic cleavage of cellulose, are composed of a complex mixture of enzymes with different specificities to hydrolyse glycosidic bonds. Cellulases can be grouped into three major enzyme classes viz. endoglucanase, exoglucanase and β-glucosidase. Endoglucanases, often called carboxy methyl cellulases (CMCase), are proposed to initiate random attack at multiple internal sites in the amorphous regions of the cellulose fiber to open up sites for subsequent attack of cellobiohydrolases. Exoglucanase, better known as cellobiohydrolase, is the major component of the microbial cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyse highly crystalline cellulose. It removes mono-and dimers from the end of the glucose chain. β-glucosidase hydrolyse glucose dimers and in some cases cello-oligosaccharides to release glucose units. Generally, the endo- and exoglucanase work synergistically in cellulose hydrolysis but the underlined mechanism is still unclear. Microorganisms generally appear to have multiple distinct variants of endo- and exoglucanases. A diverse spectrum of cellulolytic microorganisms have been isolated and identified over the years and this list still continues to grow. Cellulases play a paramount role in natural carbon cycle by hydrolysing the lignocellulosic structures. Besides their applications in pharmaceutical industry, cellulases are also widely used in textile industry, in laundry detergents and in pulp and paper industry for various purposes. The cost of enzyme preparation is a major impediment in its commercial application. Recombinant DNA technology (RDT) and protein engineering have great potential for making significant improvements in increased production and higher specific activity of cellulases. The role of cellulases holds the key for transformation of organic wastes especially agricultural residue into biofuels through fermentation. although the process is at its infant stage, this is an important aspect for sustainable development.

Chapter 3 - Cellulases are well established in different industrial areas, and are currently the third largest industrial enzyme worldwide, by dollar volume, mainly because of their use in cotton processing and paper recycling, as detergent industry enzymes, and in juice extraction and animal feeding additives as well. Nowadays the cellulases are the most important enzyme group for studies aiming at the so called second generation ethanol production and others chemicals products. The cellulase group involves three different enzymes: β-1,4-endoglucanase (EC 3.2.1.4), β-1,4-exoglucanase (EC 3.2.1.91) and cellobiase (EC 3.2.1.21), that are produced by an array of microorganisms, including bacteria and fungi. For cellulase production economically viable the raw material needs to be cheap. There are many types of low cost carbon sources that could be used for cellulases production, such as sugar cane bagasse, sugar cane straw, wheat straw, wheat bran, corn cobs, etc, reducing the costs effects and being friendly environmentally. In this chapter, the importance of using agro-industrial by-products as raw material for cellulase production will be addressed, as well as its biotechnological application in industry.

Chapter 4 - Cellulose present in renewable lignocellulosic material is considered to be the most abundant organic substrate on earth for the production of hexoses and pentoses, for fuel and other chemical feed stock. Research on cellulase has progressed very rapidly in the past few decades, emphasis being on enzymatic hydrolysis of cellulose to hexose sugars. The enzymatic hydrolysis of cellulose requires the use of cellulase [1,4-(1,3:1,4)-β-D-glucan glucanohydrolase, EC 3.2.1.4], a multiple enzyme system consisting of endo-1,4,-β-D-glucanases [1,4-β-D-glucanases (CMCase, EC 3.2.1.4)], exo-1,4,-β-D-glucanases [1,4-β-D glucan cellobiohydrolase, FPA, EC 3.2.1.91] and β – glucosidase (cellobiase) (β-D-glucoside glucanohydrolase, EC 3.2.1.21). Major impediments to exploiting the commercial potential of cellulases are the yield, stability, specificity, and the cost of production. In the past few decades focus has been on submerged fermentation (SmF) and very little attention has been given to solid-state fermentation (SSF). SSF refers to the process whereby microbial growth and product fermentation occurs on the surface of the solid materials. This process occurs in the absence of "free" water, where the moisture is absorbed to the solid matrix. The direct applicability of the product, the high product concentration, lower production cost, easiest product recovery and reducing energy requirement make SSF a promising technology for cellulase production. This review highlights the research carried out on the production of cellulase in SSF using various lignocellulosic substrates, microorganisms, cultural conditions, process parameters (i.e., moisture content and water activity, mass transfer processes: aeration and nutrient diffusion, substrate particle size, temperature, pH, surfactant, etc), bioreactor design, and the strategies to improve enzyme yield. Also, the biotechnological potentials of microbial cellulases produced in SSF for bioconversion of agricultural wastes –providing a means to a "greener" technology, have been discussed.

Chapter 5 - Mild alkali pretreatment of lignocellulosic biomass is an effective pretreatment method which improves enzymatic saccharification. Alkaline pretreatment successfully delignifies biomass by disrupting the ester bonds cross-linking lignin and xylan, resulting in cellulose and hemicellulose enriched fractions. Here the authors report the use of dilute alkaline (NaOH) pretreatment followed by enzyme saccharification of cereal crop residues for their potential to serve as feedstock in the production of next-gen biofuels in Australia. Specifically, the authors discuss the impacts of varying pretreatment parameters on enzymatic digestion of residual solid materials. Following pretreatment, both solids and lignin content were found to be inversely proportional to the severity of the pretreatment process. Higher temperatures and alkali strength were also shown to be quintessential for maximising sugar recoveries from enzyme saccharifications. Essentially, pretreatment at elevated temperatures led to highly digestible material enriched in both cellulose and hemicellulose fractions. Increasing cellulase loadings and tailoring enzyme activities with additional β-glucosidases and xylanases delivered greater rates of monosaccharide sugar release and yields during saccharification. Sugar conversion efficiency of alkali treated sorghum and wheat straw residues following enzyme saccharification, approached 80 and 85%, respectively. Considering their abundance and apparent ease of conversion with high sugar yield, cereal crop residues are ideally suited for the production of second generation biofuels and/or use as feedstock for future biorefineries.

Chapter 6 - The plant cell wall consists of cellulose, hemicelluloses and pectin as well as the phenolic polymer lignin. Cellulose is the most abundant polysaccharide in nature and the major constituent of a plant cell wall providing its rigidity. Cellulose consists of β-1,4 linked D-glucose units that form linear polymeric chains of about from 8000 to 12000 glucose units.

In crystalline cellulose, these polymeric chains are packed together by hydrogen bonds to form highly insoluble structures. Hemicelluloses, the second most abundant polysaccharides in nature, have a heterogeneous composition of various sugar units. Hemicelluloses are usually classified according to the main sugar residues in the backbone of the polymer such as xylan, (galacto)glucomannan, arabinan, galactan found in cereals and hardwood, softwood and hardwood, The main chain sugars of hemicelluloses are modified by various side groups such as 4-O-methylglucuronic acid, arabinose, galactose, and acetyl, making hemicelluloses branched and variable in structure. Pectins are a family of complex polysaccharides containing a backbone of α-1,4 linked D-galacturonic acid. Pectins contain two different types of regions. In the region of pectin classified as a smooth region, D-galacturonic acid residues can be methylated or acetylated, whereas the region classified as a hairy one consists of two different structures, D-xylose substituted galacturonan and rhamnogalacturonan to which long arabinan and galactan chains are linked via rhamnose. The cellulose wall is strengthened by lignin, a highly insoluble complex branched polymer of substituted phenylpropane units joined together by carbon–carbon and ether linkages forming an extensive cross-linked network within the cell wall.

Chapter 7 - Cellulases are key industrial enzymes used to breakdown agriculture biomass to fermentable sugars. Cellulase has been used on the market as an industrial enzyme preparation and used as a main component of various products, such as detergents, fiber treating agents, paper pulp, additives for feed, and digestants. Cellulase is also used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Due to increasing environmental concerns and constraints being imposed on textile industry, cellulase treatment of cotton fabrics is an environmentally friendly way of improving the property of the fabrics. Furthermore, Cellulases are being used also in textiles for removing excess dye from denim fabric in pre-faded blue jeans (biostoning), also in removing the microfibirle which stick out from cotton fabrics after several washing. Restoring the softness and color brightness of cotton fabric could be achieved by using the cellulases. Cellulases can be used as a supplement in animal feed to decrease the production of fecal waste by increasing the digestibility of the feed. Cellulases can also be used to increase the efficiency of alcoholic fermentations (e.g., in beer brewing) by converting undigestible biomass into fermentable sugars. Ethanol is an alcohol made by fermenting and distilling simple sugars. As a result, ethanol can be produced from any biological feedstock that contains appreciable amounts of sugar or materials that can be converted into sugar such as starch or cellulose. Biofuels are liquid fuels produced from agriculture biomass using cellulases and other different enzymes. Agriculture biomass is available on a renewable or recurring basis, including agricultural crops and trees, wood and wood wastes and residues, plants (including aquatic plants), grasses, residues, fibers, and animal wastes, municipal wastes, and other waste materials. Biofuels (Types of biofuels include ethanol, biodiesel, methanol, and reformulated gasoline components) are primarily used as transportation fuels for cars, trucks, buses, airplanes, and trains. As a result, their principal competitors are gasoline and diesel fuel. Cellulase produced by the organisms isolated from Rumen Fluid of Cattle was used for biopolishing.

Cellulases are used also, in removing microbial slime in slime covered surfaces and maintaining a slime-free surface as in exposed cooling tower surfaces and in waste water treatment and paper making. This method comprises utilizing an enzyme blend in 2 to 100

parts per million (ppm) of cellulase, α-amylase and protease. Such enzyme blends have been found specifically to digest microbial slime and reduce microbial attachment and biofilm.

Chapter 8 - Cellulose is the most abundant component of plant biomass found in nature that is almost exclusively in plant cell walls, whereas it cannot be effectively converted into the usable sugars due to the lower cellulase activity. Although various classes of cellulase have been isolated and the synergism between them has been studied in detail, the thorough degradation of natural cellulose cannot be observed in the depolymerization by cellulase system, presumably it is due to the cellulase assay methods and substrates used for determining cellulases. Assay using cellulose as substrate is useful for assessing the potential enzyme system but it cannot be used for searching novel individual cellulase because the total activity is determined with such substrates. Considering that the natural cellulose can be degraded by living microbes whereas cannot by the secreted cellulases, the authors can conclude that there are the true cellulases degrading natural cellulases not to be isolated yet. It is obvious that the substrate is the vital determinant for cellulase assay and the key problem for seeking the true cellulase is how to obtain single cellulose chains. It is believed that the thoughtful substrate for cellulase assay should be amorphous cellulose molecule in the form of single chains.

Chapter 9 - Cellulases are celluloytic enzymes (EC 3.2.1.4) produced mainly by microbes including fungi, bacteria, and also by protozoans. However, plants and animals also produce cellulases. Several different kinds of cellulases differing in structure and mechanism of action are known. Cellulases catalyze the hydrolysis of 1, 4-beta-D-glycosidic linkages in cellulose, lichenin and cereal beta-D-glucans. Other names of cellulase are endoglucanase, endo-1,4-beta-glucanase, carboxymethyl cellulase (CMCase), endo-1,4-beta-D-glucanase, beta-1,4-glucanase, beta-1,4-endoglucan hydrolase and celludextrinase. Excocellulases and beta-glucosidases are other types of cellulases. Avicelase refers to the total cellulase activity of a given sample of the enzyme(s). The cellulase activity may be the consequence of the action of more than one type of enzymes.

Chapter 10 - To evaluate the synergism of cellulases from animal and microorganism, mixture of cellulases from snail (CES) and Trichoderma reesei (CET) was used to enzymatic hydrolysis and ethanol fermentation of lignocellulose. When the mixed cellulase was used to enzymatically hydrolyze Pennisetum hydridum, the optimal ratio of CES and CET was 3:1, and the glucose yield using the mixed enzyme was 100.3% and 50.2% higher than that produced individually by CES and CET, respectively. For ethanol fermentation of lignocellulose, the optimal ratio of CES and CET was 1:3, the ethanol yield using the mixed enzyme was 42.5% and 20.1% higher than that produced individually by CES and CET, respectively. Our results showed that mixed cellulase from animal and microorganism is a potential approach for improving enzymatic hydrolysis and ethanol fermentation of lignocellulose.

Chapter 11 - The importance of cellulases in the production of fuels from biomass makes understanding their catalytic mechanisms on crystalline cellulose important in order to design more active enzymes. Seven modular cellulases from Thermobifida fusca have been purified and characterized; of which, three inverting cellulases: endocellulase Cel6A, exocellulase Cel6B and processive endocellulase Cel9A have been studied extensively. Each one has an atypical catalytic mechanism: two Asp residues hold the nucleophilic water in Cel9A while no single catalytic base was found in the family-6 enzymes, suggesting that several residues might be involved in catalysis and form a network that functions as the catalytic base in these

enzymes. Site-directed mutagenesis and removal of domains demonstrate the important role of cellulose-binding modules in crystalline substrate hydrolysis and processivity. To investigate if independent enzymes could function effectively in a cellulosome, the catalytic domains of the two family-6 T. fusca cellulases were attached to dockerin domains and then the chimeric enzymes were used to form designer cellulosomes. Additionally, Cel6B enzymes have been fluorescence-labeled, providing another way to measure binding and processivity. These studies have created several enzymes with higher activity on crystalline cellulose; however, better strategies are necessary to produce more active engineered cellulases that will be able to lower the cost of cellulases for biomass hydrolysis.