Mycobiology 34(4): 159-165 (2006)

Bioconversion of Lignocellulose Materials

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(Received September 6, 2006)

One of the most economically viable processes for the bioconversion of many lignocellulosic waste is represented by white rot fungi. *Phanerochaete chrysosporium* is one of the important commercially cultivated fungi which exhibit varying abilities to utilize different lignocellulosic as growth substrate. Examination of the lignocellulolytic enzyme profiles of the two organisms *Phanerochaete chrysosporium* and *Rhizopus stolonifer* show this diversity to be reflected in qualitative variation in the major enzymatic determinants (ie cellulase, xylanase, ligninase and etc) required for substrate bioconversion. For example *P. chrysosporium* which is cultivated on highly lignified substrates such as wood (or) sawdust, produces two extracellular enzymes which have associated with lignin deploymerization. (Mn peroxidase and lignin peroxidase). Conversely *Rhizopus stolonifer* which prefers high cellulose and low lignin containg substrates produce a family of cellulolytic enzymes including at least cellobiohydrolases and β-glucosidases, but very low level of recognized lignin degrading enzymes.

KEYWORDS: Bioconversion, Bio-fuel, Cellobiohydrolases, Lignocellulosic enzymes, White rot fungi

Lignocellulose

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" are generated through forestry and agricultural practices, paper-pulp industries, timber industries, and many agro industries. But, they pose an environmental pollution problem. Sadly, much of the lignocellulose waste is often disposed 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 bio fuels, 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, laundry, pulp and paper and agriculture.

This review's main focus is to highlight significant aspects of lignocellulolytic 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 lignocellullose derived high value bioproducts; solid state fermentation processing as a 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 by extension and any large mass of biological matter. A wide variety of biomass resources are available on our planet for conversion into bioproducts. These may include whole plants, plant parts (eg. seeds, stalks), plant constituents (eg. 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 be required 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,

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benzene, toluene and xylene which are used to synthesis of 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.

Based upon the predicted catabolic pathway and the known metabolism of of lignin by *Phanerochaete chrysosporium*. 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 kilo gram 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.

Hemicelluloses are 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, remineralization, and as an antimicrobial agent, it is used in chewing gum and toothpaste formulations (Roberto *et al*., 2003). 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 (Montane *et al*., 2002). Over 200,000 tones of furfural with a market price of about $1700 per ton is annually produced (Zeitch, 2000).

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). They produces ethanol from the fermentation of cane juice in Brazil whereas corn is used in the USA. 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 amount 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 technological focus for the ethanol production has shifted towards the utilization of residual lignocellulosic materials to have lower production costs. Other high value byproduct 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 "lignocellulosic waste".

Production of extracellular enzymes by fungi: Extensive studies have been made in the production of extracellular enzymes by fungi. Further the potential applications of such enzymes in the bioconversion of lignocelluloses to economically are important useful products. The production of the cellulases, microbial protein and reducing sugars (released) were also submerged culture than in solid state fermentation of wheat straw by *Aspergillus terreus* (Eyini *et al*., 0000). 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, 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 ,pectinases and 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).

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 glucomannan in pulp fibres to enhance chemical delignification (Suurnakki *et al*., 1997). A patented lignozyme process is effective in delignifying wood in a pilot pulp- and paper process (Call and Muck, 1997). In bio-pulping where lignocellulytic enzymes were used the following was achieved: tensile, tear and burst indexes of the resultant paper were improved, brightness of the pulp was increased and an improved energy saving of 30~38% was realized (Scott *et al*., 1998). Laccases can degrade a wide variety of synthetic dyes making them suitable for the treatment of wastewa-

ter 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 utilization, milk yield and body weight gain have attracted considerable interest. Steers fed with an enzymes 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 4~16% of various commercial fibrolytic enzyme treated forages (Beauchemin *et al*., 2001).

Degradation of Lignocellulose: Lignocellulose consists of lignin, hemicellulose and cellulose and compiled from Bettes *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 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, acid while, cellulose is a homogenous polymer of glucose. 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 (Grethlein and Converse, 1991; Nguyen, 1993) hydrolysis methods have been used to degrade lignocellulose. **Weak acids are tend to remove lignin but result in poor hydrolysis of cellulose whereas strong acid treatment occur under relatively extreme corrosive conditions at 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 associate with the disposal of spent acid and alkaline. For many processes enzymes are preferred to acid or alkaline processes since they are specific biocatalysts, they can operate under much milder reaction conditions,and they 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 byproducts at prices which can compete with current production costs. The feedstock costs can be minimized by focusing on agricultural residues and waste materials initially. 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 constitutents into bioproducts entails three steps, process design, system optimization and model development. Processing involves the use of biocatalysts, whole microorganisms or their organisms to synthesize or bioconvert raw materials into new products; recover/purify such bioproducts and subsequently any needed downstream modifications.

Solid state fermentation: 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; Jech, 2000). SSF process 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 status form with the solid matrix and the substrate (Canel 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 (Jech, 2000). SSF are practical for complex substrates including agricultural, forestry, 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 (cellulose) 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 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/outline.cfm) 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: Various lignolytic waste materials such as hay, barley straw, bagasse, rye straw, newspaper, saw dust, and coconut fibre were used for lignolytic degradation under aerobic digestion. Fungal degradation by *Phanerochaete chrysosporium Polystictus sanguineus*, *Poria subacida* and *Trametes versicolor* have been studied in fair detail (Kirk and Fenn, 1982).Palmer and Evans (1983) have reported that few actinomycetes such as *Streptomycetes* and *Nocardia* also degrade lignin.

A diverse spectrum of lignocellulolytic microorganisms, mainly fungi (Baldrian and Gabriel, 2003; Falcon *et al*., 1995) and bacteria (McCarthy, 1987; Zimmermann, 1990; Vicuna, 1988) have been isolated and identified over the years and this list still continues to grow rapidly. Already an impressive collection of more than 14,000 fungi which were active against cellulose and other insoluble fibres were collected by 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; Jorgensen *et al*., 2003; Nieves *et al*., 1998). This is so, partly because *T. reesei* was one of the first cellulolytic organisms isolated in the 1950s and because extensive strain improvement and screening programs, and cellulose 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 celluloytic enzymes but is unable to degrade lignin.

The white-rot fungi belonging to the basidimoycetes 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 production of lignin-degrading enzymes or direct application in lignocellulose bioconversion processes (Ruggeri and Sassi, 2003; Bosco *et al*., 1999). Less know, white-rot fungi such as *Daedalea flavida*, *Phlebia facicularia*, *P. floridensis* and *P. radiate* have been found to selectively degrade lignin in wheat straw and hold out prospects for bioconversion biotechnology. The aim is just to remove the lignin leaving the other components (Arora *et al*., 2002). Less prolific lignin-degraders among bacteria such as those belonging to the genera *Cellulomonas*, *Pseudemonas* and the actinomycetes *Thermomonospora* and *Microbispora* and bacteria with surface-bound cellulose-complexes such as *Clostridium thermocellum* and *Ruminococcus* are beginning to receive attention as representing a gene pool with possible unique lignocellulase engineering (Vicuna, 1988; McCarthy, 1987; Miller Jr. *et al*., 1996; Shen *et al*., 1995; Eveleigh, 1987; Perestelo *et al*., 1994).

Degradation of lignin and hemicellulose was also achieved by Pal *et al*. (1995) during the cultivation of mushroom *Flammulina velutipes* and the white rot fungus *Trametes versicolor* on sugarcane bagasse for 40 days. *Trametes versicolor* produced laccase and manganese-peroxidase and showed a simultaneous degradation of lignin and holocellulose. However, only phenoloxidase activity was found with *Flammulina velutipes*, which exhibited a greater reduction in the ratio of weight to lignin loss than *Trametes versicolor*. They also proved the laccase and manganese-peroxidase activity in both organisms. The maximum laccase activity was showed by *Trametes versicolor* on 5th day, but it decreased in subsequent days. *Flammulina velutipes* showed far less laccase activity than *Trametes versicolor* under the assay conditions used.

A bacterial strain of the *Pseudomonas putida*, is isolated from decomposing plant material, was capable of degrading lignin related compounds and also observed the ability of this bacterium to degrade Kraft-lignin and radio-

labelled lignins.

Lignases

Fungi can breakdown lignin aerobically through the use of a family of extracellular enzymes collectively termed "lignases". Two families of lignolytic enzymes are widely considered to play a key role in the enzymatic degradation: phenol oxidase (laccase) and 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 (Barbonnais and Paice, 1988), methanol oxidase (Nishida and Eriksson, 1987) and oxidoreductase (Bao and Renganathan, 1991). Enzymes involved in lignin breakdown are too large to penetrate the unaltered cell wall of plants so the question arise, how to lignases affect lignin and biodegradation. Suggestions are that lignases employ low-molecular, diffusible reactive compounds to affect initial changes to the lignin substrate (Call and Mucke, 1997)

Cellulases

In most lignocellulosic materials, cellulose forms the major part of the three components. Cellulose is composed of insoluble, linear chains of β-(1-4)-linked glucose units with an average degree of polymerization of about 10000 units but could be as low as 15 units (Eveleigh, 1987). Cellulases, responsible for the hydrolysis of cellulose, are composed of a complex mixture of proteins with different specificities to hydrolyze glycosidic bonds. Cellulases can be divided into three major enzyme activity classes (Goyal *et al*., 1991; Robinovich *et al*., 2002a, b). These are endoglucanases or endo-1,4-β-glucanase, cellobiohydrolase, and β-glucosidase. 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). A cellulase with exo-and endo-activities from *Caldocellum saccharolyticum* was identified (Saul *et al*., 1990).

Xylanase

Hemicellulase was able to degraded by xylanase enzyme. Rabinovich *et al.* (2002a) and Shallom and Shoham, (2003) present recent reviews covering the types, structure, function, classification of microbial hemicellulases. 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. 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 hydrolyzed into single xylose units by β-xylosidase.

Conclusion

The energy and environmental crises which the world is experiencing is forcing us, among other things, to re-evaluate the efficient utilization 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 lignocelluloses 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 utilization 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 meager 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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