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Review

Economic potential of the bioprocessing of residual plant materials

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This review is written from the perspective of scientists working in lignocellulose bioconversion in a developing country and the aim of this review is to remind ourselves and other scientists working in related areas of lignocellulose research of the enormous economic potential of the bioprocessing of residual plant materials generally regarded as "waste", and secondly to highlight some of the modern approaches which potentially could be used to tackle one of the major impediments, namely high enzyme cost, to speed-up the extensive commercialisation of the lignocellulose bioprocessing.

Key words: lignocellulose, bioconversion, enzyme cost.

INTRODUCTION

Lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose and cellulose. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003). Large amounts of lignocellulosic "waste" (Table 1) are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries and they pose an environmental pollution problem. Sadly, much of the lignocellulose waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon (Levine, 1996). However, the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different value-added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients. Lignocellulytic enzymes also have significant potential applications in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture.

This review's main focus is to highlight significant aspects of lignocellulose biotechnology with emphasis on demonstrating the potential value from an application rather than basic research perspective. Aspects which will be reviewed in this article include: an overview of some of the major potential lignocellulose derived high-value bioproducts; solid state fermentation processing as a relevant, initial approach to lignocellulose bioconversion relevant for developing countries; some back-ground on lignocellulolytic organisms and their enzymes, and finally looking at cost of enzymes and potential of modern approaches which could be employed to reduce cost.

POTENTIAL BIOPRODUCTS AND THEIR APPLICATIONS

Biomass can be considered as the mass of organic material from any biological material, and by extension,

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Table 1. Types of lignocellulosic materials and their current uses.

Lignocellulosic material	Residues	Competing use
Grain harvesting		
Wheat, rice, oats barley and corn	Straw, cobs, stalks, husks,	Animal feed, burnt as fuel, compost, soil conditioner
Processed grains		
Corn, wheat, rice, soybean	Waste water, bran,	Animal feed
Fruit and vegetable harvesting	Seeds, peels, husks, stones, rejected whole fruit and juice	Animal and fish feed, some seeds for oil extraction
Fruit and vegetable processing	Seeds, peels, waste water, husks, shells, stones, rejected whole fruit and juice	Animal and fish feed, some seeds for oil extraction
Sugar cane other sugar products	Bagasse	Burnt as fuel
Oils and oilseed plants Nuts, cotton seeds, olives, soybean etc.	Shells, husks, lint, fibre, sludge, presscake, wastewater	Animal feed, fertiliser, burnt fuel
Animal waste	Manure, other waste	Soil conditioners
Forestry-paper and pulp		
Harvesting of logs	Wood residuals, barks, leaves etc.	Soil conditioners, burnt
Saw-and plywood waste	Woodchips, wood shavings, saw dust	Pulp and paper industries, chip and fibre board
Pulp & paper mills	Fibre waste, sulphite liquor	Reused in pulp and board industry as fuel
Lignocellulose waste from communities	Old newspapers, paper, cardboard, old boards, disused furniture	Small percentage recycled, others burnt
Grass	Unutilised grass	Burnt

any large mass of biological matter. A wide variety of biomass resources are available (Table 1) on our planet for conversion into bioproducts. These may include whole plants, plant parts (e.g. seeds, stalks), plant constituents (e.g. starch, lipids, protein and fibre), processing byproducts (distiller's grains, corn solubles), materials of marine origin and animal byproducts, municipal and industrial wastes (Smith et al., 1987). These resources can be used to create new biomaterials and this will require an intimate understanding of the composition of the raw material whether it is whole plant or constituents, so that the desired functional elements can be obtained for bioproduct production.

There are some excellent and comprehensive literature (Bhat, 2000; Sun and Cheng, 2002; Wong and Saddler, 1992a,b; Beauchemin et al., 2001, 2003; Subramaniyan and Prema, 2002; Beg et al., 2001) available on the different potential bioproducts and their many applications but only a few of the high-value products will be reviewed.

Chemicals

Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals. Over 75% of organic chemicals are produced from five primary base-chemicals: ethylene, propylene, benzene, toluene and xylene which are used to synthesis other organic compounds, which in turn are used to produce various chemical products including polymers and resins (Coombs, 1987). 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. Table 2 shows estimations

of total demands for chemicals which could be made by fermentation.

Table 2. Annual production of chemicals which could potentially be made from fermentation (modified from Coombs, 1987).

Products	World demand (thousand of tonnes)
Ethanol	16 000
Acetone	1659
Butanol	1400
Glycerol	414
Acetic acid	2539
Citric acid	300
Fumaric acid	60

Bio-fuel

The demand for ethanol has the most significant market where ethanol is either used as a chemical feedstock or as an octane enhancer or petrol additive. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050 (Campbell and Laherrere, 1998). Brazil produces ethanol from the fermentation of cane juice whereas in the USA corn is used. In the US, fuel ethanol has been used in gasohol or oxygenated fuels since the 1980s. These gasoline fuels contain up to 10% ethanol by volume (Sun and Cheng, 2002). It is estimated that 4540 million litres of ethanol is used by the US transportation sector and that this number will rise phenomenally since the US automobile manufacturers plan to manufacture a significant number of flexi-fueled engines which can use an ethanol blend of 85% ethanol and 15% gasoline by volume (Sun and Cheng, 2002). 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 the technology development focus for the production of ethanol has shifted towards the utilisation of residual lignocellulosic materials to lower production costs.

Other high-value bioproducts

Currently a number of products such as organic acids, amino acids, vitamins and a number of bacterial and fungal polysaccharides such as xanthan are produced by fermentation using glucose as the base substrate but theoretically these same products could be manufactured from “lignocellulose waste”. Based upon the predicted catabolic pathway and the known metabolism of *Phanerochaete chrysosporium* of lignin, Ribbons (1987) presented a detailed discussion of the potential value-added products which could be derived from lignin. Vanillin and gallic acid are the two most frequently discussed monomeric potential products which have attracted interest. Vanillin extraction from *Vanilla* pods costs between $ 1200 to $4000 per kilogram whereas synthetic vanillin costs less than $15 per kilogram (Walton et al., 2003). 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 (Walton et al., 2003). The high price and limited supply of natural vanillin have necessitated a shift towards its production from other sources (Priefert et al., 2001).

Hemicelluloses are of particular industrial interest since these are a readily available bulk source of xylose from which xylitol and furfural can be derived. Xylitol used instead of sucrose in food as a sweetner, has odontological applications such as teeth hardening, remineralisation, and as an antimicrobial agent, it is used in chewing gum and toothpaste formulations (Roberto et al., 2003; Parajó et al., 1998). The yield of xylans as xylitol by chemical means is only about 50-60% making xylitol production expensive. Various bioconversion methods, therefore, have been explored for the production of xylitol from hemicellulose using microorganisms or their enzymes (Nigam and Singh, 1995). Furfural is used in the manufacture of furfural-phenol plastics, varnishes and pesticides (Montané et al., 2002). Over 200 000 tones of furfural with a market price of about $1700 per ton (Montané et al., 2002) is annually produced (Zeitch, 2000).

Enzymes

Cellulases and hemicellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Bhat, 2000; Sun and Cheng, 2002; Wong and Saddler, 1992a,b; Beauchemin et al., 2001, 2003). It is estimated that approximately 20% of the >1 billion US dollars of the world’s sale of industrial enzymes consists of cellulases, hemicellulases and pectinases and that the world market for industrial enzymes will increase in the range of 1.7-2.0 billion US dollars by the year 2005 (Bhat, 2000).

In the baking industry xylanases are used for improving desirable texture, loaf volume and shelf-life of bread. A xylanase, Novozyme 867, has shown excellent performance in the wheat separation process (Christopherson et al., 1997). Hemicellulases are used for pulping and bleaching in the pulp and paper industry where they are used to modify the structure of xylan and

Table 3. Lignocellulose contents of common agricultural residues and wastes.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0
S32 rye grass (early leaf)	21.3	15.8	2.7
S32 rye grass (seed setting)	26.7	25.7	7.3
Orchard grass (medium maturity)	32	40	4.7
Grasses (average values for grasses)	25-40	25-50	10-30

NA, data not available.

glucomannan in pulp fibres to enhance chemical delignification (Suurnäkki et al., 1997). A patented Lignozyme® process is effective in delignifying wood in a pilot pulp- and paper process (Call and Mücke, 1997). In bio-pulping where lignocellulytic enzymes were used the following was achieved: tensile, tear and burst indexes of the resultant paper improved, brightness of the pulp was increased and an improved energy saving of 30-38% was realised (Scott et al., 1998). Laccases can degrade a wide variety of synthetic dyes making them suitable for the treatment of wastewater from the textile industry (Rosales et al., 2002). Organisms such as the white rot fungi producing lignases could be used for the degradation of persistent aromatic pollutants such as dichlorophenol, dinitrotoluene and anthracene (Gold and Alic, 1993).

There is a huge potential market for fibre- degrading enzymes for the animal feed industry and over the years a number of commercial preparations have been produced (Beauchemin et al., 2001, 2003). The use of fibre-degrading enzymes for ruminants such as cattle and sheep for improving feed utilisation, milk yield and body weight gain have attracted considerable interest. Steers fed with an enzyme mixture containing xylanase and cellulase showed an increased live-weight gain of approximately 30-36% (Beauchemin et al., 1995). In dairy cows the milk yield increased in the range of 4 to 16% on various commercial fibrolytic enzyme treated forages (Beauchemin et al., 2001).

DEGRADATION OF LIGNOCELLULOSE

Lignocellulose consists of lignin, hemicellulose and cellulose and Table 3 compiled from Betts et al. (1991); Sun and Cheng, (2002) shows the typical compositions of the three components in various lignocellulosic materials. Because of the difficulty in dissolving lignin without destroying it and some of its subunits, its exact chemical structure is difficult to ascertain. In general lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl and p-coumaryl). In addition, grass and dicot lignin also contain large amounts of phenolic acids such as p-coumaric and ferulic acid, which are esterified to alcohol groups of each other and to other alcohols such as sinapyl and p-coumaryl alcohols. Lignin is further linked to both hemicelluloses and cellulose forming a physical seal around the latter two components that is an impenetrable barrier preventing penetration of solutions and enzymes.

Hemicellulose macromolecules are often polymers of pentoses (xylose and arabinose), hexoses (mostly mannose) and a number of sugar acids while cellulose is a homogenous polymer of glucose.

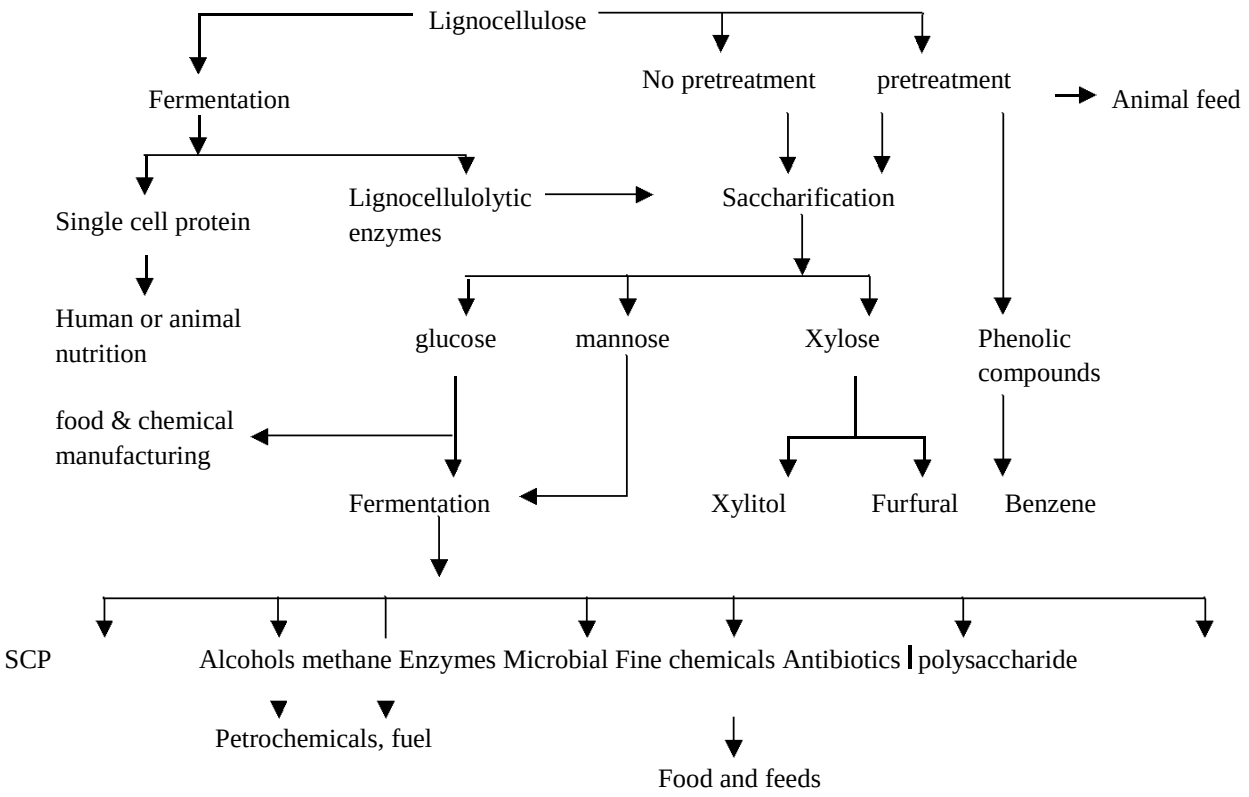

Figure 1. Generalised process stages in lignocellulose bioconversion into value-added bioproducts.

Of the three components, lignin is the most recalcitrant to degradation whereas cellulose, because of its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose. Alkaline (Chahal, 1992) and acid (Nguyen, 1993; Grethlein and Converse, 1991) hydrolysis methods have been used to degrade lignocellulose. Weak acids tend to remove lignin but result in poor hydrolysis of cellulose whereas strong acid treatment occurs under relatively extreme corrosive conditions of high temperature and pH which necessitate the use of expensive equipment. Also, unspecific side reactions occur which yield non-specific by-products other than glucose, promote glucose degradation and therefore reduce its yield. Some of the unspecific products can be deleterious to subsequent fermentation unless removed. There are also environmental concerns associated with the disposal of spent acid and alkaline. For many processes enzymes are preferred to acid or alkaline processes since they are specific biocatalysts, can operate under much milder reaction conditions, do not produce undesirable products and are environmentally friendly.

Bioprocessing of lignocellulosic materials

Technologies are currently available for all steps in the bioconversion of lignocelluloses to ethanol and other chemical products. However, these technologies must be improved and new technologies developed to produce renewable biofuel and other bioproducts at prices which can compete with current production costs. The feedstock costs can be minimized by initially focusing on agricultural residues and waste materials. Other process steps, which are particularly expensive, include pretreatments to improve the bioconversion, the production of enzymes for depolymerization of the complex raw materials and capital costs associated with bioconversions.

In general the technology of bioprocessing of raw materials or their constituents into bioproducts entails three steps: process design, system optimization and model development. Processing involves the use of biocatalysts, whole microorganisms or their enzymes or enzymes from other organisms to synthesize or bioconvert raw materials into new products; recover/purify such bioproducts and subsequently any needed downstream modifications.

Bioconversions of lignocellulosic materials to useful, higher value products normally require multi-step processes (Figure 1) which include: (i) pretreatment (mechanical, chemical or biological) (Grethlein and Converse, 1991; Grethlein, 1984), (ii) hydrolysis of the polymers to produce readily metabolizable molecules (e.g. hexose or pentose sugars), (iii) bio-utilization of these molecules to support microbial growth or to produce

chemical products and (iv) the separation and purification (Smith et al., 1987). To date, the production of cellulase and other lignocellulosic enzymes have been widely studied in submerged culture processes in the laboratory, ranging from shake flask to 15 000-l fermentations (Haltrich et al., 1996; Kim et al., 1997; Xia and Len, 1999). A stirred-tank reactor, widely used for the production of cellulose and other lignocellulosic enzymes, is known to have shear problems which rupture mycelial cells and may deactivate the enzymes (Wase et al., 1985). Alternative bioreactors such as the air-lift or bubble-column, which have a lower shear stress, seem to produce better results. For example, studies on cellulase and xylanase production by *A. niger* in various bioreactors showed that in general, better yield and productivity were shown in a bubble-column and an air-loop air-lift than in the stirred-tank reactor (Kim et al., 1997). However, the relatively high cost of enzyme production has hindered the industrial application of the enzymatic process (Xia and Len, 1999).

It has been reported that the solid state fermentation (SSF) is an attractive alternative process to produce fungal microbial enzymes using lignocellulosic materials from agricultural wastes due to its lower capital investment and lower operating cost (Chahal et al., 1996; Haltrich et al., 1996; Jecu, 2000) . SSF process, for the reasons stated, will be ideal for developing countries. Solid-state fermentations are characterized by the complete or almost complete absence of free liquid. Water, which is essential for microbial activities, is present in an absorbed or in complexed-form with the solid matrix and the substrate (Cannel and Moo-Young, 1980). These cultivation conditions are especially suitable for the growth of fungi, known to grow at relatively low water activities. As the microorganisms in SSF grow under conditions closer to their natural habitats they are more capable of producing enzymes and metabolites which will not be produced or will be produced only in low yield in submerge conditions (Jecu, 2000). SSFs are practical for complex substrates including agricultural, forestry and food-processing residues and wastes which are used as carbon sources for the production of lignocellulolytic enzymes (Haltrich et al., 1996). Compared with the two-stage hydrolysis-fermentation process during ethanol production from lignocellulosics, Sun and Cheng (2002), reported that SSF has the following advantages: (1) increase in hydrolysis rate by conversion of sugars that inhibit the enzyme (cellulase) activity; (2) lower enzyme requirement; (3) higher product yield; (4) lower requirement for sterile conditions since glucose is removed immediately and ethanol is produced; (5) shorter process time; and (6) less reactor volume. In a recent review (Malherbe and Cloete, 2003) reiterated that the primary objective of lignocellulose treatment by the various industries is to access the potential of the cellulose encrusted by lignin within the lignocellulose matrix. They expressed the opinion that a combination of SSF technology with the ability of an appropriate fungus to selectively degrade lignin will make possible industrial-scale implementation of lignocellulose-based biotechnologies.

Like all technologies, SSF has its disadvantages and these have received the attention by Mudgett (1986). Problems commonly associated with SSF are heat build-up, bacterial contamination, scale-up, biomass growth estimation and control of substrate content. However, the process has been used for the production of many microbial products and the engineering aspects and the scale-up will depend on bioreactor design and operation (Lonsane et al., 1992). A recent technical report in 2002 (http://www.lgu.umd.edu/project/outline.cfm?trackID=1934) on “The Science and Engineering for a Bio-based Industry and Economy” has adequately discussed some of the strategies in lignocellulose bio-conversion processes. Other lignocellulose bioprocessing strategies include anaerobic treatment, composing, production of single cell protein for ruminant animal feeding and mushroom cultivation. These processes have been extensively reviewed (Smith et al., 1987) and will not be further discussed in this review.

MICROORGANISMS AND THEIR LIGNOCELLULYTIC ENZYMES

A diverse spectrum of lignocellulolytic microorganisms, mainly fungi (Baldrian and Gabriel, 2003; Falcón et al., 1995) and bacteria (McCarthy, 1987; Zimmermann, 1990; Vicuň a, 1988) have been isolated and identified over the years and this list still continues to grow rapidly. Already by 1976 an impressive collection of more than 14 000 fungi which were active against cellulose and other insoluble fibres were collected (Mandels and Sternberg, 1976). Despite the impressive collection of lignocellulolytic microorganisms only a few have been studied extensively and mostly *Trichoderma reesei* and its mutants are widely employed for the commercial production of hemicellulases and cellulases (Esterbauer et al., 1991; Jørgensen et al., 2003; Nieves et al., 1998). This is so, partly because *T. reesei* was one of the first cellulolytic organisms isolated in the 1950’s and because extensive strain improvement and screening programs, and cellulase industrial production processes, which are extremely costly, have been developed over the years in several countries. *T. reesei* might be a good producer of hemi-and cellulolytic enzymes but is unable to degrade lignin. The white-rot fungi belonging to the basidiomycetes are the most efficient and extensive lignin degraders (Akin et al., 1995; Gold and Alic, 1993) with *P. chrysosporium* being the best-studied lignin-degrading fungus producing copious amounts of a unique set of lignocellulytic enzymes. *P. chrysosporium* has drawn considerable attention as an appropriate host for the

Table 4a. List of fungi with the highest specific activity (µmol.min^{-1}.mg^{-1}) for lignases.

Enzyme	Organism	Substrate	Specific activity	Opt. Temp (^{O}C)	Opt. pH
manganese peroxidase	*Stropharia coronilla*	Mn^{2+} + H^{+} + H_2O_2	692	25	NA
Laccase	*Botrytis cinerea*	1,2,4-benzenetriol + O_2/1-naphthol + O_2/2-naphthol + O_2/3,5-dimethoxy-hydroxy-benzaldazine + O_2/4,5-dimethyl-o-phenylenediamine + O_2/4-amino-N,N'-dimethylaniline + O_2/4-methylcatechol + O_2/ascorbate + O_2/caffeic acid + O_2/catechol + O_2/ferrocyanide + O_2/gallic acid + O_2/guaiacol + O_2	5778	55	4
Diarylpropane peroxidase (ligninase)	*Phanerochaete chrysosporium*	1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2/1-(3,4-diethoxyphenyl)-1,3-dihydroxy-2-(4-methoxy-phenyl)propane + O_2 + H_2O_2/1-(4-ethoxy-3-methoxyphenyl)-1,2-propene + O_2 + H_2O_2/1-(4-ethoxy-3-methoxyphenyl)propane + O_2 + H_2O_2/2-keto-4-thiomethylbutyric acid + H_2O_2/3,4-dimethoxybenzyl alcohol + H_2O_2	28	23/37	3/4.5

Table 4b. List of bacteria with the highest specific activity (µmol.min^{-1}.mg^{-1}) for cellulases.

Enzyme	Organism	Substrate	Specific activity	Opt. temp (^{O}C)	Opt. pH
mannan endo-1,4-β-mannosidase	*Bacillus subtilis*	Galactoglucomannan/glucomannans/ mannans	514	50-60	5-7
cellulase	*Clostridium thermocellum*	Avicel/carboxymethylcellulose/cellulose cellopentaose/cellotetraose/cellotriose/	428	75	7
1,3-β-glucan glucohydrolase	*Streptomyces murinus*	laminarin	6.7	50	6
1,3-1,4-β-D-glucan glucanohydrolase	*Bacillus macerans*	β-D-glucan/ lichenan	5030	60-65	6
1,3-β-D-glucan glucanohydrolase	*Bacillus sp.*	3-O-β-D-Glc-D-Glc-D-Glc-D-Glc/ laminarin	369.6	60	9

production of lignin-degrading enzymes or direct application in lignocellulose bioconversion processes (Ruggeri and Sassi, 2003; Bosco et al., 1999). Less known, white-rot fungi such as *Daedalea flavida*, *Phlebia fascicularia*, *P. floridensis* and *P. radiate* have been found to selectively degrade lignin in wheat straw and hold out prospects for bioconversion biotechnology were the aim is just to remove the lignin leaving the other components almost intact (Arora et al., 2002). Less prolific lignin-degraders among bacteria such as those belonging to the genera *Cellulomonas*, *Pseudomonas* and the actinomycetes *Thermomonospora* and *Microbispora* and bacteria with surface-bound cellulase-complexes such as *Clostridium thermocellum* and *Ruminococcus* are beginning to receive attention as representing a gene pool with possible unique lignocellulolytic genes that could be used in lignocellulase engineering (Vicuña, 1988; McCarthy, 1987; Miller (Jr) et al., 1996; Shen et al., 1995; Eveleigh, 1987). Table 4, compiled from the Brenda Enzyme Data Base (http://www.brenda.uni-koeln) shows the microorganisms with the highest specific activity under the appropriated conditions.

It is conventional to consider lignocellulose-degrading enzymes according to the three components of lignocellulose (lignin, cellulose and hemicellulose) which they attack but bearing in mind such divisions are convenient classifications since some cross activity for these enzymes have been reported (Kumar and Deobagkar, 1996). The exact mechanism by which lignocellulose is degraded enzymatically is still not fully understood but significant advances have been made to gain insight into the microorganisms, their lignocellulolytic genes and various enzymes involved in the process.

Lignases

Identifying lignin degrading microorganisms has been hampered because of the lack of reliable assays, but significant progress has been made through the use of a ^{14}C-labelled lignin assay (Freer and Detroy, 1982). Fungi breakdown lignin aerobically through the use of a family of extracellular enzymes collectively termed “lignases”. Two families of lignolytic enzymes are widely considered

Table 4c. List of fungi with the highest specific activity (µmol.min^{-1}.mg^{-1}) for cellulases.

Enzyme	Organism	Substrate	Specific activity	Opt. temp. (^{O}C)	Opt. pH
mannan endo-1,4-β-mannosidase	*Sclerotium rolfsii*	Galactoglucomannan/ galactomannans/ glucomannans/ mannans	475	72 - 74	3.3
cellulase	*Aspergillus niger*	Carboxymethylcellulose/ cellohexaose/ cellopentaose/ cellotetraose/ cellotriose/ cellulose	194	70	5
1,3-β-glucan glucohydrolase	*Achlya bisexualis*	Glucan/ laminarin/ neutral glucan/ phosphoglucan	7840	30	6
1,3-1,4-β-D-glucan glucanohydrolase	*Orpinomyces sp.*	β-D-glucan/ lichenin	3659	45	5.8
1,3-β-D-glucan glucanohydrolase	*Rhizopus chinensis*	β-glucan	4800	NA	NA
1,6-β-D-glucan glucanohydrolase	*Penicillium brefeldianum*	β-glucan/ gentiobiose/ pachyman	405	50	4.2

Table 4d. List of bacteria with the highest specific activity (µmol.min^{-1}.mg^{-1}) for hemicellulases.

Enzyme	Organism	Substrate	Specific activity	Opt. temp. (^{O}C)	Opt. pH
Feruloyl esterase	*Clostridium stercorarium*	Ethyl ferulate	88	65	8
Endo-1,4-β-xylanase	*Bacillus pumilus*	B-1,4-D-xylan	1780	40	6.5
β-1,4-xylosidase	*Thermoanaerobacter ethanolicus*	o-nitrophenyl-β-D-xylopyranoside	1073	93	6
Exo-β-1,4-mannosidase	*Pyrococcus furiosus*	p-nitrophenyl-β-D-galactoside	31.1	105	7.4
Endo-β-1,4-mannanase	*Bacillus subtilis*	Galactoglucomannan/ glucomannans/mannan	514	50	5/7
Endo-α-1,5-arabinanase	*Bacillus subtilis*	1,5-α-L-arabinan	429	60	6/8
α-L-arabinofuranosidase	*Clostridium stercoarium*	alkyl-α-arabinofuranoside/ aryl-α-arabinofuranoside/ L-arabinogalactan/ L-arabinoxylan/ methylumbelliferyl-α-L-arabinofuranoside	883	70	5
α-Glucuronidase	*Thermoanaerobacterium saccharolyticum*	4-O-methyl-glucuronosyl-xylotriose	9.6	50	6
α-Galactosidase	*Escherichia coli*	raffinose	27350	60	6.8
Endo-galactanase	*Bacillus subtilis*	arabinogalactan	1790	48	6
β-Glucosidase	*Bacillus polymyxa*	4-nitrophenyl-β-D-glucopyranoside	2417	NA	NA
Acetyl xylan esterase	*Fibrobacter succinogenes*	Acetylxylan/ α-naphthyl acetate	2933	47	7

to play a key role in the enzymatic degradation: phenol oxidase (laccase) and peroxidases (lignin peroxidase (LiP) and manganese peroxidase (MnP) (Krause et al., 2003; Malherbe and Cloete, 2003). Other enzymes whose roles have not been fully elucidated include H_2O_2-producing enzymes: glyoxal oxidase (Kersten and Kirk, 1987), glucose oxidase (Kelley and Reddy, 1986), veratryl alcohol oxidases (Bourbonnais and Paice, 1988), methanol oxidase (Nishida and Eriksson, 1987) and oxido-reductase (Bao and Renganathan, 1991). Enzymes involved in lignin breakdown are too large to penetrate the unaltered cell wall of plants so the question arises, how do lignases affect lignin biodegradation. Suggestions are that lignases employ low-molecular, diffusible reactive compounds to affect initial changes to the lignin substrate (Call and Mücke, 1997).

Hemicellulases

Hemicellulose is a collective term referring to those polysaccharides soluble in alkali, associated with cellulose of the plant cell wall, and these would include non-cellulose β-D-glucans, pectic substances (polygalacturonans), and several heteropolysaccharides such as those mainly consisting of galactose (arabinogalactans), mannose (galactogluco-and glucomannans) and xylose (arabinoglucurono-and glucuronoxylans). However, only

Table 4e. List of fungi with the highest specific activity (µmol.min^{-1}.mg^{-1}) for hemicellulases.

ENZYME	Organism	Substrate	Specific activity	Opt. temp. (^{o}C)	Opt. pH
Feruloyl esterase	*Aspergillus niger*	Methyl sinapinate	156	55	5
Endo-1,4-β-xylanase	*Trichoderma longibrachiatum*	1,4-β-D-xylan	6630	45	5
β-1,4-xylosidase	*Aspergillus nidulans*	p-nitrophenyl-β-D-xylopyranoside	107.1	50	5
Exo-β-1,4-mannosidase	*Aspergillus niger*	β-D-Man-(1-4)-β-D-GlcNAc-(1-4)-β-D-GlcNAc-Asn-Lys	188	55	3.5
Endo-β-1,4-mannanase	*Sclerotium rolfsii*	Galactoglucomannan/mannans galactomannans/glucomannans/	380	72-74	2.9/3.3
Endo-α-1,5-arabinanase	*Aspergillus niger*	1,5-α-L-arabinan	90.2	50-55	4.5-5.0
α-L-arabinofuranosidase	*Aspergillus niger*	1,5-α-L-arabinofuranohexaose/ 1,5-α-L-arabinotriose/ 1,5-L-arabinan/ α-L-arabinofuranotriose	396.6	50-60	3.4-4.5
α-Glucuronidase	*Phanerochaete chrysosporium*	4-O-methyl-glucuronosyl-xylobiose	4.5	50	3.5
α-Galactosidase	*Mortierella vinacea*	melibiose	2000	60	4
Endo-galactanase	*Aspergillus niger*	NA	6593	50-55	3.5
β-glucosidase	*Humicola insolvens*	(2-hydroxymethylphenyl)-β-D-glucopyranoside	266.9	50	5
Acetyl xylan esterase	*Schizophyllum commune*	4-methylumbelliferyl acetate/ 4-nitrophenyl acetate	227	30	7.7

the heteropolysaccharides, those with a much lower degree of polymerisation (100-200 units) as compared to that of cellulose (10000-14000 units), are referred to as hemicelluloses. The principal sugar components of these hemicellulose heteropolysaccharides are: D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, D-galacturonic acid, and to a lesser extent, L-rhamnose, L-fucose, and various O-methylated sugars.

Rabinovich et al. (2002a) and Shallom and Shoham, (2003) present recent reviews covering the types, structure, function, classification of microbial hemicellulases. For details on the catalytic mechanism and structure of glycoside hydrolases, refer to other articles (Withers, 2001; Rabinovich et al., 2002b).

Hemicellulases like most other enzymes which hydrolyse plant cell polysaccharides are multi- domain proteins (Henrissat and Davies, 2000; Prates et al., 2001). These proteins generally contain structurally discrete catalytic and non-catalytic modules. The most important non-catalytic modules consist of carbohydrate binding domains (CBD) which facilitate the targeting of the enzyme to the polysaccharide, interdomain linkers, and dockerin modules that mediate the binding of the catalytic domain via cohesion-dockerin interactions, either to the microbial cell surface or to enzymatic complexes such as the cellulosome (Shallom and Shoham, 2003; Prates et al., 2001). Based on the amino acid or nucleic acid sequence of their catalytic modules hemicellulases are either glycoside hydrolases (GHs) which hydrolyse glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyse ester linkages of acetate or ferulic acid side groups and according to their primary sequence homology they have been grouped into various families (Henrissat and Bairoch, 1996; Rabinovich et al., 2002a,b) (Table 5). A comprehensive classification of hemicellulases and other hydrolases is available at http://afmb.cnrs-mrs.fr/CAZY and Table 5 shows the various hemicellulases, the substrate they hydrolyse and their classification into families.

Xylan is the most abundant hemicellulose and xylanases are one of the major hemicellulases which hydrolyse the β-1,4 bond in the xylan backbone yielding short xylooligomers which are further hydrolysed into single xylose units by β -xylosidase. Most known xylanases belong to the GH 10 and 11 families and β-xylosidases are diustributed in families 3, 39, 43, 52 and 54. Bifunctional xylosidase-arabinosidase (Lee et al., 2003) enzymes are found mainly in families 3, 43 and 54. β-mannanases hydrolyse mannan-based hemicellulose and liberate short β-1,4-manno-oligomers which can further be hydrolysed to mannose by β-mannosidases. About 50 mannases are found in GH families 5 and 26, and about 15 β-mannosidase in families 1, 2 and 5. α-L-Arabinofuranosidases and α-L-arabinanases hydrolyse arabinofuranosyl-containing hemicellulose, and are distributed in GH families 3, 43, 51, 54 and 62. Some of these enzymes exhibit broad substrate specificity, acting on arabinofuranoside moieties at O-5, O-2 and /or O-3 bonds as a single substituent, as well as from O-2 and O-3 doubly substituted xylans, xylooligomers and arabinans (Saha, 2000). Other xylanases are α-D-glucuronidases which hydrolyse the α-1,2-glycosidic bond of the

Table 5. The major hemicellulases and their classification.

Enzymes	Substrates	EC number	Family
Exo-β-1,4-xylosidase	β-1,4-Xylooligomers xylobiose	3.2.1.37	GH 3 39 43 52 54
Endo- β-1,4 xylanase	β-1,4-Xylan	3.2.1.8	GH 5 8 10 11 43
Exo- β-1,4-mannosidase	β-1,4-Mannooligomers mannobiose	3.2.1.25	GH 1 2 5
Endo- β-1,4-mannanase	β-1,4-Mannan	3.2.1.78	GH 5 26
Endo-α-1,5-arabinanase	α-1,5-Arabinan	3.2.1.99	GH 43
α – L-arabinofuranosidase	α-Arabinofuranosyl (1→ 2) or (1→ 3) xylooligomers α-1,5-arabinan	3.2.1.55	GH 3 43 51 54 62
α-Glucuronidase	4-O-Methyl- α-glucuronic acid (1→2) xylooligomers	3.2.1.139	GH 67
α-Galatosidase	α-Galactopyranose (1→6) mannooligomers	3.2.1.22	GH 4 27 36 57
Endo-galactanase	β-1,4-Galactan	3.2.1.89	GH 53
β-Glucosidase	β-Glucopyranose (1→4) mannopyranose	3.2.1.21	GH 1 3
Acetyl xylan esterases	2-or 3-O-Acetyl xylan	3.1.1.72	CE 1 2 3 4 5 6
Acetyl mannan esterase	2-or 3-O-Acetyl mannan	3.1.1.6	CE 1
Ferulic and p-cumaric acid esterases	2-or 3-O-Acetyl mannan	3.2.1.73	CE 1

4-O-methyl-D-glucuronic acid sidechain of xylans and are found in family 67. Hemicellulolytic esterases include acetyl esterases which hydrolyse the acetyl substitutions on xylose moieties, and feruloyl esterase which hydrolyse the ester bond between the arabinose substitutions and ferulic acid. Feruloyl esterases aid the release of hemicellulose from lignin and renders the free polysaccharide product more amenable to degradation by the other hemicellulases (Prates et al., 2001).

Cellulases

In most lignocellulosic materials cellulose forms the major part of the three components (Table 3). Cellulose is composed of insoluble, linear chains of β-(1→4)-linked glucose units with an average degree of polymeraisation of about 10 000 units but could be as low as 15 units (Eveleigh, 1987). It is composed of highly crystalline regions and amorphous (non-crystalline) regions forming

Table 6. Classification of cellulases as enzymes capable of hydrolysing 1,4-β-glucosidic bonds (modified from Rabinovich et al., 2002a).

Type and origin of the enzyme	EC number	Family
Fungal endo-1,4-β-mannases, and aerobic and anaerobic bacteria endo-glucanases	3.2.1.78 3.2.1.4	5/A1
Endoglucanases of actinomycetes, and aerobic and anaerobic bacteria, as well as nematodes	3.2.1.4	5/A2
Exo-1,3-β-glucanases; endoglucanases/1,3-1,4-β-glucanases and 1,3- β-glucanases (yeast, *Clostridium*)	3.2.1.58 3.2.1.4/73 3.2.1.39	5/A3
Endoglucanases and mannanases of actinomycetes, aerobic and anaerobic bacteria, and anaerobic fungi	3.2.1.4 3.2.1.78	5/A4
Endoglucanases of filamentous fungi and aerobic bacteria	3.2.1.4	5/A5
Endo-1,6- β-glucanases	3.2.1.75 3.2.1.123	5

a structure with high tensile strength that is generally resistant to enzymatic hydrolysis, especially the crystalline regions (Walker and Wilson, 1991).

Cellulases, responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. Cellulases can be divided into three major enzyme activity classes (Goyal et al., 1991; Rabinovich et al., 2002a,b). These are endoglucanses or endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) . Endoglucanases, often called carboxymethylcellulose (CM)-cellulases, are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre opening-up sites for subsequent attack by the cellobiohydrolases (Wood, 1991). Cellobiohydrolase, often called an exoglucanase, is the major component of the fungal cellulase system accounting for 40-70% of the total cellulase proteins and can hydrolyse highly crystalline cellulose (Esterbauer et al., 1991). Cellobiohydrolases remove mono- and dimers from the end of the glucose chain. β-glucosidase hydrolyse glucose dimers and in some cases cello-oligosaccharides to glucose. Generally, the endoglucanases and cellobiohydrolases work synergistically in the hydrolysis of cellulose but the details of the mechanism involved are still unclear (Rabinovich et al., 2002b). Microorganisms generally appear to have multiple distinct variants of endo- and exo glucanases (Beldman et al., 1987; Shen et al., 1995). Similar to hemicellulases most cellulases are multi-domain proteins. There is still uncertainty in the current definition and classification of “true” cellulase families (Rabinovich et al., 2002a). Table 6 shows the current classification of cellulases (Rabinovich et al., 2002a). This classification includes family 10/F xylanases with a broad specificity and xylanases belonging to family 11/G which are strictly specific for xylan but excludes families 16 and 17 which include 1,3(4)-β-glucanases which have similar activity towards 1,4-β-glucanases.

Multifunctionality of glucanases

Kumar and Deobagkar (1996) provide a comprehensive discussion on multifunctional glucanases including some cellulases and hemicellulases, demonstrating that the concept of “one enzyme one activity” might not hold in all cases, especially with glucanases. A multifunctional protein is a protein consisting of a single type of polypeptide chain, but has multiple catalytic or binding activities. For example, XylA from *Neocallimastrix patriciarum* has two identical catalytic domains (Zhou et al., 1994). A cellulase with exo-and endo-activities from *Caldocellum saccharolyticum* was identified (Saul et al., 1990).

ENZYMES COST

Enzyme cost is considered to be a major impediment in extensive commercialisation of enzymatic cellulose hydrolysis (Walker and Wilson, 1991; Eveleigh, 1987). Enzyme cost is estimated to represent approximately 50% of the total hydrolysis process cost. A study conducted by Lee in 1981 (cited in Walker and Wilson, 1991) puts the enzyme cost into stark monetary terms.

The study showed that cellulose free of hemicellulose and lignin could be produced for 55 US dollars mg^{-1} while the cellulase cost was $2665 US dollars mg^{-1}. However the cost of enzymes has decreased over the last twenty years but is still considered to be very high. The low cellulase activity which is approximately 10-100-fold less than those of amylases depending on the cellulose pre-treatment and hydrolysis process conditions (Eveleigh, 1987) demands substantial amounts of lignocellulytic enzymes. This raises the question, what should the target enzyme production levels be to make lignocellulose hydrolysis commercially cost feasible? A review article (Persson et al., 1991) provides a comprehensive discussion on the latter issue with reference to fungal cellulases. It has been estimated that an enzyme concentration of 10-20 Filter Paper Units (FPU) g^{-1} dry weight would be required to achieve 90% hydrolysis of 100 g l^{-1}dry weight lignocellulose slurry within 70 -110 hours (Persson et al., 1991). To supply sufficient amount of this enzyme for the latter process requires an enzyme production rate of approximately 200 FPU l^{-1} h^{-1} (Persson et al., 1991; Szakacs and Tengerdy, 1997; Esterbauer et al., 1991). The nearest to the target benchmark was obtained with fed- batch cultures using mutants derived from the original *T. reesei* QM 6a strain. On purified cellulose, enzyme concentration above 20 FPU ml^{-1} produced at a rate exceeding 150 FPU has been reported (Persson et al., 1991). However, the production cost is still prohibitive since the increase was obtained at the expense of using more costly purified cellulose. Much research efforts have been focused on lowering the cost of enzymes. The approaches which have been considered in achieving the latter falls into three groups: (1) Screening for organisms with novel enzymes; (2) Strain improvement of existing industrial organisms and enzyme engineering; (3) production and operations related factors such as choice of substrate, culturing conditions, recycling of enzymes and redesigning of processes.

Persson et al. (1991) has covered issues of strain improvement, choice of substrate and culture conditions in relation to improvement of fungal cellulolytic enzyme production. Other investigators have looked at various aspects including aspects of using cheaper substrates (Reddy et al., 2003; Szakacs and Tengerdy, 1997), immobilising enzymes (Woodward, 1989; Dourado et al., 2002), recycling enzymes (Lee et al., 1995) and improvements related to process design (Rivela et al., 2000; Galbe and Zacchi; 1993). Although all these latter approaches present opportunities to reduce cost, we will focus on screening for organisms with novel enzymes, strain improvement of existing industrial organisms and enzyme engineering. Since strain improvement has already delivered significant improvement in increasing cellulase production yields, it has been a method of choice in enzyme biotechnology. Enzyme engineering opens-up numerous possibilities for designer enzymes with various engineered properties.

Screening for organisms with novel enzymes

For more than fifty years one of the main areas of biotechnology research into lignocellulose has been driven by the need to isolate and identify organisms which are either hyper-producers and/or sufficiently robust to withstand conditions of the intended application and/or are producers of novel lignocellulolytic enzymes. In terms of enzyme novelty from an applications perspective, interest is focused on not only finding enzymes which could break down lignocellulose much more rapidly but also enzymes which could withstand pH, temperature and inhibitory agents more resiliently depending on the intended application. Mutant strains of *T. reesei* have been selected that produce extracellular cellulases up to 35 g/l (Esterbauer, 1991; Jørgensen et al., 2003). It has been suggested that cellulase production is probably as high as it would get and it is highly unlikely that it would increase much further (Béguin, 1990) and that increasing the specific enzyme activity is the most likely approach to improving the commercial prospects of lignocellulose hydrolysis. Questions have been raised concerning the continued screening of the environment for lignocellulolytic microorganisms given that on average less than 1% of the potential microbes in the biosphere have been identified using traditional methods of culturing (Amann et al., 1995; Rondon et al., 2000; Pace, 1997; Handelsman et al., 1998) and that genomics and metagenomics might be more productive approaches in biomining for unique lignocellulolytic genes which could then be cloned and expressed in industrial strains. Genomics offer the potential to obtain the complete blueprint of an organism and to assess its genetic potential in a comparative and functional manner. Metagenomics describes genomics associated with the functional analysis of organisms at community level. Although this technology holds out the best opportunity for discovering unique genes, the technology is still in its infancy and issues such as representative cloning, quantitative analysis, and expression still require refining (Gupta et al., 2002).

Strain improvement of existing industrial organisms and enzyme engineering

Table 7 shows the specific activities of commercial cellulase preparations from major USA cellulase manufacturers/suppliers (Nieves et al., 1998). In comparison, Table 4 shows the microorganisms with the highest specific activities. There is a wider and bigger range of specific activity found in different hemicellulases and cellulases from different organisms thus presenting opportunities for developing industrial commercial

Table 7. Specific activity of commercial cellulase preparations (modified from Nieves et al., 1988).

Preparation	Microbial source	FPU*/mg	β-GlucosidaseU/mg	CMCU/mg	CellobiaseU/mg
Biocellulase TRI	*T. reesei*	0.24	0.72	5.5	0.059
Biocellulase A	*A. niger*	0.01	1.4	3.6	ND
Cellulast 1.5L	*T. reesei*	0.37	0.16	5.1	0.018
Cellulase TAP10	*T. viride*	0.13	5.2	14	ND
Cellulase AP30K	*A. niger*	0.03	10	21	ND
Cellulase TRL	*T. reesei*	0.57	1.0	13	0.016
Econase CE	*T. reesei*	0.42	0.48	8.5	0.038
Multifect CL	*T. reesei*	0.42	0.20	7.1	0.015
Multifect GC	*T. reesei*	0.43	0.39	13	0.025
Spezyme #1	*T. reesei*	0.54	0.35	15	0.026
Spezyme #2	*T. reesei*	0.57	0.42	15	0.029
Spezyme #3	*T. reesei*	0.57	0.46	25	0.031
Ultra-low Microbial	*T. reesei*	0.48	0.96	ND	ND

*FPU (filter paper units), 1 FPU = 1 µmole min^{-1} glucose released.
ND, not determined.

enzymes with greater specific activities than commercial enzymes from *T. reesei* which dominate the lignocellulolytic enzyme market. There are a number of possible approaches to creating enzymes with greater activities and these approaches are discussed in detail in some recent review articles and other articles (Schülein, 2002; Levy et al., 2002; Planas, 2000). This review will only highlight some aspects of these approaches which will dominate the lignocellulolytic research for sometime into the future resulting in a diminishing research focus on screening and isolation of lignocellulytic organisms, which still dominate research in developing countries. These approaches have the potential to create organisms or designer enzymes more rapidly and cost effectively than the continued screening for organisms which are likely to be novel.

Enormous amount of data and information have been gathered over the years on the different organism's lignocellulolytic genes and controlling elements, their sequences and organisation, protein sequence data, identification of catalytic amino acids and protein structural data including an increase in three-dimensional modular protein structures obtained from crystallographic studies (Shallom and Shoham, 2003; Martinez, 2002). Recently, the entire genome of *P. chrysosporium* has been sequenced opening-up new possibilities for a variety of molecular studies (Martinez, 2002). This is the first basidiomycete genome that has been sequenced and is now available for genomic and proteomic studies (http://www.jgi.doe.gov). All of this information is useful from a biotechnology applications approach for engineering lignocellulolytic enzymes with different properties for various industrial commercial applications.

Optimising cellulase mixtures

Enzyme mixtures with interesting properties including a possible increase in enzyme specific activity could be created by combining purified individual proteins or individual domains either from the producing organisms or from recombinant organisms expressing a single cloned lignase, hemicellulase or cellulase encoding gene. These combinations could either consist of pure enzymes from different organisms or the supplementation of crude enzymes with a pure enzyme or supplementing the pure enzyme with a purified cellulose-binding domain from another organism or with specific co-factors. Addition of pure *T. reesei* cellobiohydrolase I to a *Thermonospora fusca* crude cellulase preparation increased the rate of filter paper hydrolysis approximately two-fold greater than when each component was used separately (Wilson and Walker, 1991). A laccase- mediator system (lignozyme®-process) consisting of a combination of laccase and mediators with either NO, NOH or HRNOH groups effectively delignify wood in a pilot pulp and paper process (Call and Mücke, 1997). CBDs have been found in a wide range of hydrolase proteins as part of the muti-domian complex (Levy and Shoseyov, 2002). The CBD exerts non-hydrolytic disruption of cellulose. More than 200 putative sequences in over 40 different organisms have been identified. CBDs from the same organism or different organism can differ in their binding specificity and even two CBDs located on the same enzyme may differ (Carrard and Linder, 1999; Brun et al., 2000). A pure CBD derived from CBHII of *T. reesei* added to the *T. harzianum* chitinase increased the hydrolytic activity of the insoluble substrate (Limon et al., 2001). Replacing

the CBD of endo-1,4-β-glucanase from *B. subtilis* with the CBD of the *T. viride* exoglucanase resulted in higher binding and enhanced hydrolytic activity on microcrystalline cellulose (Kim et al., 1998).

Genetics and recombinant DNA technology

This section of the review will not cover the extensive body of knowledge dealing with the molecular biology of lignocellulolytic organisms; readers interested in the latter should consult the numerous available literature. Béguin, (1990), Knowles et al., (1987), Gold and Alic, (1993) are some suggested literature which deal with the various genes identified and controlling sequences from various organisms, gene organisation, gene classification, and expression and regulation that could serve as a starting point for readers interested in this area. The initial work on the genetics of lignocellulolytic organisms was dominated by random mutagenesis, and selection of hyper-cellulase producing mutants and mutants insensitive to catabolite repression. Most of the *T. reesei* mutants such as RUT C 30, CL-847, VTT-D which are used for commercial production of cellulases were derived from the wild strain *T. reesei* QM 6a that was originally isolated by the US Army Laboratories (Esterbauer, 1991). The first catabolite repressed *Bacillus pumilus* with cellulase yield four times higher than the wild type strain was created through mutagenesis (Kotchoni et al. 2003).

Attempts have also been made to identify genomic heterogeneticity within individual strains of *P. chrysosporium* and between different strains classified as belonging to this species with the expectations that their genetic differences may also translate into variations in their lignocellulolytic genes and efficiency in lignocellulose degradation. These different strains could then be used for breeding purposes to generate novel hybrids strains (Broda et al., 1989).

While significant progress has been made using physical and chemical mutagens to increase production of lignocellulolytic enzymes, recombinant DNA technology and protein engineering used apart or in combination are powerful modern approaches holding-out the greatest potential for making significant improvements in several aspects of lignocellulolytic enzymes such as increased production, increased specific activity, pH and temperature stability and also creating “synthetic” designer enzymes for specific applications. Computer programs permit the researcher to identify catalytic-sites, residues which are essential for protein stability under various pH and temperature
conditions and protein folding patterns. This accumulated knowledge is then used to simulate enzymatic behaviour upon any changes in any one or a combination of sites. Then a specific gene is cloned, subjected to site-directed mutagenesis to give rise to a protein with properties similar to the simulated model. The gene-product’s enzymatic behaviour is then experimentally verified. Equally so DNA technology offers the possibility of fusing different lignocellulolytic genes or sections of genes from different organisms to give rise to novel chimeric gene-products with altered properties. A few examples will briefly be discussed to demonstrate the value and potential of these approaches in lignocellulolytic enzyme improvement. Unique lignocellulolytic enzymes with multiple activities can be created by artificial gene-fusion. A bifunctional exoglucanase-endoglucanase (Warren et al., 1987) and a chimeric xylanases-endoglucanase (Tomme et al., 1994) were successfully constructed. A novel thermostable hybrid (1,3-1,4)-β-glucanases expressed from a gene fusion consisting of the beta-glucanase encoding genes from *Bacillus amyloliquefaciens* and *Bacillus macerans* was created (Borris et al., Patent abstracts). 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 two *T. reesei* cellobiohydrolase and a *T. reesei* endoglucanase (Aylward et al., 1999). The active carboxylic residues in *T. reesei* endoglucanse was identified by site-directed mutagenesis (Okada et al., 2000) opening up future research prospects for modifying these to improve activity. A South African research team led by Pretorius have for years been active in engineering *Saccharomycetes cerevisiae* for efficient cellulose hydrolysis (Van Rensburg et al., 1998).

A number of designer enzymes, also called glycosynthases, including cellulases and hemicellulases have been engineered by replacing the nucleophilic residue thus resulting in higher yields of different oligosaccharides (Palcic, 1999; Fairweather et al., 2002). Substituting two tryptophan residues in the heme cavity by two phenylalanines in a cytochrome-c peroxidase resulted in improved activity (Gengenbach, 1999). Increasing the thermostability of recombinant manganese peroxidase has been attempted by engineering a disulfide bridge contiguous to the distal Ca^{+2} binding site (Reading and Aust, 2000). Attempts have also been made to create a bifunctional hybrid of manganese-lignin peroxidase (Timofeevski et al., 1999).

Using a combination of genetic and DNA technology approaches, a few strains with increased specific activities have been reported (Montenecourt et al., 1983; Sheir-Neiss and Montenecourt, 1984). A *T. reesei* strain KY 746 showed a 50% increase in filter paper activity with only a 25% increase in endoglucanase activity over the wild type QM 9414 (Morikawa et al., 1985), while *T. reesei* RUT-P 37 showed a doubling of specific activity in both filter paper and endoglucanse units compared with the wild type QM 6a (Montenecourt et al., 1983). A genetically improved *T. reesei* mutant strain CL 847, resistant to catabolite repression, was generated that

exhibited a four-fold increase in cellulose productivity compared with QM 9414 and increased β- D-glucosidase specific activity (Durand and Clanet, 1988). A heterologous expressed *Cellulomonas fimi* exoglucanase with increased specific activity from *E. coli* was over-produced (Lam et al., 1997). Although some strides have been made in increasing lignocellulolytic activity, there is an enzyme activity ceiling beyond which it would not be possible to move since the rate and extent of breakdown of lignocellulose is influenced by the complex structural nature of the substrate, as previously discussed, including structural features which prevent enzyme access and binding.

CONCLUSION

The energy and environmental crises which the world is experiencing is forcing us, among other things, to re-evaluate the efficient utilisation or finding alternative uses for natural, renewable resources, especially organic "waste", using clean technologies.

The same strategic imperatives, economic growth and developmental issues which drove Western countries research into lignocellulose since the 1970's are of even greater and pressing relevance to developing countries. Developing countries are still grappling with socio-economic issues including meeting the massive energy-shortage demands, food security and developing technological solutions in the agriculture, agro-processing and other related manufacturing sectors. Lignocellulose biotechnology offers significant opportunities to developing countries for addressing some of the issues highlighted since most of the technology is based on the utilisation of readily available residual plant biomass considered as "waste" to produce numerous value-added products. Brazil's success in bio-fuel, is often a show-case of but one example of the economic potential for developing countries in the area of lignocellulose biotechnology. On the other hand neglecting this technology could be immensely costly. Already patterns of production and trade are significantly affected by the emergence of biotechnologically produced goods some which may reduce or eliminate the demand by Western countries for agrarian products from developing countries. For example, sugar from cane can be replaced by enzyme produced sugar–syrups, xylitol,
glucose and fructose sweeteners. Lignocellulose technology may be transferred to developing countries but at exorbitant prices and only after its technological and business cycles have been fully exploited. Lignocellulose biotechnology from a capital costs investment perspective is an attractive technology for developing countries since its biodegradation could follow solid-state fermentation comparable to silage or mushroom production, thus making such technology suitable for farms and small industrial plants without the need for large engineering infrastructure.

It is also important to emphasize that in order for lignocellulose biotechnology to make meaningful impact on developing countries; suitable bioconversion processes need to be developed on a much wider scale and these countries should begin to pull their meagre resources and biological science expertise in a cooperative and integrated manner towards modern, advance genomics and proteomics technologies for identifying novel lignocellulolytic enzymes and engineering enzymes with improved activities suitable for industrial-scale application.

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