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REVIEW

Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives

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Abstract In view of rising prices of crude oil due to increasing fuel demands, the need for alternative sources of bioenergy is expected to increase sharply in the coming years. Among potential alternative bioenergy resources, lignocellulosics have been identified as the prime source of biofuels and other value-added products. Lignocelluloses as agricultural, industrial and forest residuals account for the majority of the total biomass present in the world. To initiate the production of industrially important products from cellulosic biomass, bioconversion of the cellulosic components into fermentable sugars is necessary. A variety of microorganisms including bacteria and fungi may have the ability to degrade the cellulosic biomass to glucose monomers. Bacterial cellulases exist as discrete multi-enzyme complexes, called cellulosomes that consist of multiple subunits. Cellulolytic enzyme systems from the filamentous fungi, especially *Trichoderma reesei*, contain two exoglucanases or cellobiohydrolases (CBH1 and CBH2), at least four endoglucanases (EG1, EG2, EG3, EG5), and one β-glucosidase. These enzymes act synergistically to catalyse the hydrolysis of cellulose. Different physical parameters such as pH, temperature, adsorption, chemical factors like nitrogen, phosphorus, presence of phenolic compounds and other inhibitors can critically influence the bioconversion of lignocellulose. The production of cellulases by microbial cells is governed by genetic and biochemical controls including induction, catabolite repression, or end product inhibition. Several efforts have been made to increase the production of cellulases through strain improvement by mutagenesis. Various physical and chemical methods have been used to develop bacterial and fungal strains producing higher amounts of cellulase, all with limited success. Cellulosic bioconversion is a complex process and requires the synergistic action of the three enzymatic components consisting of endoglucanases, exoglucanases and β-glucosidases. The co-cultivation of microbes in fermentation can increase the quantity of the desirable components of the cellulase complex. An understanding of the molecular mechanism leading to biodegradation of lignocelluloses and the development of the bioprocessing potential of cellulolytic microorganisms might effectively be accomplished with recombinant DNA technology. For instance, cloning and sequencing of the various cellulolytic genes could economize the cellulase production process. Apart from that, metabolic engineering and genomics approaches have great potential for enhancing our understanding of the molecular mechanism of bioconversion of lignocelluloses to value added economically significant products in the future.

Keywords Lignocelluloses · Bioconversion · Cellulases · β-Glucosidase · Metabolic engineering

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Introduction

The amount of solar energy received at the earth's surface is 2.5×10^{21} Btu/year [1 British thermal unit (Btu) = 055.05585 joules], more than 12,000 times the present human requirement of 2.0×10^{17} Btu/year, and approximately 4,000 times the energy humans are projected to use

in 2050 [18]. The amount of energy from the sun which is stored as carbon via photosynthesis is 10 times the world usage. On a worldwide basis, terrestrial plants produce 1.3×10^{10} metric tons (dry weight basis) of wood per year, which has the energetic equivalent of 7×10^9 metric tons of coal or about two-thirds of the world's energy requirement. Available cellulosic feedstocks from agriculture and other sources are about 180 million tons per year [18].

The cost of various fermentation products (sugars, organic acids, tensides, glues, solvents or drink softeners etc.) largely depends on the cost of the carbohydrate raw material, and lignocellulosic residues from forests and agriculture still comprise the prominent carbohydrate source. Technologies need to be developed that are capable of handling a billion tons of biomass per year for the production of biofuels. According to the DOE-USDA Billion-Ton Study, corn stover and perennial crops such as switchgrass and hybrid poplar could provide about 1.3 billion tons of biomass by the mid-twenty-first century for utilization in bioenergy generation [87].

Estimated global wood consumption is around 3.5 billion metric tons/year, and has increased more than 65% since 1960. Wood and other lignocellulosics are composed of cellulose (insoluble fibres of β-1,4-glucan), hemicellulose (noncellulosic polysaccharides, including xylans, mannans, and glucans), and lignin (a complex polyphenolic structure). Wood in angiosperm trees generally contains 42–50% cellulose, 25–30% hemicelluloses, 20–25% lignin, and 5–8% extractives. This lignocellulosic pool is a major carbon sink in the forest ecosystems and accounts for roughly 20% of the terrestrial feed stock carbon storage, offering an enormous, renewable source of feedstock for biofuels production.

Agricultural resources of lignocellulosic waste are quite abundant as estimated by the Food and Agriculture Organization (FAO [25]), USA. Around 2.9×10^3 million tons from cereal crops and 1.6×10^2 millions tons from pulse crops, 1.4×10 million tons from oil seed crops and 5.4×10^2 million tons from plantation crops are produced annually worldwide [90]. Apart from the aforementioned lignocellulosic waste, approximately 6.0×10^2 million tons of harvestable palm oil biomass is being produced worldwide annually. However, only 10% of it is used as finished products such as palm oil and palm kernel oil. The remaining 90% (empty fruit bunches, fibres, fronds, trunks, kernels, palm oil mill effluent) is discarded as waste. The various types of lignocellulosic raw materials include wheat straw, rice straw, palm, corncobs, corn stems and husk etc. have varying amounts of cellulosic components. It has been estimated that the yearly biomass production of cellulose is 1.5 trillion tons, making it an essentially inexhaustible source of raw material for environmentally friendly and biocompatible products [54]. Therefore, the bioconversion of large amounts of lignocellulosic biomass into fermentable sugars has potential application in the area of bioenergy generation. Although extensive studies have been carried out to meet the future challenges of bioenegy generation, there is no self-sufficient process or technology available to convert the lignocellulosic biomass for bioenegy generation. The present review focuses on the processes or technologies currently under trial as well as their limitations. Possible future advances in the area of cellulosic bioconversion is also discussed.

Cellulose bioconversion

Cellulose is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1→4)-glycosidic bonds. Cellobiose is the smallest repetitive unit of cellulose and can be converted into glucose residues. The cellulose-hydrolysing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases, cellobiohydrolases (exoglucanases), and β-glucosidases. The endoglucanases catalyse random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasing cellobiose. β-glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose, for instance (Fig. 1).

Bioconversion of cellulose into fermentable sugars is a biorefining area that has invested enormous research efforts, as it is a prerequisite for the subsequent production of bioenergy. Sugars and starch comprise the feedstock for 90% of the produced ethanol today, but the most prevalent forms of sugar in nature are cellulose and hemi-cellulose. Lignocellulosic biomass can be converted to ethanol by hydrolysis and downstream fermentation processing. This process is much more complicated than just fermentation of C6 sugar [17] and is still far from being cost effective as compared to the production of bioethanol from starch or sugar crops. In hydrolysis, the cellulosic part of the biomass is converted into sugars, and fermentation converts these sugars to ethanol. Lignocellulosic biomass consists of 10–25% lignin, which contains no sugar, and therefore impossible to convert into sugars. Lignin is therefore a residue in ethanol production, and it represents a big challenge to convert it into a value-added product.

Hemicellulose conversion

Hemicellulose is the second most abundant renewable biomass and accounts for 25–35% of lignocellulosic biomass [98]. Hemicelluloses are heterogeneous polymers built up by pentoses (D-xylose, D-arabinose), hexoses (D-mannose,

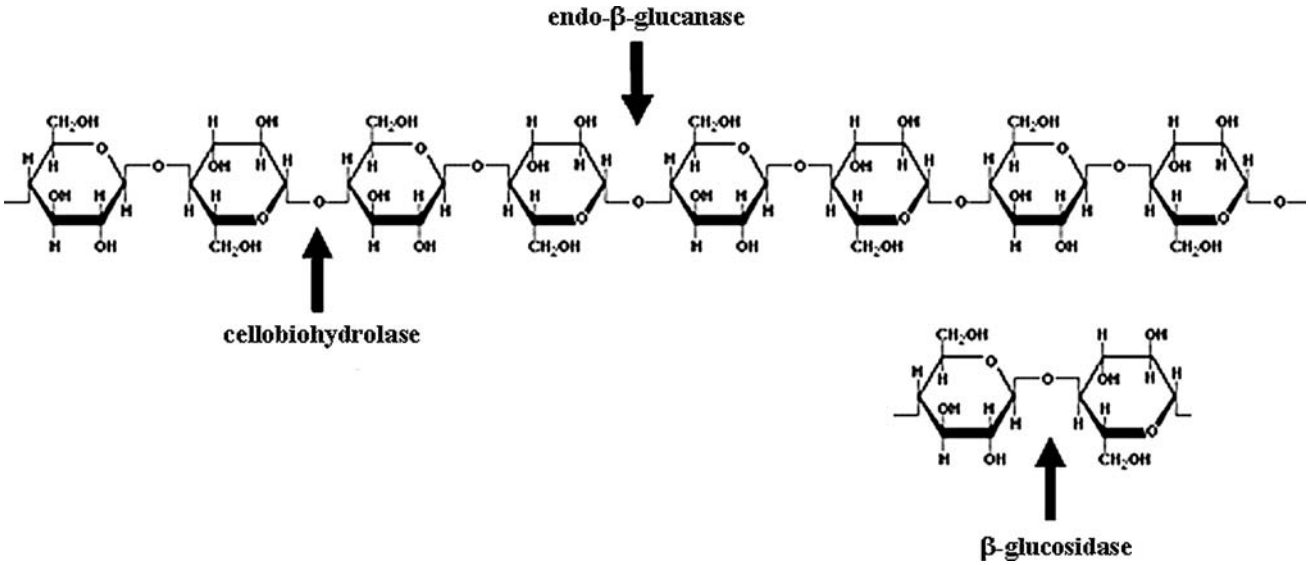

Fig. 1 Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and *β*-glucosidase

D-glucose, D-galactose) and sugar acids. Hemicelluloses in hardwood contained mainly xylans, while in softwood glucomannans are most common.

There are various enzymes responsible for the degradation of hemicellulose (Fig. 2). In xylan degradation, for instance, endo-1,4-*β*-xylanase, *β*-xylosidase, α-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase all act on the different heteropolymers available in nature. In glucomannan degradation, *β*-mannanase, and *β*-mannosidase cleave the polymer backbone. Like cellulose, hemicellulose is also an important source of fermentable sugars for biorefining applications. Xylanases are being produced and used as additives in feed for poultry and as additives to wheat flour for improving the quality of baked products at the industrial scale [80].

Conversion of pectins

Pectins are the third main structural polysaccharide group of plant cell walls, abundant in sugar beet pulp and fruits, e.g. citrus and apple fruit, where it can form up to half of the polymeric content of the cell wall [11]. The pectin backbone consists of homo-galacturonic acid

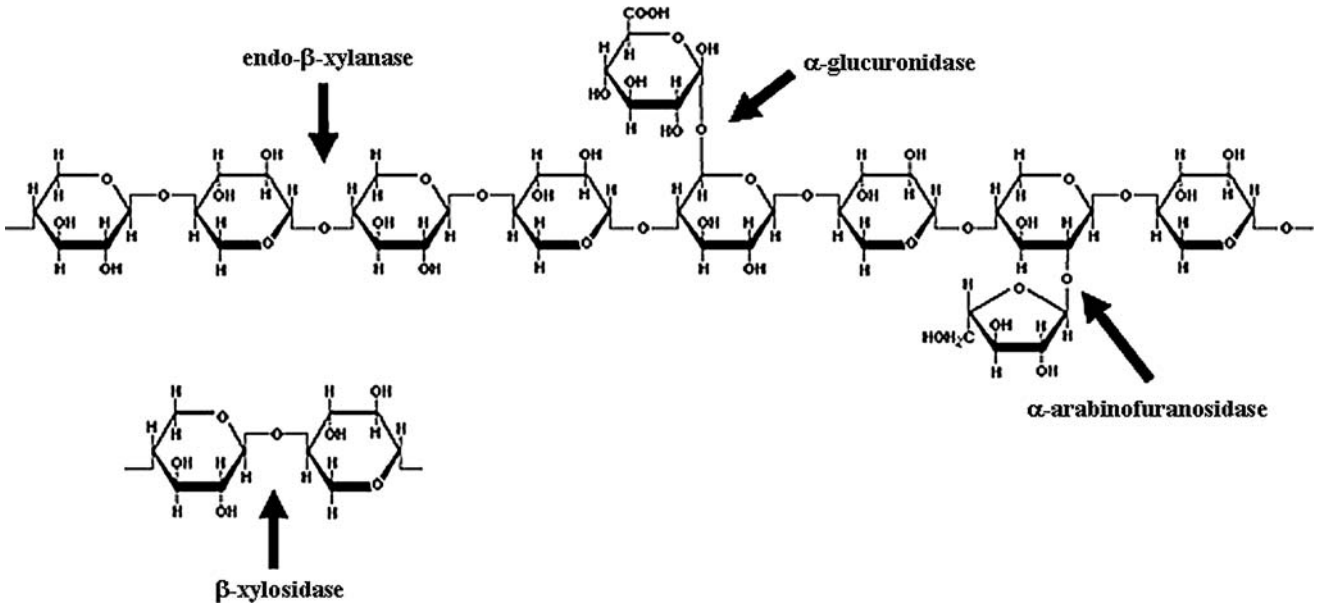

Fig. 2 Polymeric chemical structure of hemicellulose and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation

regions with neutral sugar side chains made from L-rhamnose, arabinose, galactose and xylose. L-rhamnose residues in the backbone carry sidechains containing arabinose and galactose. Pectin has found widespread commercial use, especially in the textile industry and in the food industry as a thickener, texturizer, emulsifier, stabilizer, filler in confections, dairy products, and bakery products, etc [65]. Despite these applications, pectins are similar to cellulose and hemicelluloses, common waste materials that can be converted to soluble sugars, ethanol, and biogas [21, 37]. Many enzymes are involved in pectin degradation (Fig. 3). They may be acting either by hydrolysis or by trans-elimination; the latter performed by lyases. Pectin-degrading enzymes i.e. polymethylgalacturonase, (endo-) polygalacturonase pectin depolymerase, pectinase, exopolygalacturonase, and exopolygalacturanosidase hydrolyse the polygalacturonic acid chain of the pectin polymer by the addition of a water molecule [41]. α-L-rhamnosidases hydrolyse rhamnogalacturonan in the pectic backbone. α-L-Arabinofuranosidases hydrolyse the L-arabinose side-chains, and endo-arabinase act on arabinan side-chains in pectin [112]. These two enzymes operate synergistically in degrading branched arabinan to yield L-arabinose. Polysaccharide lyases (PL) cleave the galacturonic acid polymer by β-elimination and comprise e.g. polymethylgalacturonate lyase (pectin lyase), polygalacturonate lyase (pectate lyase), and exopolygalacturonate lyase (pectate disaccharide-lyase).

Sources of cellulolytic enzymes

The search for potential sources of cellulolytic enzymes is continuing in the interest of successful bioconversion of lignocellulosic biomass. Although various microorganisms of bacterial as well as fungal origin have been evaluated for their ability to degrade cellulosic substrates into glucose monomers, relatively few microorganisms have been screened for their cellulase production potential [16, 125]. In addition, some microorganisms secrete either endoglucanase or β-glucosidase (components of cellulase complex). Only those organisms, which produce appropriate levels of endoglucanase, exoglucanase and β-glucosidase, would effectively be capable of degrading native lignocellulose. As discussed earlier, several strains of *Trichoderma* produce an extracellular cellulase complex degrading native cellulose [120]. Since then, many microorganisms have been isolated but only a few have been shown to produce adequate levels for their meaningful utilization [18, 67]. As, observed, all components of the extracellular cellulase complex (endogucanase, exoglucanase and β-glucosidase) are essential for cellulose hydrolysis and in general, β-glucosidase that catalyses cellobiose hydrolysis is either lacking or present in relatively small amounts in the extracellular cellulase complex. Thus, the sugars that are the end product of hydrolysis do not accumulate quickly, because cellobiose inhibits the endo and exoglucanases synthesis by feedback inhibition [7]. One of the ways to meet this deficiency is to add β-glucosidase to the reaction mixture

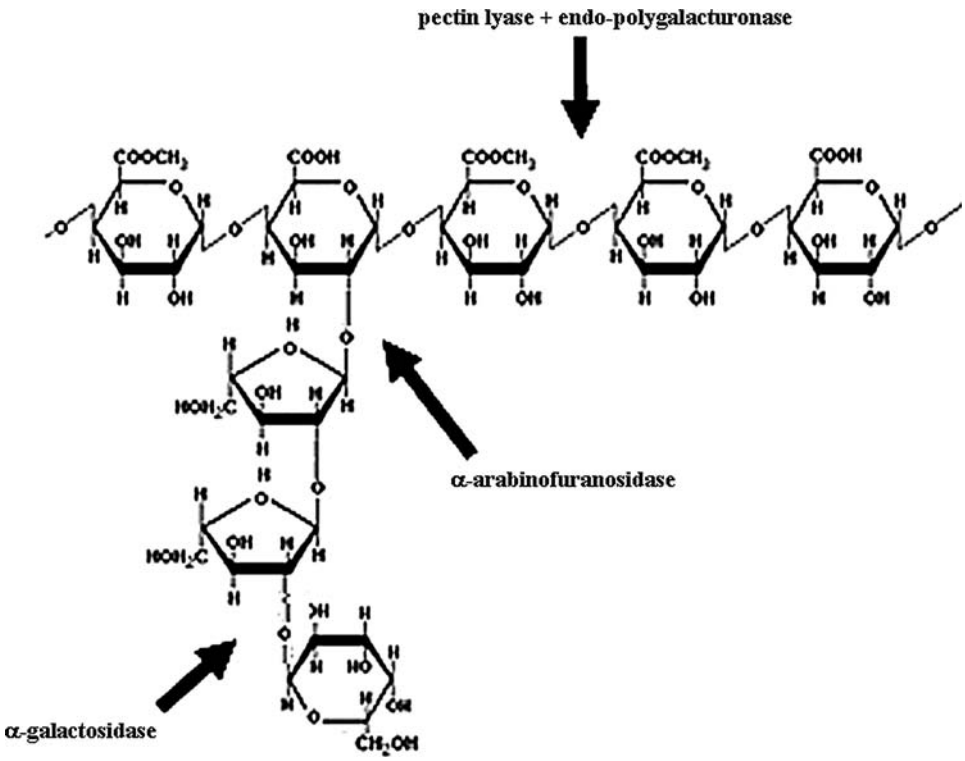

Fig. 3 Structure of pectin and enzymatic sites for pectin lyase, endo-β-polyglucturonase, α-arabinofuranosidase, α-galactosidase

containing other cellulase components. Another approach might be the design of a suitable bioreactor in which cellobiose is removed continuously from the reaction mixture and treated in a separate reactor to yield glucose.

The decay of lignocellulosic material catalysed by enzymes from cellulolytic fungi is of great significance in our ecosystem. Not surprisingly, these fungal cellulases have been the major subjects of major investigation over the years. The primary interest in fungal cellulases stems from the fact that several fungi produce extracellular cellulases in significant amounts. Like bacterial cellulases, fungal cellulases act synergistically with endoglucanases, exoglucanases and β-glucosidases for cellulosic hydrolysis [126]. Apart from the cellulolytic fungus *Trichoderma viride*, many other fungi produce cellulases and degrade treated cellulosic material or soluble cellulose derivatives such as carboxymethylcellulose. However, they are not very effective on crystalline cellulosic substrates. Besides *Trichoderma viride*, the other mesophilic strains producing cellulases are *Fusarium oxysporium*, *Piptoporus betulinus*, *Penicillium echinulatum*, *P. purpurogenum*, *Aspergillus niger* and *A. fumigatus*, have also been reported [73, 100, 104, 111, 114]. The cellulases from *Aspergillus* usually have high β-glucosidase activity but lower endoglucanase levels, whereas, *Trichoderma* has high endo and exoglucanase components but lower β-glucosidase levels, and hence has limited efficiency in cellulose hydrolysis. Thermophillic fungi such as *Sporotrichum thermophile*, *Scytalidium thermophillum Clostridium straminisolvens* and *Thermonospora curvata* also produce the cellulase complex and can degrade native cellulose [37, 48, 49]. Such thermophilic organisms may be valuable sources of thermostable cellulases.

Similarly, various bacterial strains have the ability to produce cellulase complexes aerobically as well as anaerobically. Some of the bacterial strains producing cellulases are *Rhodospirillum rubrum*, *Cellulomonas fimi*, *Clostridium stercorarium*, *Bacillus polymyxa*, *Pyrococcus furiosus*, *Acidothermus cellulolyticus*, and *Saccharophagus degradans* [16, 47, 113, 118].

Bacterial cellulosome: structure and function

Bacterial cellulases exist as discrete multi-enzyme complexes, called cellulosomes that consist of multiple subunits that interact with each other synergistically and degrade cellulosic substrates efficiently [6] (Fig. 4). The major components and their catalytic action are shown in Table 1.

The cellulosome is believed to allow concerted enzyme activity in close proximity to the bacterial cell, enabling optimum synergism between the cellulases presented on the cellulosome. Concomitantly, the cellulosome also minimizes the distance over which cellulose hydrolysis products must diffuse, allowing efficient uptake of these oligosaccharides by the host cell [99]. Cellulosome preparations from *C. thermocellum* are very efficient at hydrolyzing microcrystalline cellulose [61]. The cellulosome structure of *C. thermocellum* consists of a large non-catalytic scaffoldin protein (CipA) that is multi-modular [1]. It includes nine cohesins, four X-modules and cellulose binding module (CBM). The scaffoldin is anchored to the cell wall via type II cohesin domains. There are 22 catalytic modules such as 9 exhibit endoglucanase activity (CelA, CelB, CelD, CelE, CelF, CelG, CelH, CelN, and CelP), 4 exhibit exoglucanase activity (CbhA, CelK, CelO, CelS), 5 exhibit hemicellulase activity (XynA, XynB, XynV, XynY, XynZ), 1 exhibits chitinase activity (ManA) and 1 exhibits lichenase activity (LicB). These modules have dockerin moieties that can associate with the cohesins of the CipA protein to form the cellulosome [6] (Table 1).

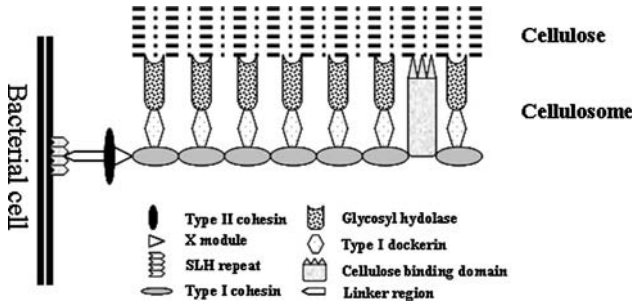

Fig. 4 Components of bacterial cellulosome structure and their adsorption pattern on cellulosic fibre

Table 1 Components of the cellulosome of *Clostridium thermocellum*

Cellulosome components	Description	Cellulosome components	Description
CipA (c)	Scaffoldin	XynA, XynU	Xylanase
CelJ	Cellulase	CelD	Endoglucanase
CbhA	Cellobiohydrolase	XynC	Xylanase
XynY	Xylanase	XynD	Xylanase
CelH	Endoglucanase	ManA	Mannanase
CelK	Cellobiohydrolase	CelT	Endoglucanase
XynZ	Xylanase	CelB	Endoglucanase
CelE	Endoglucanase	CelG	Endoglucanase
CelS (c)	Exoglucanase	CseP	Unknown
CelF	Endoglucanase	ChiA	Chitinase
CelN	Endoglucanase	CelA	Endoglucanase
CelQ	Endoglucanase	XynB, XynV	Xylanase
CelO	Cellobiohydrolase	LicB	Lichenase

Conversion of lignocellulosic biomass into value-added products

Bio-fuel

Over-utilization of Earth's available fossil energy (hydrocarbons) is a major challenge for the twenty-first century. Alternative energy sources based on sustainable, regenerative and ecologically friendly processes are important resources with which to address this challenge. Bioconversion energy products including ethanol, methane, hydrogen etc. are being considered as integral constituents of biofuels. Ethanol presently has the largest market due to its use as a chemical feedstock or as a fuel additive or primary fuel [51]. Ethanol constitutes 99% of biofuels in the USA [26]. The production of ethanol from sugars or starch impacts negatively on the economics of the process, thus making ethanol more expensive compared with fossil fuels. Hence, several attempts are being made for the production of ethanol using lignocellulosic materials to lower the production costs [26]. Various crop residues rich in lignocellulosics, like wheat straw, rice straw, corn cob, sunflower stalks, sunflower hulls and water-hyacinth have been exploited for ethanol production [81, 95, 101]. However, rapid and efficient fermentation of hydrolysates is limited because a range of inhibitory compounds in addition to monomeric sugars is generated during the hydrolysis of lignocellulosic materials.

Similarly, bio-methane has the potential to yield more energy than any other current type of bio-fuel (e.g. bio-diesel, bio-ethanol). Bio-methane can be produced from a wide range of conventional lignocellulosic biomass [3, 63]. The experimental evidences suggested that maize, wheat, rye, sunflower and other variety of lignocellulosic biomass can be utilized efficiently to produce biomethane [2]. For example, the typical yield of methane was observed to be 1,500 to 2,000 metric tons per hectare per year when maize was used as a lignocellulosic substrate. Methane yields of cereal crop wastes were achieved in a range from 3,200 to 4,500 metric ton per hectare per year. Apart from that other lignocellulosic materials obtained from sunflowers and alpine grass have also been reported as potential substrate for methane production (2,600–4,550 metric ton per hectare per year) [2]. Hydrogen has also been regarded as a viable energy option. It has been demonstrated that the indigenous microbes were capable of producing significant amounts of hydrogen by fermentation of aqueous hydrolysates of the steam-pretreated hemicellulosic fraction of corn stover [96].

Chemicals and other high-value bioproducts

Bioconversion of lignocellulosic biomass could make a significant contribution to the production of organic chemicals. Biomass-derived sugars can be readily fermented to fuel ethanol and commodity chemicals by the appropriate microbes. *B. coagulans* have been described that can ferment lignocellulosic hexoses and pentoses to lactic acid [85]. More than 75% of organic chemicals are produced from five primary base-chemicals: ethylene, propylene, benzene, toluene and xylene which are used to synthesize other organic compounds [36]. The aromatic compounds might be produced from lignin, whereas the low molecular mass aliphatic compounds can be derived from ethanol produced by fermentation of sugar generated from the cellulose and hemicellulose degradation. Vanillin and gallic acid are the two most frequently discussed monomeric potential products which have attracted interest [117]. Vanillin is used for various purposes including being an intermediate in the chemical and pharmaceutical industries for the production of herbicides, anti-foaming agents or drugs such as papaverine, L-dopa and the anti microbial agent, trimethoprim. It is also used in household products such as air-fresheners and floor polishes [117]. Hemicelluloses are of particular industrial interest because these are a readily available bulk source of xylose from which xylitol and furfural can be derived. Xylose produced from palm waste can be used for the production of xylitol [89]. Xylitol is used in place of sucrose in food as a sweetener, has odontological applications such as teeth hardening, remineralisation, and as an antimicrobial agent, plus it is used in chewing gum and toothpaste formulations [94]. Various bioconversion methods, therefore, have been explored for the production of xylitol from hemicellulose using microorganisms or their enzymes [82]. Furfural is used in the manufacture of furfural phenol plastics, varnishes and pesticides [77]. Glutamic acid produced from palm waste hydrolysate by fermentation process with high yield as compared to that produced from pure glucose as a carbon source [15]. Recently, conversion of lignocellulosic biomass into edible protein by *Pleurotus sajor-caju* has also been reported [9, 71].

Factors affecting cellulosic bioconversion

Physical factors

pH

Different physical parameters influence the cellulose bioconversion, and pH is an important factor affecting cellulase production [84]. The effect of pH on cellulase production was analysed using *Aspergillus niger*, and it was observed that pH 5.5 was optimal for maximum cellulase production. On the other hand the pH range of 5.5–6.5 was optimal for *β*-glucosidase production from *Penicillium*

rubrum [75]. Eberhart et al. [23] had reported that production and release of cellulase depended on the pH of the medium. His observations indicated that extracellular release of cellulase from *Neurospora crass* occurred at pH 7, whereas the enzyme remained accumulated in the cell at pH 7.5. Similarly, pH 7 was suitable for extracellular production of cellulase from the *Humicola fuscoatra* [91]. Further, the adsorption behaviour of cellulases was also affected by the pH of the medium. Kim et al. [53] had reported that maximum adsorption of cellulase from *Aspergillus phoenicus* occurred at pH of 4.8–5.5. The pH range 4.6–5.0 was found suitable for CMCase, FPase and β-glucosidase production with *Aspergillus ornatus* and *Trichoderma reesei* AYCC-26921 [78].

Temperature

Temperature has a profound effect on lignocellulosic bioconversion. The temperature for assaying cellulase activities are generally within 50–65 °C for a variety of microbial strains e.g. *Thielavia terrestris*-255, *Mycelieopthora fergussi*-246C, *Aspergillus wentii, Penicillum rubrum*, *Aspergillus niger, Aspergillus ornatus* and *Neurospora crassa* [75, 91, 110], whereas growth temperature of these microbial strains was found to be 25–30 °C [68]. Similarly, a native strain of *Penicillium purpurogenum*, *Pleurotus florida* and *Pleurotus cornucopiae* showed higher growth at 28 °C but maximum cellulase activities at 50 °C [110] and about 98, 59 and 76% of the CMCase, FPase and β-glucosidase activities, respectively, retained after 48 h at 40 °C. Temperature also has been shown to influence the cellulase adsorption. A positive relationship between adsorption and saccharification of cellulosic substrate was observed at temperature below 60°C. The adsorption activities beyond 60°C decreased possibly because of the loss of enzyme configuration leading to denaturation of the enzyme activity [115]. Bronnenmeier and Staudenbauer reported that extracellular as well as cell bound β-glulcosidase from *Clostridium stercorarium* required an identical temperature of 65°C for activity [10]. Further increase in the temperature led to a sharp decrease in the enzyme activity. Some of the thermophilic fungi, having maximum growth at or above 45–50 °C had produced cellulase with maximum activity at 50–78 °C [120].

Chemical factors

Carbon source

Many different substrates that are agro or industrial wastes, synthetic or naturally occurring have been evaluated as the carbon source for the process. Among the cellulosic materials, sulfite pulp, printed papers, mixed waste paper, wheat straw, paddy straw, sugarcane bagasse, jute stick, carboxymethylcellulose, corncobs, groundnut shells, cotton, ball milled barley straw, delignified ball milled oat spelt xylan, larch wood xylan, etc. have been used as the substrates for cellulase production [20, 30, 105].

The observations indicated that the production of cellulases increased with increase in substrate concentration up to 12% during solid state fermentation using *Aspergillus niger*. Further increase in substrate concentration resulted in decreased production levels. This might have been due to limitation of oxygen in the central biomass of the pellets, and exhaustion of nutrients other than energy sources. Similar to Menon et al. [75] and Steiner et al. [110] also demonstrated that carboxymethycellulose or cereal straw (1%, w/w) would be the best carbon source compared to sawdust for CMCase and β-glucosidase production using *Chaectomium globosum* as the cellulolytic agent.

Apart from that 3% malt extract or water hyacinth was found to be optimum for CMCase, FPase and β-glucosidase as observed with lactose as the additional carbon sources [78]. However, the saccharification of alkali-treated bagasse at higher substrate levels (up to 4% w/v) was also reported [105]. Interestingly, higher concentrations (2.5–6.2% w/v) of carbon source were observed to be suitable for maximum saccharification when cellobiose was supplemented into the medium containing delignified rice straw, news print or other paper wastes as substrates [44, 121].

Nitrogen source

The effect of different nitrogen sources such as ammonium sulfate, ammonium nitrate, ammonium ferrous sulfate, ammonium chloride and sodium nitrate have been studied. Among these, ammonium sulfate (0.5 g l^{-1}) led to maximum production of cellulases [106]. In contrast to this finding Menon et al. [75] observed a significant reduction in enzymatic levels in the presence of ammonium salts as the nitrogen source. However, an increase in the level of β-glucosidase was reported when corn steep liquor (0.8% v/v) was added. Corn steep liquor also resulted into a threefold to fivefold induction into endoglucanase and exoglucanase levels with synthetic cellulose (Sigma cell type-20), wheat straw and wheat bran as the substrates. Enzyme production was sensitive to corn steep liquor (0.88 g l^{-1}), and production increased significantly when mixed nitrogen sources (corn steep liquor and ammonium nitrate) were used [110]. However, additional incorporation of nitrogen sources into the medium scale up the cost of the process.

Phosphorus sources

Phosphorus is an essential requirement for fungal growth and metabolism. It is an important constituent of

phospholipids involved in the formation of cell membranes. Besides its role in effecting the linkage between nucleotides forming the nucleic acid strands, it is also involved in the formation of numerous intermediates, enzymes and coenzymes that are essential to the metabolism of carbohydrates, as well as for many other oxidative reactions and intracellular processes [106]. Different phosphate sources such as potassium dihydrogen phosphate, tetra-sodium pyrophosphate, sodium β-glycerophosphate and dipotassium hydrogen phosphate have been evaluated for their effect on cellulases production [28]. It has been widely demonstrated that potassium dihydrogen phosphate is the most favourable phosphorus source for cellulase production.

Phenolic compounds

The phenolic compounds have the ability to induce laccase that in turn stimulates the cellobiose-quinone-oxidoreductae enzyme; this enzyme possibly is involved in cellobiose (CMCase and FPase inhibitor) oxidation to cellobionic acid and thus effecting the cellulase synthesis indirectly [4, 43]. Among various phenolics, e.g. gallic acid, tannic acid, maleic acid, salicylic acid and β-nepthnol used, salicylic acid was observed to be a better inducer of cellulases [103]. Other phenolic compounds however, had shown an inhibitory effect. Mullar et al. [79] had also proposed a similar mechanism of cellulase regulation in *Trametes versicolor* in the presence of phenolics. His observations indicated that the vanillin had a stimulatory effect on cellulase biosynthesis and its regulation was possibly due to cellobiono-lactone formed by the interactions of laccase, phenol, cellobiose and cellobiose-quinone-oxidoreductase. This lactone seemed to influence cellulase production. Thus, cellobiose formed during cellulose hydrolysis was continuously withdrawn from the system by oxidation.

Sugars

Several investigations so far have indicated that cellulases are inducible enzymes, and different carbon sources have been analysed to find their role in effecting the enzymatic levels. Cellobiose (2.95 mM) may act as an effective inducer of cellulases synthesis in *Nectria catalinensis* [84]. An increased rate of endoglucanase biosynthesis in *Bacillus* sp. was reported in the presence of cellobiose or glucose (0.2%) added to the culture medium [86]. Xylanase biosynthesis was also induced by xylose or cellobiose added to the culture medium during growth. Yeoh et al. [124] had reported that cellobiose, gentibiose at higher concentration inhibited about 80% of the β-glucosidase activity; similarly, laminaribiose and glucose also led to a 55–60% inhibition in the enzymatic activity. Shiang et al. [102] described a possible regulation mechanism of cellulase biosynthesis and proposed that sugar alcohols, sugar analogues, xylose, glucose, sucrose, sorbose, cellobiose, methylglucoside etc. at a particular concentraton may induce a cellulose regulatory protein called cellulase activator molecule (CAM). The level and yield of CAM is possibly affected due to substrate concentration and some unknown factors imparted by moderators.

Limitations of lignocellulose bioconversion

The creation of a new industry on a large scale will require much basic and applied work on methods used to convert lignocellulose to value-added products, because several significant problems must be overcome to make the process ready for large-scale use [108]. Various serious obstacles in the utilization of lignocellulosic biomass have been explored and are being discussed as follows:

Crystallinity of cellulose

X-ray diffraction analysis revealed that cellulose exists in several crystalline forms [8]. The crystalline form is highly resistant to microbial and enzymatic degradation while amorphous cellulose is hydrolysed much faster. The rate of enzymatic hydrolysis of cellulose is greatly affected by its degree of crystallinity [14]. Dunlap et al. [22] had analysed the relationship between the cellulose crystallinity and its digestibility by cellulases. Cellulases degrade readily the accessible amorphous regions of regenerated cellulose but are unable to attack the less accessible crystalline region. Caulfied and Moore [12] measured the degree of crystallinity of the ball milled cellulose before and after partial hydrolysis and observed that mechanical action (ball milling) increased the susceptibility of both the amorphous and crystalline components of cellulose. Therefore, crystallinty of natural lignocellulosic is the major obstacle to its utilization to produce fermentable sugar economically.

Pretreatment of lignocellulosic material

The major obstacle in effective lignocellulose utilization is its crystalline unreactivity and in particular its resistance to hydrolysis. A wide spectrum of pretreatment protocols have been investigated for hydrolysis and only a few of these have been developed sufficiently to be called technologies [7, 53]. A variety of pretreatment procedures have been evaluated for their effectiveness towards cellulose biodegradation and possibly the suitability of pretreatment procedures may vary depending on the raw material selected.

Different chemical pretreatments that are generally practiced include sodium hydroxide, perchloric acid, peracetic acid, acid hydrolysis using sulfuric and formic acids, ammonia freeze explosion, and organic solvent e.g.

n-propylamine, ethylenediamine, *n*-butylamine etc. [72, 119]. Besides these, steam or acid/alkali-steam pretreatment have also been found suitable. However, utilization of various chemicals in the pretreatment procedures is a major drawback and affects the total economy of the bioconversion of the lignocellulosic biomass.

Physical treatment

Physical methods of pretreatment like ball milling, compression milling, cryomilling or attrition milling and steam treatment using poplar, wheat straw, newspaper, oat straw etc. [107, 119] will reduce particle sizes thereby increasing the available surface area for enzymatic attack. Steam explosion loosens the cellulose-hemicellulose-lignin complex and also removes the pentose while increasing the surface area. However, the drawback of the process is that steam treatment may generate certain cellulase inhibitors which can interfere with the enzymatic hydrolysis of the cellulosic substrate [33].

Biological delignification

Biological delignification is another interesting and alternative pretreatment, which utilizes white rot fungi that selectively, degrades lignin and leaves cellulosic biomass. The biological delignification of paddy straw, corn (zea mays), sugarcane bagasse and aspen wood has been attemped by *Cyathus* sp., *Streptomyces viridosporus*, *Phelebia tremellosus*, *Pleurotus florida* and *Peurotus cornucopiae* strain, respectively [13, 58]. Such processes have potential advantages such as low-capital cost, low-energy input and high yields without generating polluting byproducts. However, the long treatment time and degradation of the residual carbohydrates are some of the drawbacks of such processes.

Adsorption–desorption of cellulose

One of the significant aspects of cellulose hydrolysis is the adsorption of cellulolytic enzymes by the active components of the cellulosic substrates. A positive correlation between the adsorption of cellulase and the relative enzymatic hydrolysis of cellulose was observed by Klyosov et al. [55]. It has been shown that the available surface area of the cellulose polymer plays an important role in the interaction between the cellulases and the cellulose and is an essential step for the hydrolysis to proceed. It has also been demonstrated that cellulose strongly absorbs cellulases under optimal conditions for enzymatic action, and the extent of adsorption is proportional to the initial cellulose concentration. It was reported that 50% of endo and exoglucanase and ~80% of β-glucosidase was adsorbed on delignified bagasse and rice straw within 15 min of exposure of the substrates [29].

Analysis of various factors e.g. pH, ionic strength, temperature and surface area have indicated that maximum adsorption of cellulases on microcrystalline cellulose occurred at 50 °C and Vanderwall's interaction might be responsible for the adsorption phenomenon [45, 60]. However, Reinikainen et al. [92] had reported that maximum adsorption occurred at pH 6.5 and suggested that electrostatic repulsion between the bound proteins may regulate the level of adsorption. The binding of enzyme with cellulose was significantly affected by high-ionic strength suggesting that hydrophobic interaction may also contribute towards adsorption. Van-wyk [115] had demonstrated that the relative rate of adsorption and saccharification increases with temperature and showed the increase in adsorption at 60 °C while enzyme activity decrease.

Analysis of the adsorption behaviour of cellobiohydrolases indicated that cleavage of the cellulose binding domain of cellobiohydrolase-I led to a 76.5% decrease in the adsorption affinity at 25 °C and similarly a 20.7% decrease in the adsorption affinity for cellobiohydrolase-II [52]. The synergism between these two cellobiohydrolases may be due to formation of a partial complex between binding domain of CBH-I and core protein of CBH-II, which have higher adsorption affinities and tightness than those of the individual components. Recently, a linear relationship between the production of soluble sugar and the adsorption was observed for CBH [74]. Thus, the major problem to be overcome is the physiological conditions because maximum enzyme production was reported at ~30–35 °C temperature, pH 6.0. However, proper adsorption of the cellulases was reported at significantly higher temperature. This disparity represents a potentially significant limitation of the lignocellulosic conversion rate.

Biotechnological aspects of lignocellulose bioconversion

Since huge quantities of lignocellulosic biomass are available, its utilization to produce biofuel is reasonable. Using modern biotechnological approaches, the conversion of lignocellulosic biomass into commodity products is of fundamental significance. Due to various hurdles and technological gaps, sufficient utilization of renewable energy resources is still pending. However various methods or procedures to increase cellulase production are discussed subsequently.

Co-cultivation

Bioconversion of cellulosic substrates into first precursor products, such as glucose, is a complex process. It requires the synergistic action of all three enzymatic components i.e.

endo/exo and β-glucosidase. The ability of major cellulolytic members of microbial strains including fungi or bacteria identified so far produced limiting levels of one or the other enzymatic components. For assistance, *Trichoderma reesei*, a cellulolytic fungus, was reported to have lower levels of β-glucosidase, whereas, *Aspergillus niger* fungi have limited levels of the endoglucanase component [69, 70]. Therefore, attempts have been made to increase the levels of the enzymatic components either by genetic manipulation [59] or by co-cultivation approach [109]. Recently, co-cultivation of the cellulolytic organisms complementing the desired cellulolytic component has been attempted for achieving an increased rate of lignocellulosic bioconversion. *Trichoderma reesi* Qm 9123 and *Aspergillus niger* were co-cultured for cellulase production using paper mill sludge as a cellulosic substrate [70]. Similarly Gupte and Madamwar [31] cultivated *Aspergillus ellipticus* and A. *fumigatus* and reported improved hydrolytic activities as compared to separate cultures in a solid-state fermentation system. Improved enzyme levels were also achieved by Madamwar and Patel [69] when *Trichoderma reesei* was co-cultured with *Aspergillus niger* using bagasse, corncobs and saw dust, as the substrates in solid state fermentation.

Mutagenesis

Major producers of fermentation products extensively utilize mutation and selection. The production of cellulases by the microbial cell is regulated by genetic and biochemical controls that include induction and catabolite repression, or end product inhibition. These controls are operative under cellulase production conditions, thus resulting in limited yields of the enzymatic constituents. The first catabolite repressed *Bacillus pumilus* with cellulase yields four times higher than the wild type strain that was created through mutagenesis [56]. Mutagenic treatments of *Trichoderma reesei* Qm 6a, a wild type strain isolated at US Army Natick Research and Development Command, Natick, USA led to the development of mutants with higher cellulolytic activity [7]. A hypercellulolytic mutant NTG-19 from *Fusarium oxysporum* was developed by Kuhad et al. [59] by ultraviolet treatment followed by chemical mutagenesis using NTG (100 μg ml^{-1}). The resultant mutant strain had substantially higher (80%) cellulolytic activity than its parent strain. NTG treatment of *Cellulomonas flavigena* also produced four mutants (M4, M9, M11 and M12) with improved xylanolytic activities [93]. A mutant $creA^{d}30$ with the end product inhibition resistance and that showed improved levels of D-glucose metabolism was constructed from *Aspergillus nidulans* [116]. However, this effort did not result in robust strains that consistently produce ethanol at high yields under a broad range of conditions and in the hands of different investigators [67].

Genetic manipulation techniques

Engineering of cellulolytic microorganisms for cellulase production will benefit from the observations obtained over the past two decades pursuant to engineering of an end product metabolism in noncellulolytic anaerobes. Examples of these results include enhancement of ethanol production in *E. coli* and *K. oxytoca* [38], solvent production in *C. acetobutylicum* [76], and lactic acid production in yeasts [88]. In these and other cases, metabolic flux is altered by blocking undesirable pathways, typically via homologous recombination-mediated "gene knockout" [57] and/or overexpression of genes associated with desirable pathways [19, 32]. Various microbial strains have been metabolically engineered to produce lactic acid, succinic acid, ethanol and butanol [40, 62, 97]. *Corynebacterium glutamicum* was metabolically engineered to broaden its lignocellulosic substrate utilization for the production of fermentable sugar. Two recombinant *C. glutamicum* strains were also constructed by cloning the *Escherichia coli* gene *xylA* and *xylB* encoding xylose isomerase to enable the utilization of xylose as the sole carbon source [50].

While significant progress has been made using physical and chemical mutagens to increase production of lignocellulolytic enzymes, recombinant DNA technology and protein engineering are also being used as a powerful modern approach for efficient lignocellulosic bioconversion. Recombinant DNA technology offers significant potential for improving various aspects of lignocellulolytic enzymes such as production, specific activity, pH and temperature stability as well as creating "synthetic" designer enzymes for specific applications [34, 46]. It may also prove possible to fuse different lignocellulolytic genes or sections of genes from different organisms to produce novel chimeric proteins/enzymes with altered properties. For example, a heterologously expressed *Neocallimastrix patriciarum* CelD encoding a multi-domain, multi-functional enzyme possessing endoglucanase, cellobiohydrolase and xylanase activity exhibited higher specific activities on Avicel than cellobiohydrolase and endoglucanase of *T. reesei* [5]. A number of designer enzymes, also called glycosynthases, including cellulases and hemicellulases, have been engineered by replacing nucleophilic residues resulting in higher yields of different oligosaccharides [24].

Recombinant DNA technology can improve our understanding of the molecular mechanisms of lignocellulose degradation and the development of the bioprocessing potential of lignocellulolytic microorganisms. It can also aid the study of regulation and catalytic function of cellulases and ligninases, the nature of synergistic interactions

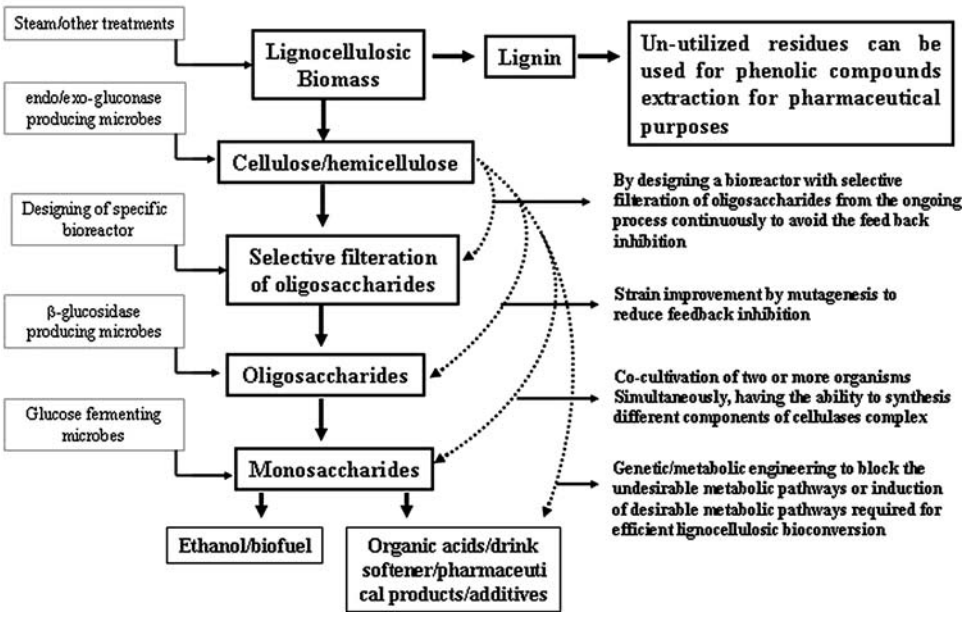

Fig. 5 Schematic presentation of novel integrated approaches for efficient lignocellulosic bioconversion into industrially significant products

among different enzymes and the development of economically feasible systems for the efficient conversion of waste biomass into value-added products. It is expected that for industrial applications, cellulases must have high adsorption capacities and catalytic efficiencies, high thermal stabilities and lower end product inhibition. It is therefore essential that efforts should be made for cloning cellulase genes with desirable molecular properties. A large number of fungal and bacterial genes have been cloned in *E. coli* in the recent years [27, 122, 123]. In addition, cellulase genes have also been expressed efficiently in other microbial systems such as *Penicillium crysogenum*, *Trichoderma reesei*, *Pseudomonas fluorescens* and yeast [34, 35, 64, 83]. The cloning and sequencing of the various cellulolytic genes will help in characterizing the potential systems for economizing the process of lignocellulosic conversion in future.

Genomics of cellulolytic microorganisms

The yeast *Pichia stipitis* can digest lignocellulose and can transform xylose (a component of lignocellulose) into ethanol [42]. Genome sequence analysis of *P. stipitis* revealed that the whole genome (15.4 Mb) contains 5,841 predicted genes, including a group of cellulases and xylanases and a number of genes encoding putative xylose transporters. Similarly, the *Phanerochaete chrysosporium* genome (30 Mb) was sequenced. The *P. chrysosporium* genome revealed an impressive array of genes encoding secretary oxidases, peroxidases and hydrolytic enzymes that cooperate in wood decay. Further analysis of the genome data could enhance our understanding of lignocellulose degradation [72]. The thermophilic soil bacterium *Thermobifida fusca* appears to produce extracellular glycoside hydrolases (cellulases and xylanases) capable of degrading all major plant cell wall polymers except lignin and pectin [66]. *T. fusca* has been identified as a source organism for isolating and studying multiple secreted cellulases and other carbohydrate-degrading enzymes. Using classical biochemical methods, six different cellulases have been identified from *T. fusca* including four endocellulase and two exocellulases genes [39]. Genome analysis of *T. fusca* revealed the existence of 29 putative glycoside hydrolases in addition to the previously identified cellulases and xylanases. Secreted cellulases have great biotechnological promise for the utilization of agricultural products and waste to produce sugars that can be subsequently converted to ethanol.

Conclusion and future perspectives

With the increasing demands for energy and the shrinking energy resources, the utilization of lignocellulosic biomass for the production of biofuel offers a renewable alternative. Apart from biofuels, other value-added products such as fermentable sugars, organic acids, solvents and drink softeners etc. may also be produced from lignocellulosic biomass using appropriate technologies. Theoretically this is all quite possible; however, technologically it is not an easy task because of various technological gaps. Morphological complexity and crystallinity of the lignocellulosic biomass is one of the major hurdles in the bioconversion processes. Cellulosic bioconversion is a multi-step process requiring a multi-enzyme complex for efficient bioconversion into fermentable sugars. However, there is no known organism capable of producing all the necessary enzymes

in sufficient quantities. Apart from that, physical and chemical conditions required for efficient enzymatic adsorption and hydrolysis of lignocellulosic biomass are somewhat different (i.e. higher temperature) than the optimum for enzyme biosynthesis. Most of the lignocellulose degrading organisms have end product inhibition which reduces the rate of enzyme synthesis resulting in incomplete utilization of lignocellulosic biomass. Various biotechnological approaches are being used for efficient biomass conversion with limited success. Co-cultivation of organisms has the ability to produce different components of cellulase complexes in adequate quantity but have been tried with only limited success due to induced end product/feedback inhibition. Therefore, to combat the problem, various mutant strains are being developed and used at the laboratory scale. Metabolic engineering including blocking of undesirable pathways and induction of gene expression associated with desirable pathways to enhance the production of biofuels and organic acids using lignocellulosic biomass is under progress. However, no single cost effective and efficient technology is currently available to meet the challenges of large-scale utilization of lignocellulosic biomass. Therefore, we suggest here an integrated approach (Fig. 5) including efficient bioreactor design, selection and optimization of physical and chemical conditions for several organisms may be used under co-cultivation conditions. Further, strain improvement for enhanced cellulases biosynthesis using mutagenesis, metabolic engineering and genomics approaches, should be used for the lignocellulosic bioconversion processes into a powerful technology to produce the value added and industrially significant products in future.

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