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Biotechnology in the degradation and utilization of lignocellulose

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Abstract

Lignocellulose is the predominant renewable resource. Its uses include fuel, as the feedstock for the pulp and paper industry, and for animal nutrition. It also constitutes a large proportion of agricultural and urban waste. Biotechnology has roles in its efficient production and utilisation. The types of lignin substrates available for study of lignin biodegradation are described. The white rot fungus *Phanerochaete chrysosporium* is the archetypal system for the study of lignocellulose degradation, since it mineralises lignin and degrades both cellulose and hemicellulose. The salient features of the *P. chrysosporium* system are described. The lignin peroxidases are a family of proteins, and it is shown that expression of their genes is differential. *P. chrysosporium* is heterokaryotic with two gene equivalents that have abundant RFLPs. A set of basidiospore-derived strains with genetic compositions defined by such RFLPs provided the potential basis for a strain improvement programme for lignin degradation. However, analysis of this system using radiolabelled synthetic lignin (DHP) as the substrate confirmed previous evidence that both the substrate and the fungal cultures displayed much variation, so that it was difficult to quantify performance for this property. The cellobiohydrolase I enzymes are also coded for by a family of genes, and evidence is also presented for allelic variants, for differential expression and for differential splicing. In contrast, the cellobiohydrolase II function is encoded at a unique genetic locus. Approaches to an homologous integrative transformation system are discussed. Some actinomycete bacteria represent an alternative system for lignin solubilisation in which strains differ in their spectra of activities on lignocellulose substrates. The xylanase system of *Streptomyces cyaneus* is shown to include three enzymes, two of which are inducible by xylan. A novel assay method was developed and used to demonstrate that the third is constitutive and also non-repressible by glucose. It is proposed that this acts as a sensor for xylans in the environment that can yield breakdown products that are taken up and can then act as inducers of the other two enzymes. The studies on microbial lignocellulose degradation from different laboratories have allowed the formulation of specific biotechnological goals, and some of the problems and opportunities in this area are identified.

Introduction

Lignocellulose is the principal source of biomass and therefore of renewable resources; it is a complex of three classes of polymer. Cellulose, which consists of repeating glucose units largely in crystalline fibres, is chiefly what is sought in chemical pulping processes. The hemicelluloses are a more mixed group of polysaccharides, in which the monomers are mainly pentoses. In the case of lignin the monomers are three cinnamyl alcohols. Both cellulose and lignin are difficult to disrupt, but for fundamentally different reasons. Because cellulose is largely crystalline, it is substantially more difficult to attack than, for instance, starch or the hemicelluloses; in contrast, lignin is assembled

by an oxidative polymerisation of free radical species of its monomers to yield a highly irregular three-dimensional polymer with a number of intermonomeric bond types. The predominant class is the β aryl ether bond, but others include direct linkages and ethers between aromatic rings.

The objective of this article is to survey existing forms of lignocellulose utilisation, which have little biotechnological input, and to identify aspects where biotechnology might in the future make contributions to improved utilisation. It will be shown that such innovations will depend upon improved understanding of the biodegradation of these polymeric components, and work from the author's laboratory will be described in this context.

Utilization of whole lignocelluloses

The range of biomass materials includes trees and other crops, and also wastes from forests, agriculture, stock raising and towns (Coombs 1986). Much lignocellulose is and will be used in an unfractionated form. Thus, mechanical pulping involves the milling of the total lignocellulose to give high yields that can be used for newsprint and for brown papers and board, e.g. for packaging materials; this process requires a very high energy input. Production of lignocellulose as an energy crop is an alternative to the utilisation of fossil fuels that has the merit of being CO_2-neutral. Crude burning is likely to be supplanted by chemical processes that yield various mixtures of gas, liquid and solid waste; a potential return for investment comparable to that for oil is suggested. There will be changes in the appropriateness of different technologies depending on economic, resource-availability and environmental considerations. Nevertheless, there are processes that are being developed and it will be for biotechnology to find a competing or complementary role in such processes (Hartley et al. 1987; Anon. 1992).

Biological utilization of whole lignocellulose

It should be recognised that at least three biologically based processes already exist that utilise whole lignocellulose. First, it is a major component of animal feeds such as grasses, hay and straw. Its precise composition varies between species and varieties of plant, and also in its proportion of the total material during the life cycle of the plant. Whereas cellulose can be degraded by microorganisms in the ruminant gut to glucose, the hemicelluloses have an as yet poorly defined nutritional role (Williams 1989). The lignin component is especially recalcitrant to microbial degradation and constitutes a physical barrier to the extraction and utilization of the other components of plant material. Biotechnology might thus improve the nutritional value of lignocellulose for instance through upgrading of silage and ultimately by depolymerisation of the polysaccharides so that such materials provide a feed for non-ruminants such as pigs and poultry (Coughlan 1989).

The second biologically based process is the production of biogas, which has been practised for centuries in China. There are substantial efforts to develop such technology in India, and it has a future in the utilization of wastes in landfills and on farms in developed countries (Ferrero et al. 1984). A particular problem with industrial farming is the disposal of animal waste, especially from pig production.

The third process is production of mushrooms in solid-substrate fermentation systems (Flegg et al. 1985). The major problems for the process are in the physiology of the organisms and in protection against disease. For some species, e.g. *Agaricus bisporus*, the preparation and management of the starting compost and the casing layer substrates are critical (Derikx et al. 1990), but a very wide range of lignocellulosic starting materials might be used for other species, e.g. *Pleurotus ostreatus* (Hadar et al., this issue). There is much work to be done at the physiological and process engineering levels on the analysis of the conditions that could be employed for different fungal strains and substrates. This is therefore a model that is already operating on a large scale (Reid 1989; Karem et al. 1992).

Fractionation of lignocellulose

By far the largest process currently in operation is chemical pulping for both the paper industry and the production of cellulose fibre for textiles. There are a number of pulping methods, but in each what is obtained is cellulose, with a proportion of the hemicelluloses. In the Kraft process (the dominant process worldwide) the bulk of the hemicelluloses are retained in a modified form whereas lignin is converted into soluble products (Kraft lignins) that as yet have little value other than for burning; however, this means that the process requires no external energy source and there is extra material that could be made available for other uses. Thus in the overall balance sheet principally the cellulose and the hemicellulose emerge from the process. Furthermore, the bleaching and subsequent papermaking processes create major environmental problems to do with effluents and requirement for water (Shoham et al., this issue).

Industrial lignins surplus to the needs of chemical pulping processes have been considered as a source of aromatic chemicals such as phenols and catechols, and have been used for fibreboard, as an emulsion stabilizer, as an additive in oil drilling, and for cement processing, but none of these is a high value product. Novel uses must be sought for derivatives of lignin that would match the enormous scale on which they are produced in nature and then industrially.

Possible processes for utilization of the monomers of the different components of lignocellulose, whether chemical or biological, are visualised in three stages: pretreatment to disrupt the gross structure, depolymerization, and utilization of the monomers. In the pretreatment, mechanical chipping is followed in a number of schemes that are in the pilot stage by either steam explosion or solvent extraction. Such methods render the mixtures of lignin, cellulose and hemicellulose amenable to further treatment. The methods used to fractionate the carbohydrates include treatment with dilute acid, which removes the hemicelluloses from the celluloses, or with concentrated acids, which have high material costs. Both steam explosion and solvent extraction yield hemicellulose that could serve as a substrate for enzymatic hydrolysis (Overend & Chornet 1987; Ragg & Fields 1987). Relatively mild treatments with ammonia are used to upgrade the digestibility of straw by ruminants.

It seems that pretreatment is likely to be common to all processes, but that biological methods can then be considered in competition with chemical and physical methods (or as complements to them). In general, biological methods will operate at lower temperatures and pressures, with cheaper starting materials and the possibility of operating on a smaller scale; on the other hand, the throughput time may be much slower.

Biologically based processes

There are at least six areas in which scientific progress might lead to biologically based processes. Three of these are concerned with the polymers, namely disruption of the crystallinity of the cellulose allowing more rapid action by cellulases, the depolymerisation of hemicellulose, and the solubilization of lignin. The other three are concerned with the utilization of monomers, i.e. the fermentation of pentose sugars, the production of ethanol in high yield together with its separation from the aqueous medium, and the conversion of solubilized lignin-derived monomers into high added-value aromatics. The solubilization of the polymers is the key to biological processes and therefore these will be discussed. Considerable effort has been expended on cellulases, which now represent a commercial product (Coughlan 1985), and as will be described in this issue and elsewhere (Shoham et al. 1992; Visser et al. 1992), hemicellulases are assuming importance in the pulp and paper industry. In contrast, no industrial processes yet exist involving biological delignification (but see Kirk & Chang 1990).

Cellulose biodegradation

Cellulose in lignocellulose is a much larger resource than starch for breakdown to sugars. But it is also represents a much more complex problem

because of its crystallinity and its admixture with hemicellulose and lignin. Most work on its biodegradation has been done with the filamentous fungus *Trichoderma reesei*, which, given optimum culture conditions, can produce yields of 20 g extracellular cellulase protein from 60 g cellulose (Coughlan 1985). It is now evident that in this organism cellulase is a complex of at least eight proteins that act synergistically and are subject to complex controls (Eveleigh 1987). *T. reesei* produces five or more endo β-glucanases which convert the crystalline cellulose to oligocellodextrins and amount to 20–35% of the protein by weight. In addition, there are at least two cellobiohydrolase components (65–80% of total protein), which convert these to cellodextrins and cellobiose. Both classes of enzymes are subject to end product inhibition by cellobiose. Finally there is a cellobiase that converts the cellodextrins and cellobiose to glucose; this enzyme constitutes a mere 1% of the protein, and its action is inhibited by glucose. The whole complex is also subject to catabolite repression by glucose.

The pilot studies show that with preparations of cellulase that contain these various activities it is possible in stirred tanks, under realistic conditions, to hydrolyze various types of cellulose to soluble sugars with substrate concentrations of 10–30% and yields of 30–60%. One could convert 1000 kg of lignocellulose of substrate (wood chips, straw, corn stover, or fibre from urban waste) to 350 kg of glucose in 24 h. But the economic barrier is the very large amounts of enzyme complex needed to hydrolyze the cellulose (perhaps a hundred times more than the amylase needed to break down starch).

The reasons have to do with the recalcitrant and impure nature of cellulose, with the requirement for a synergistic complex, with product inhibition, and with the difficulty of desorbing the enzyme complex from the substrate. However, it seems premature to dismiss the biological attack of cellulose in bulk materials on grounds of present economics. The appropriate view to take is that the methods of molecular genetics, allowing cloning of individual enzymes, will result in a far better understanding of how, in one organism, the system works. There will then be opportunities for site-directed mutagenesis to remove product inhibition, to improve the rate at which the enzymes are produced (very little is secreted while the fungus has sufficient cellulose for growth; it occurs as cellulose and nitrogen are approaching depletion), perhaps to improve the activities of the enzymes themselves, and for the different synergistic enzymes to be produced in the optimum ratio.

Other fungi have also been studied, but none is qualitatively more attractive than *T. reesei*, and this organism is an excellent model for this difficult system. However, other organisms should also be studied further. Among these is the bacterial species *Clostridium thermocellum*, which is the topic of a separate article (Bayer & Lamed, this issue).

Lignin biodegradation

Discussions on lignin breakdown and utilization are still primarily based on chemical engineering approaches (Ragg & Fields 1987) with the lignin being recovered in polymeric form. The achievement of this step by biological methods would require the efficient release of the cellulose and hemicelluloses and then the lignin might in principle be recovered either in polymeric form or as monomers that could be converted into useful chemicals. In nature the function of lignin solubilization may be primarily to allow the degrading organism to obtain access to the carbohydrates since the energetic investment in lignin solubilization is rather substantial; thus the basidiomycete white-rot fungus *Phanerochaete chrysosporium* will only degrade lignin if it is depleted for carbon or nitrogen sources (Keyser et al. 1978).

We must recognise that the natural substrate is lignocellulose and hence it is more complex even than lignin. Nevertheless, lignocellulose degradation is a unitary problem because the materials involved (e.g. wood, straw and sugar cane bagasse) are as a first approximation similar to each other. It is the interactions between degrader organisms and the substrate that must be studied, analogous to the cases of microbe-plant and microbe-animal host interactions. These interactions must be under-

stood at different levels that include biochemistry, gene expression and physiology. This involves studying the synergy between different enzymes attacking different components, and indeed between those attacking individual components. As stated above, for cellulose it is already evident that all microbial systems being studied involve a number of synergistic enzymes (Coughlan 1985), and this is likely to be the case for the other components as well. With respect to the substrates, better analytical techniques will allow greatly improved analysis of both the substrates and the products (Kirk & Farrell 1987; Chesson & Orskov 1989).

There is a need to continue to concentrate on a few organisms, so as to obtain a complete description of the enzymes involved and how they act together, their control and topographical relationships. The white rot fungi, e.g. *P. chrysosporium*, remain the only organisms for which the mineralisation of lignin to CO_2 is known to be the primary process; nevertheless other organisms, notably the actinomycetes, also have important roles in lignocellulose degradation (Broda et al. 1989; Zimmerman 1990; Magnuson & Crawford 1992) and should therefore also be studied. The recent results from our own laboratory that will be described are concerned with both *P. chrysosporium* and actinomycete bacteria.

Different groups use different assays for lignin biodegradation. The problems are two-fold: first, in nature lignin is intimately associated with polysaccharides, probably being covalently bound to hemicellulose. Therefore any extensively purified lignin is different from lignin in native lignocellulose. Industrial lignin isolated from the pulping process has substantial chemical modifications. Second, purified lignin is insufficient as a growth substrate: some other carbon co-substrate, e.g. glucose, must be provided, so that growth cannot be used as an assay for lignin degradation. For these reasons a critical discussion on the substrates and assays that are used is an essential component of any review of lignin degradation. Wood & Kellogg (1988a,b) is an invaluable source of information on this area.

Lignocellulose substrates

Studies designed to screen for organisms that are capable of degrading and/or utilizing lignin have employed either synthetic or natural substrates. Synthetic model compounds of lignin include ethoxy and/or methoxy substituted phenols and aromatic alcohols joined together to form dimers either linked via an oxygen (ether bond) or directly through the beta group (Kirk & Farrell 1987). Such substrates are appropriate for the study of the mechanism of purified enzymes, such as the lignin peroxidases of white rot fungi that have been implicated in lignin degradation. However degradation of such model compounds is not by itself proof of degradation of lignin in lignocellulose. The oxidation of veratryl alcohol (3,4 dimethoxybenzyl alcohol) has been used as the standard assay for lignin peroxidases.

In an attempt to reflect the complexity of the lignin molecule, the degradation of polymers obtained from the *in vitro* polymerization of radiolabelled aromatic alcohols (e.g. coniferyl alcohol; Haider & Trojanowski 1975; Tanahashi & Higuchi 1981; Kirk & Brunow 1988) have also been investigated. Such 'dehydrogenative polymerizate' (DHP) has a variety of bonds and is regarded as a good model for lignin; the molecular weights of these compounds are in the range 2 to 10×10^3. These studies have been designed to measure their mineralization to $^{14}CO_2$ during incubation with the organisms.

Radiolabelled natural substrates have also been used; they have been prepared either from cereal grasses (e.g. wheat and barley) grown in the presence of ^{14}C phenylalanine (McCarthy et al. 1984; Mason et al. 1990), or ^{14}C cinnamic acid (Benner et al. 1984), or by dipping cut stems of woody plants in a buffer containing ^{14}C ferulic acid (Crawford & Crawford 1978). In each case the radiolabelled acids provide a precursor for lignin biosynthesis. The removal of non-lignocellulose materials using inorganic and then organic solvents results in a lignocellulose substrate which contains 85% of the radioactivity in the lignin component of the complex, and is therefore sometimes referred to as (^{14}C lignin) lignocellulose; such determinations are

based on the Klason definition of lignin, namely the material that is insoluble in 72% H_2SO_4 (Effland 1977). The proportion of ^{14}C lignin may be improved to 90% by the removal of some residual proteinaceous material using proteases. The resulting substrates have a specific activity (approximately 10^3 dpm/mg) sufficient to allow the screening of organisms for the ability to degrade lignin (Phelan et al. 1979; Haider & Trojanowski 1980; McCarthy & Broda 1984; Agosin et al. 1985). The use of such substrates has revealed two quite different types of activity: one is the solubilization of the ^{14}C lignin, giving rise to soluble radiolabelled products in culture supernatants of actinomycetes (McCarthy & Broda 1984); Mason et al. 1988), and the other is the complete degradation of the ^{14}C lignin to $^{14}CO_2$, as exhibited by cultures of *P. chrysosporium* (McCarthy et al. 1984; Agosin et al. 1985).

Given the complexity of lignocellulose and indeed of lignin itself, it is likely that both solubilization and degradation require that a number of enzymes act synergistically. As a result of this synergism, factors affecting the composition of the lignocellulose substrate, such as plant maturity, will determine the contribution that each enzyme makes in the fate of lignin from lignocellulose. This was shown in a recent study (Mason et al. 1990) in which (U-^{14}C) phenylalanine was injected into spring barley plants at frequent intervals during 140 d growth. Autoadiography of the plants revealed that, apart from high concentrations around the injection sites, radioactivity was evenly distributed throughout the plants. (^{14}C lignin) lignocellulose was prepared from plants cropped at 30, 58, 82 and 140 d after sowing, using organic and aqueous solvents followed by enzymic hydrolysis with commercial protease; portions of each were then also treated with polysaccharidase preparations. Weight loss due to solvent and enzymic treatments was greatest in the youngest plants, resulting in a preparation of ^{14}C lignocellulose of specific activity 2-8-fold higher than that of preparations from more mature plants.

When subjected to crude preparations of extracellular protein from either *P. chrysosporium* or the actinomycete *Streptomyces cyaneus*, the substrates derived from the youngest plants (30 d) were particularly susceptible to solubilization of lignocellulose by polysaccharidases (determined by comparison of their action on substrates with and without previous polysaccharidase treatment). Substrates prepared from plants after 58 d growth (i.e. fully grown) showed significant levels of lignocellulose solubilization that were not due to the action of polysaccharidases. When used in similar assays the oldest crops (82 and 140 d) were solubilized at very low levels. Thus three different types of result were obtained, depending on the age of the cropped material.

The *Phanerochaete chrysosporium* lignin degradation system

A dozen years ago the situation was that it had been shown that the white rot fungi could mineralise lignin to CO_2 and that *P. chrysosporium* had been identified as a good experimental organism. Two major facts were known about such degradation (Kirk & Farrell 1987).

1. Overall, it is an oxidative rather than a hydrolytic process. Since no enzyme mechanism had yet been identified one hypothesis was that lignin degradation depended upon the production of free radicals (e.g. singlet oxygen or hydroxyl radicals) that would effect a chemical degradation (Hall 1980).
2. As mentioned earlier, lignin degradation is not inducible by lignin itself but is triggered by severe carbon or fixed nitrogen limitation (Keyser et al. 1978). The biological rationale is that lignin is degraded as a stress response, so that the organism can obtain access to further sources of nutrients and energy previously made inaccessible by the presence of the lignin. Note that lignin does not contain nitrogen.

A number of groups were already involved in studies on *P. chrysosporium* and other became involved at this time. In the intervening years the following major developments have occurred:

1. The groups of Kirk and Gold have described

lignin peroxidases; these extracellular haem-containing enzymes can cleave model dimers representing some of the different bond types present in lignin. *P. chrysosporium* strain BKM-F-1767 produces a family of such enzymes; they have molecular weights of around 40,000 and are glycosylated (Kirk & Farrell 1987).

2. The discovery of these enzymes immediately raised the issue of how the H_2O_2 that they require is generated. This could be derived from the breakdown of carbohydrates and/or of the products of lignin de-polymerisation; therefore the process of lignin degradation is likely to be coupled to the degradation of cellulose and/or hemicellulose.
3. As a development of the discovery of lignin peroxidases, a mechanism involving the formation of substrate radical intermediates was proposed (Kirk & Farrell 1987); such radicals might invade the lignin molecule and be the immediate effectors of its degradation. A mechanism of this type would account for the range of bonds cleaved, and makes these enzymes of particular interest.
4. A second class of enzymes, the manganese peroxidases, have been defined (Kuwahara et al. 1984). These oxidise Mn(II) to Mn(III) and it is proposed that such Mn(III), chelated to organic acids, functions as an active radical that can mediate oxidative depolymerisation of lignin in wood (Glenn et al. 1986).
5. Both cDNA and genomic sequences of lignin peroxidase genes from *P. chrysosporium* have been published by many groups. It has been shown that there are families of lignin peroxidase and Mn peroxidase-related genes (Brown et al. 1988; Godfrey et al. 1990).
6. It has been suggested that peroxidases can depolymerize lignin more effectively in non-aqueous solvents (Dordick et al. 1986). If this is indeed the case (Lewis et al. 1987), it has relevance to the technological exploitation of lignin peroxidases and lignins.
7. Since peroxidases are implicated in polymerisation as well as depolymerisation of lignin, it has been particularly important to establish whether lignin peroxidases and Mn peroxidases do indeed have *in vitro* depolymerising activity. This has now been established using DHP and each of these enzymes (Hammel & Moen 1991; Wariishi et al. 1991).
8. It has been possible to elucidate the sexual cycle, and establish a mating system, using auxotrophic markers (Alic & Gold 1991). A genetic map has been constructed for *P. chrysosporium* using RFLP analysis (Raeder & Broda 1986; Raeder et al. 1989a), and it has been shown that the lignin peroxidase-related sequences are clustered (Raeder et al. 1989a). This has been largely confirmed using pulse field gel electrophoretic methods (Gaskell et al. 1991).
9. *P. chrysosporium* and *T. reesei* clearly have different strategies of action on lignocellulose, since *T. reesei* does not attack lignin. However, is *P. chrysosporium* typical among the white rot fungi? Interest exists in the range of extracellular enzymes produced by other fungal species such as *Phlebia radiata*, (Niku-Paavola et al. 1990), *Coriolis versicolor* (Eriksson et al. 1990), *Rigidosporus lignosus* (Galliano et al. 1991) and *Dichomitus squalens* (Pham et al. 1990).

The present model for lignin degradation in *P. chrysosporium* is that the system consists of manganese and lignin peroxidase enzymes together with the H_2O_2 generation system that they require, and that this is coupled to carbohydrate breakdown. This model is useful in that it is open to a number of *in vitro* and *in vivo* tests.

Lignin peroxidase genes: multiple sequences and differential expression

As stated above, a number of groups have reported lignin peroxidase and Mn-peroxidase gene sequences. In our case, the cloned sequences carrying putative lignin peroxidase genes were found as a set among clones isolated from *P. chrysosporium* strain ME446 because they carried genes expressed specifically in secondary metabolism (Raeder & Broda 1986). In order to establish which of this set were expressed, oligonucleotide probes were made

that would distinguish between them by hybridising differentially to their mRNA transcripts. The surprising result obtained was that none of the four sequences that we had characterised was expressed (James et al. 1992).

An alternative approach was then used to resolve this paradox; this was to construct a cDNA library (using mRNA prepared from mycelium grown on ball milled straw), and partially sequence lignin peroxidase related cloned sequences identified within it. It was found that only one class of cDNA was present, with a sequence that was different from any of those previously identified. The conclusion that only one of the sequences is expressed under our conditions is different from the situation found by others, with another strain of *P. chrysosporium* (Leisola et al. 1987; Stewart et al. 1992). However, it is consistent with our own finding that only a single lignin peroxidase protein peak is present on chromatography of supernatants of strain ME446 (Birch 1988), another observation that has been different from that published by others (Kirk & Farrell 1987). In a situation where only a single gene is expressed, in principle it should be possible to test the hypothesis that lignin peroxidase is necessary for lignin degradation. This requires the disruption of the expressed sequence, which in turn depends upon the development of homologous integrative transformation (see later).

Genetic factors affecting extracellular lignin peroxidase activity in P. chrysosporium

Earlier work referred to above had shown that *P. chrysosporium* ME446 has two genome equivalents with abundant RFLPS; 53 strains derived from basidiospores obtained from the parental strain each had a single genome equivalent, with different combinations of alleles for the RFLP markers. Analysis of the RFLP composition of this set of strains yielded a genetic map, the first RFLP-based map for a fungus (Raeder et al. 1989a).

Is the extensive polymorphism at the DNA level (evidenced by RFLPs) mirrored by polymorphism at the level of performance? The basidiospore-derived strains that had been used for map construction now provided a set (with known compositions of alleles) with which this question could be asked. It was shown that there was indeed substantial variation in levels of extracellular lignin peroxidase levels between these strains (Raeder et al. 1989b). The amount of such enzyme activity was independent of the allele distributions of the lignin peroxidase gene clusters, but correlated with the allele distribution of another locus. This locus appears to control the spread of the lignin peroxidase-active state within the mycelial mat, and may be the mating type locus.

Other parameters examined with these strains were weight loss during the onset of the ligninolytic phase and the appearance of foci of brown coloration that spread, and that correlate with the amount of extracellular lignin peroxidase activity. A model was put forward for the onset of ligninolytic activity: this proposes two switches, the first resulting in either more than 42% or less than 28% weight loss in the mycelium, and the second (dependent on there having been extensive weight loss) resulting in more or less rapid spread of the brown coloration.

Improvement of performance

In principle, at least three routes to improvement in performance can be used separately or together. First, growth conditions can be optimised, whether it is for individual activities, e.g. extracellular lignin peroxidase production, or for the overall process of mineralisation of lignin to CO_2. For instance, we have identified conditions under which lignin, cellulose and hemicellulose are degraded simultaneously; these use ball-milled straw as the substrate, and sodium acetate buffer, pH6 (Birch 1988). Second, one can introduce mutations. This has been done for cellulase-deficiency (Eriksson et al. 1983; Eriksson et al. 1990); cellulase-deficient mutants that are still able to degrade lignin (perhaps obtaining the required H_2O_2 from hemicellulose degradation) might give a nutritionally upgraded feed for cattle since the cellulose is spared. Another class of mutant (that would require the development of transformation methods) would be to cou-

ple lignin peroxidase expression to an inducible promoter such as that for a cellobiohydrolase I (CBH I) gene. The third approach is to exploit the genetic diversity of *P. chrysosporium* strains and of their basidiospore-derived progeny in a programme of crosses. This system could provide a model for fungal strain improvement, in which the question that is asked is how far can one go exploiting such diversity, compared with the classical strain improvement programmes such as those for penicillin production and that of cellulase production by *T. reesei*, which have consisted of mutagenesis and screening in single lineages.

Such a strain improvement programme would involve crosses between basidiospore derived strains of known composition and of opposite mating types to establish strains with two genome equivalents, from which further basidiospore-derived strains would be obtained by fruiting. Correlations would then be made between levels of individual activities, overall performance and genetic composition, and strains for further crosses would then be chosen. Such a programme depends upon there being rapid, reproducible and convenient assays for the various parameters of performance, e.g. supernatant lignin peroxidase activity and lignin mineralisation.

We have found that optimal lignin mineralisation and optimal levels of extracellular lignin peroxidase are produced under different growth conditions. Indeed, under the conditions used for optimal lignin degradation, measuring $^{14}CO_2$ release from ^{14}C-labelled DHP or (^{14}C-lignin) lignocellulose (dimethyl succinate as buffer, pH4.5, and 2% glucose), no extracellular lignin peroxidase activity is found (A. Wyatt, P. Broda, unpublished results). However, this result may arise from entrapment of lignin peroxidase in the polysaccharide slime which is abundantly produced in the high glucose media found to be optimal for lignin mineralisation. Thus the measurement of extracellular enzyme levels may not be a good measure of the amount of enzyme available for lignin degradation and it may actually be misleading. Oligonucleotide probes for mRNA provide an alternative means of assaying for lignin peroxidase synthesis (James et al. 1992). An approach already being used by others is enzyme localisation studies using specific antibodies with either transmission electron microscopy or light microscopy (Blanchette et al. 1989; Daniel et al. 1989; Lackner et al. 1991).

We have asked whether there are differences with respect to ^{14}C DHP mineralisation between strains (A. Wyatt, P. Broda, unpublished results). A set of homokaryotic basidiospore-derived strains were compared with the parental strain ME446 and with a number of putative heterokaryotic strains obtained by crosses of the homokaryotic strains. There was considerable variation in the values obtained with independent cultures of each of the individual strains. A possible reason for such variation was the particulate and insoluble nature of the DHP substrate. However, such independent cultures of the same strain were more variable in performance than could be accounted for by this limitation on the experiment. Even so, there were still reproducible differences between the strains, so that in principle a strain improvement programme for DHP mineralisation is possible, but it would involve much effort because of the experimental variability in the system.

Expression of cellulase genes

One cellulase sequence from *P. chrysosporium*, from a CBH I gene, has been published (Sims et al. 1988). The argument for the nature of the CBH I sequence was based on its close homology with that of the archetypal CBH I sequence of *T. reesei*.

The genomic library of strain ME446 was also probed with endoglucanase I (EG I) DNA from *T. reesei*. The clones that were isolated and characterised contained CBH I-like sequences rather than EG I-like sequences; this result can be explained on the basis that in *T. reesei* there is a sequence that is common to the genes for these two types of enzyme (Knowles et al. 1987). These sequences would code for a second class of CBH I enzyme, different both from that previously reported by us and those described by another group (Covert et al. 1992). Probing with the two classes of CBH I DNA sequences revealed yet more related sequences in *P. chrysosporium* DNA digests. A similar result

was obtained with DNA digests from a monokaryotic derivative of strain ME446, excluding an explanation for such complexity based on any RFLPs between allelic sequences in the heterokaryotic parent (P.F.G. Sims, unpublished).

Sequences of both CBH I classes were expressed when ball-milled straw was used as substrate, but not when glucose was used. A cDNA clone of the original (CBH I.1) type had 12 nucleotide differences (eleven of them silent) from the archetypal genomic sequence, indicating that it was a closely related sequence, perhaps an allelic variant, that was expressed. Two internal introns and two upstream introns were demonstrated. These latter introns are therefore located within the probable regulatory region of this gene and their precise role and influence on its expression may be of considerable importance. Similarly, cDNA of a possibly allelic variant of the CBH I.2 genomic sequence was found to have multiple base differences from the original sequence (mostly silent), and an upstream intron and an internal intron had both been spliced out. In addition, in this case differential splicing was also indicated. Thus *P. chrysosporium* has a CBH I system that has multiple non-allelic sequences, possible allelic differences, differential splicing, and differential regulation. The continued failure to isolate EG I like-sequences raises the question of whether such genes are present in *P. chrysosporium*.

A CBH II probe from *T. reesei* was used to isolate homologous sequences in the *P. chrysosporium* genomic library. Probing against a digest of total DNA of *P. chrysosporium* showed that in this case there was a unique locus, unlike with CBH I.1 and CBH I.2 (see above). Nevertheless, sequencing of both genomic and cDNA clones showed that there were two variants of this sequence, which can therefore be regarded as allelic (C. Tempelaars, P.F.G. Sims, P. Broda, unpublished results). This gene contains 6 introns, all of which are in the coding region. There are 5 single base differences within the intron sequences.

Towards a transformation system

A transformation system that allows insertional mutagenesis of *P. chrysosporium* is critical if we are to fully exploit the possibilities offered by DNA manipulation techniques. A site-directed mutagenesis experiment has allowed the isolation of the CBH I.1 promoter in a form that can be precisely coupled to other coding sequences. We have taken advantage of this in the development of a transformation vector for use in *P. chrysosporium*. This contains the coding sequence of a selectable bacterial marker, phleomycin resistance, coupled to the CBH I.1 promoter (P. Birch, P.F.G. Sims, P. Broda, unpublished results). Since phleomycin is toxic to *P. chrysosporium* as well as to bacteria, we hoped that this would facilitate controllable expression of drug resistance and thus selection of transformants. Experiments using the monokaryotic *P. chrysosporium* strain referred to above showed that stable apparently transformed isolates can indeed be recovered. However, analysis of these putative transformants suggests that transforming DNA is not integrated into the genome as is typically seen in filamentous fungi, but may be maintained extrachromosomally. Other groups have reported both integrative but non-homologous transformation (Alic & Gold 1991) and autonomous transformants (Randall et al. 1991; Randall & Reddy 1992).

We have also begun to evaluate other systems for integrative transformation. We have focused on those that offer a clear route to an homologous selectable marker and for which suitable recipient strains can themselves be easily isolated by positive selection strategies. Three such systems have been or are being assessed:

1. In a number of fungi strains deficient in nitrate reductase have been positively selected by their resistance to chlorate and also the nitrate reductase genes have been identified using heterologous probes. Although mutants of *P. chrysosporium* were isolated, we were unable to demonstrate homology between the *P. chrysosporium* genome and the *A. nidulans* nitrate reductase gene; therefore work on this system was not taken further.

2. The TrpC gene of *P. chrysosporium* was sequenced (Schrank et al. 1991). Sequence analysis of this gene has defined restriction fragments that contain the whole coding sequence and the putative promoter; such fragments could therefore be incorporated into vectors for use as a selectable marker in transformation experiments. The genetic organisation of the multifunctional TrpC gene has novel features compared with ones known from other fungi. We have used Polymerase Chain Reaction technology to demonstrate that it is unique in that it contains an intron. The same technique showed that another *prima facie* candidate for an intron was in fact not one, but instead codes for an additional amino acid sequence not found in other fungi.
3. We have also isolated and characterised the acetyl-CoA synthetase (FacA) gene of *P. chrysosporium* (Birch et al. 1992) as well as mutant strains lacking this activity, which is essential for growth on acetate. This will allow the evaluation of a second homologous marker system.

Actinomycete bacteria

There have also been studies on the role of actinomycete bacteria in lignocellulose biodegradation (McCarthy 1987). Actinomycetes, which have the same invasive habit as fungi, are common in decaying lignocellulose systems such as compost. Early studies (Phelan et al. 1979) indicated that a *Streptomyces* strain, in addition to being able to degrade the cellulose from lignocellulose and thereby contributing to a substantial weight loss of the substrate, is also able to degrade lignin, although to a much lesser extent. We began a research programme by screening actinomycetes for ligninolytic activity (McCarthy & Broda 1984). Organisms of several genera, *Streptomyces*, *Micromonospora*, *Thermomonospora* and *Actinomadura*, were identified as being able to grow on grass lignocellulose (McCarthy & Broda 1984). Whereas in liquid cultures of *P. chrysosporium* 30–40% of the (^{14}C lignin) lignocellulose used was converted to $^{14}CO_2$, the levels achieved by the actinomycetes were much lower (typically 10%, McCarthy & Broda 1984). However, in addition, some strains solubilize up to 30% of the total lignin content; that part which is not mineralized to $^{14}CO_2$ accumulates in the supernatant of cultures, and may represent an end product of lignin degradation.

This soluble product has been termed acid precipitable polymeric lignin or APPL (Crawford et al. 1983; Pometto & Crawford 1986) because it is insoluble when the pH of the medium is lowered. It has been partially characterized using high performance liquid chromatography (HPLC) (Borgmeyer & Crawford 1985; Mason 1988) and nuclear magnetic resonance (NMR) spectroscopy (McCarthy et al. 1986). APPL is a population of components in the apparent molecular weight range 1 to 3 $\times 10^5$ and the size distribution of the APPL products is specific for each organism cultured on ball milled straw (Mason 1988).

Differential degradation of lignocellulose components by different actinomycete species

Do different actinomycete strains have similar profiles of activity against the different components of lignocellulose? This was studied for ground and ball milled barley straw samples in 3-week incubations in liquid cultures of *Streptomyces cyaneus*, *Thermomonospora mesophila* and *Actinomadura* sp. (Zimmermann & Broda 1989). High weight losses from both substrates were found after 3 weeks of incubation. Water-soluble components released from the substrates did not accumulate in the supernatants but were partially utilised.

Because of the partial solubility of the lignin-carbohydrate component of straw in water at higher temperatures and pressures, part of the substrate was solubilised during the initial autoclaving; such water-soluble material contains not only low molecular weight compounds but also lignin-carbohydrate complexes of higher molecular weight. Although all three strains caused further weight losses from the substrates, they differed with respect to the removal of lignin. In addition to that removed by autoclaving, only *S. cyaneus* and *T. mesophila* removed a further part of the lignin from the sub-

strate residues. This work demonstrates the importance of taking into account the effect of autoclaving and that, as with the fungi, different strains have different strategies for attacking lignocellulose.

Elemental analysis confirmed that pronounced chemical and structural changes in the lignin-carbohydrate polymer had occurred during incubation; these included oxidative reactions and the removal of methoxyl groups from the lignin. Gel chromatography of the lignin-carbohydrate components showed solubilisation of lower molecular size components, particularly by *S. cyaneus* and *T. mesophila*.

Degradation of a non-phenolic lignin model compound by S. cyaneus

Although we have not made extensive use of low molecular weight lignin model compounds, we have made one contribution in this area (Zimmermann et al. 1988a). It is known that many actinomycetes can utilise aromatic substrates including lignin-related phenols; some can perform transformations that include hydroxylations, demethylations and aromatic ring cleavages. Although an arylglycerol-β-phenyl ether was readily degraded by various actinomycetes, the presence of a substituent on the phenyl ether moiety inhibited or completely prevented its breakdown. We were able to show that cultures of *S. cyaneus* can degrade a non-phenolic dimeric β-O–4 model compound; by identifying the main products, we were able to show that the mechanism involved was cleavage of the C-α-C-β bond.

Cell-free solubilisation activities

An important step towards elucidating the system in actinomycetes was to develop a cell-free system (McCarthy et al. 1986). We showed that supernatants from a *T. mesophila* strain yielded a soluble product that was a complex of lignin, carbohydrates (especially pentoses derived from the hemicellulose) and protein. Chemical analysis and solid-state ^{13}C NMR spectroscopy both indicated similarities with humic acid, suggesting that actinomycetes might have an important role in the process of humification. The NMR spectra revealed an increase in the number of α-aryl ether linkages, one of the most common linkages in lignin, but there was no evidence for extensive demethoxylation.

It was found that *T. mesophila*'s solubilizing activity may be induced by either xylan or ball-milled straw, but not to a significant extent by indulin AT (an industrial lignin). An obvious possibility was that the solubilizing activity is primarily xylanolytic in nature. However, supernatants from strains of two other species (*Thermomonospora fusca* and *Saccharomonospora viridis*), which gave much higher levels of xylanolytic activity with xylan showed no significant levels of ^{14}C solubilization, suggesting that this is not the case, at least with these strains.

Later work involved the resolution of different activities from *S. cyaneus* MT813 that had been grown on ball-milled straw (Mason et al. 1988). This was achieved using ammonium sulphate precipitation and gel filtration of extracellular proteins. Material with an apparent molecular weight of about 20,000 accounted for almost all of the solubilization not attributed to cellulase activity. Since this had neither cellulase or xylanase activity, it was termed a lignin solubilizing activity.

Extracellular xylanases from actinomycetes

As well as examining activity against lignin in lignocellulose, we tested a number of thermophilic isolates for extracellular xylanase production. In each, xylanase activity was optimal within the temperature range 60–75 °C and was maintained between pH 5 and pH 8. In all cases, the xylan was initially hydrolysed to a mixture of oligomeric products, indicating that the main activity was of the endoxylanase type (McCarthy et al. 1985).

One xylanolytic strain of *S. viridis* was unusual in that it had no detectable cellulase activity. This was judged to be of some industrial interest, e.g. as a way of removing xylans from pulp to produce pure cellulose. It was also of value as a way of assessing

the contribution of xylans to the papermaking properties of pulp (Roberts et al. 1990). Application of crude enzyme preparations to birch wood pulp resulted in the selective removal of approximately 20% of the total xylan. Paper handsheets from pulp treated in this way demonstrated modifications in a number of physical properties, of which a decrease in burst and long-span tensile strength were the most significant. The results suggested that specific removal of xylan reduces the extent of the inter-fibre bonding but does not significantly weaken the fibres themselves.

Xylan is mainly composed of 1,4 linked β-xylopyranosyl units. The presence of various substituents including arabinofuranosyl, glucuronyl and acetyl groups has a pronounced influence on the chemical and structural properties, and also on the enzymatic degradability of xylan in lignocellulose. We screened for xylanase, acetyl esterase and α-arabinofuranosidase activities in a number of actinomycetes, using oat spelt xylan (a de-acetylated preparation) and ball milled straw (Zimmermann et al. 1988b). The latter substrate was used as the source of a more complex and native form of hemicellulose. Each of the enzyme activities could be detected in at least some of the strains after different times of incubation; their production was also dependent on the growth medium.

Xylanolytic activities of S. cyaneus

In preliminary experiments, we sought to optimise the conditions for expression of cellulase and lignin solubilisation-activities as well as xylanase activity (Wang et al. 1992a). Eighteen possible carbon sources (mono-, di- and polysaccharides, aromatic compounds and ball milled straw) were tested for their ability to elicit these activities. Of these, only ball milled straw and xylan had this effect, and xylan was chosen as the carbon source in most subsequent experiments. pH 7.0 was also found to be the optimum for the expression of all activities in cultures.

The fact that both xylan and ball milled straw can induce all three lignocellulose solubilisation systems raises questions about their regulation. Both substrates are too large and complex to enter cells, so how does induction occur? Why should xylan induce all three activities? It is possible that the xylan used (oat spelt) is not pure, but it also could indicate that xylan degradation is a key step. The prediction was made that *S. cyaneus* produces a 'sensor' xylanase constitutively and that this degrades any xylan present in the environment to smaller molecules that can act as intracellular inducers. The testing of this prediction is described below.

Demonstration of three xylanases from S. cyaneus

Supernatants from xylan-grown cultures were precipitated with ammonium sulphate, and gel filtration and SDS-PAGE allowed the resolution of three xylanases and their existence was confirmed using a zymogram method. The apparent sizes were 37 (Xylanase I), 34 (II) and 45 (III) kD. After further purification, these were characterised for their temperature optima (72 °C, 65 °C and 53 °C, respectively) and pH optima. Xylanase I and III had activity between pH 4 and 10.5 whereas xylanase II only had activity between pH 5.5–7.5.

Novel turbidimetric assay for xylanase activity

Xylanase activities are usually determined by measuring the release of reducing sugars from the substrate. However, this method cannot offer accurate results where the presence of both endo- and exo-xylanases results in synergism. Moreover, it is not sensitive enough to monitor very low levels of xylanase activity or activity in the presence in the mixture of reducing sugars. The oxidation or reduction of products can also affect the values obtained. An alternative method was required for the detection of sensor xylanase activity.

The alternative method developed (Wang et al. 1992a), involves the use of a stable xylan preparation that consists of xylan chains with sizes in the range of 70–100 residues. Such molecules are in suspension rather than solution (like for instance *E. coli* cells), and their concentration can therefore

be measured by the deflection of light (e.g. at 600 nm); that is, turbidimetrically. Hydrolysis of such particles by endoxylanase results in their disintegration into soluble products, and this can be measured as a decrease in turbidity. We have shown that, over a range, linear kinetics are obtained.

Regulation of synthesis of S. cyaneus *xylanases*

The kinetics of xylanase induction by xylan were studied over a 17-day period. It was observed that there was an increase in reducing sugar concentration before any large amount of xylanase was induced, and that at this point the reducing sugar concentration declines. A possibility is that there is induction of transport proteins at this point. Mixed incubation with xylan and glucose resulted in the delay of xylanase induction until the glucose concentration had declined to a low level, suggesting that glucose repressed xylanase induction. However, in the presence of glucose, as in its absence, there was an increase in reducing sugar concentration and a decrease in xylan concentration, suggesting the existence of a constitutively produced low level of activity. It was shown that xylanase III is detectable before xylanases I and II on induction, and the novel assay was used to show that with glucose-grown cultures this is the only xylanase activity that is present. It is therefore proposed that xylanase III is the predicted constitutively produced enzyme and that xylanases I and II are inducible.

Cloning of S. cyaneus *xylanase genes*

Investigation of the lignocellulose degrading enzymes from *S. cyaneus* has involved the preparation of genomic libraries in *E. coli* using both lambda and plasmid vectors. However, the introduction of DNA constructs containing *S. cyaneus* DNA into *E. coli* resulted in very low transformation frequencies.

Strains of *E. coli* derived from K12 contain a methylcytosine-specific restriction system encoded by *mcrA* and *mcrB* and we hypothesised that the incoming *S. cyaneus* DNA was being restricted. Strains mutated in the *mcrAB* region could be transformed by constructs containing *S. cyaneus* DNA at a much higher frequency than before, suggesting that in previous *E. coli* hosts the incoming *S. cyaneus* DNA had indeed been cleaved at frequent methylcytosine residues. The improved transformation frequencies thus obtained made possible the construction of gene libraries with adequate-sized inserts (Wang et al. 1992b).

As stated above, three xylanases are produced by *S. cyaneus*. One is expressed constitutively whereas the other two are only expressed when xylosaccharides are present in the medium. An *S. cyaneus* DNA library was constructed in Lambda gt11 by inserting *Eco*RI-digested DNA into the *Eco*RI site of the *lacZ* gene in the vector. The expression library was screened by xylanase activity using RBB-xylan as substrate. Two classes of xylanase-producing clones were identified; they contained different inserted fragments that had no DNA homology with each other. These inserts were then subcloned into pUC18, mapped with restriction enzymes and examined for xylanase production. A subclone derived from one of the classes of insert expressed xylanase at a very low level whereas a subclone derived from the other class of lambda insert expressed a very high level of xylanase activity, which was secreted from the *E. coli* host cells (Wang 1991).

The first report of cloning and sequencing of *Streptomyces* xylanase genes was that of Shareck et al. (1991). The protein sequences inferred from the DNA sequence showed similarities with those of one or other of two sets of glycanases that had already been described. DNA sequencing of the two clones that we have isolated is in progress and shows significant amino acid similarities between the derived protein sequences and conserved regions of both xylanases and cellulases from other prokaryotes.

Conclusions

Biotechnology has been primarily concerned with the health care and food industries, and agricultu-

ral biotechnology is now assuming greater importance. However, the use of biotechnology to solve environmental problems and for the rational utilization of renewable resources are still in their infancy. The reasons include problems of what the precise objectives are, the complexity of the ecosystems, concern about release of genetically manipulated organisms, the generally low value and bulk of the materials involved, the lack of public and private funding for the basic research needed, and perhaps inadequate recognition of the true environmental costs of existing processes, in terms of pollution and inefficient utilisation of resources.

There are strong arguments for improved and enhanced production and utilisation of biomass, which include minimising the release of CO_2 by switching from once-off utilisation of fossil fuels to use of renewable resources. Properly managed, biomass production can also minimise soil degradation and enhance employment. However, each of the global regions has different problems and opportunities, and in each the overall process from production to utilization and also disposal of wastes must be appraised in terms of Clean Technology. That is, solutions to pollution problems should not be 'end of pipe', but should incorporate the avoidance or minimisation of creation of pollutants within the process in the first place.

Some crops, e.g. wood and straw, will be used as fuels, especially in countries such as Denmark that have a heavy tax on the domestic use of fossil fuels, and they can be used on the farm, e.g. for grain drying and greenhouse heating. Such processes require very large storage facilities. Science and technology has already made a major input to improving productivity of biomass, e.g. in tree propagation, silviculture and cropping. However, the overall process of paper production involves many subsequent steps, including utilisation and disposal of bark, branches and roots, transport, milling, thermomechanical or chemical pulping, bleaching, and disposal of wastes, and also re-cycling of waste paper and board into fresh pulp. The reappraisal of the whole of this complex process must be in terms that allow real environmental costs to be included in the balance sheet; the same holds for the conversion and utilisation of lignocellulose for animal nutrition. Such analyses would identify the pressing aspects from the point of view of devising novel technologies, which might, but need not, have biological components.

Biotechnology has two roles: the obvious one is to design processes with a biological component, whether that involves whole organisms or enzymes; the other is to provide the understanding that is still lacking on the interaction of degrading and modifying organisms with their substrates. Specific scientific questions include how in nature is encrustation by lignin disrupted, and what are the immediate fates of lignin and the other components. Because of the progress made in a number of laboratories, including our own, each adopting its distinctive approach, this is a good time to identify some of the major problems and opportunities for the immediate future (Broda et al. 1989).

1. Because of the chemical and structural complexity of the lignocellulose substrate, which includes water-soluble and insoluble components, a description of the overall process of lignocellulose degradation does not yet exist for any substrate or organism. Thus the degree of solubilization and modification of the different components, the further fate of the material that is solubilized and to what extent particular fungi or actinomycetes have similar effects on the lignocellulose substrate are not known at the chemical level. With respect to the lignin component specifically, in this article the use of model compounds, DHP and radiolabelled lignin have been emphasized. However, it is important to complement these with other approaches such as chemical and gravimetric analysis of the fate of the substrate, improved liquid chromatography methods, and use of NMR spectroscopy and other non-destructive techniques.
2. It is well known that in both prokaryotic and eukaryotic systems degradation of cellulose involves a number of enzymes; in some organisms a large number are involved (Bayer & Lamed, this issue). Hemicellulose degradation involves enzymes for both main-chain and side-chain degradation, though little is yet known of the details, largely because hemicelluloses

themselves are so poorly understood, and model compounds are generally not yet available. The degradation of each of these classes of substrate will be better understood with the availability of individual enzymes, in a pure form, made by genetic engineering methods. It will then be possible to do experiments to determine the synergistic actions of different enzymes, confident that each is free of contaminating activities. The availability of better-defined carbohydrate substrates and assays are also essential for such studies.

3. *P. chrysosporium* is the archetypal organism for lignin degradation studies. Lignins from a number of sources (wood, straw, bagasse) are all degraded efficiently and furthermore molecular and classical genetics as well as biochemical methods can be used, as has been discussed. Moreover, it has a sharp switch from growth to the ligninolytic idiophase. In this, it may be atypical, and it will be of interest to what extent the white rot fungi have common systems with respect to the enzymes and their regulation. However, it is possible that lignin degradation by *P. chrysosporium* simply requires lignin peroxidases, manganese peroxidases and the complementary H_2O_2 generating system. It will be important to determine whether there is any spatial organization of the component enzymes of the system (see e.g. Ruel & Joseleau 1991). Other questions of importance are the nature of the coupling of lignin degradation to carbohydrate metabolism at the level of gene expression, the reason for the presence of multiple genes and the basis for the variability in performance discussed earlier.
4. The actinomycete system is at an earlier stage of study, mainly because with the available techniques lignin solubilization and degradation have been more difficult to demonstrate rigorously than with the fungal system. However, it is a promising system which is significantly different from the *P. chrysosporium* system, and where different strains have differing characteristics. Parallel studies of lignin degradation with the white-rot fungi and actinomycetes might provide interesting comparisons and suggest avenues of exploitation utilizing these differences. If the aim is the complete destruction of the lignin component of lignocellulose then the choice of a fungal system would seem obvious. However, as described above, such degradation of lignin only occurs during the idiophase, following severe nitrogen and carbon depletion. In contrast, with actinomycetes lignin solubilization occurs during primary growth and has no requirement for the presence of H_2O_2. Thus although lignin degradation may be more limited, it may have the advantage in making available solubilized products of lignin degradation (APPL) rather than its total destruction to CO_2.
5. The progress made in this area means that we can now begin to assemble a specification for a useful organism to exploit particular lignocellulose resources. One objective of particular relevance to the U.K. is the improved utilization of lignocellulose for animal nutrition. In this case, one can envisage an organism that degrades lignin and hemicellulose efficiently but has no activity against cellulose, with the ability to grow in the low pH and high temperature commonly encountered in silage. Alternatively, one might use enzyme preparations from such an organism grown under industrial fermentation conditions. However, it is still too early to predict which group of micro-organisms, e.g. white-rot fungi or actinomycetes, will produce the best candidate for exploitation in this manner.
6. Lignin biodegradation provides a good model for the biotechnology of open systems of an intermediate scale. It is thus different both from the fermenter-scale systems in use for e.g. production of interferons and also from typical large chemical engineering processes. A possible mode for lignocellulose utilization is solid substrate fermentation (comparable in complexity to mushroom composting or silage production) which would be effective on the farm and suitable for use in some developing countries. The emphasis would not be on major capital costs, but on devising robust organisms appropriate to the local context.

7. As stated at the beginning of this article, rational discussion of whether biotechnology can contribute to the utilization of lignocellulose requires a better understanding of lignocellulose biodegradation. Until this is achieved, it will not be possible to assess the economics of such processes in particular contexts. *P. chrysosporium* has served as an excellent model system; it degrades a wide range of lignocellulose substrates (hardwood, softwood, straw and bagasse) and a substantial body of biochemical and molecular genetic knowledge exists for it. Nevertheless, it is important that other fungi should be assessed for their performance on lignocellulose. Heterologous probing using genes from *P. chrysosporium* and *T. reesei* will allow the rapid development of molecular genetics for such organisms. One can envisage the production of enzymes either by the original organism or by cloning the genes into alternative hosts such as yeast or *Trichoderma*, which is able to produce exceptionally large amounts of extracellular cellulases. In some cases the objective would be to tailor the organism to produce a set of enzymes in relative amounts that have been optimized for the intended process.
8. Preliminary experiments have already been reported on the use of cellulase-defective mutants of *P. chrysosporium* as part of a pulping process for papermaking of bagasse. In this case presumably residual sugars and/or the hemicelluloses are utilized by the organisms for the production of H_2O_2. The different ways of strain improvement that are possible include genetic crosses and cloning techniques. An obvious example is to attempt to obtain mutants of *P. chrysosporium* that are able to degrade lignin in normal growth rather than only in starvation. Traditional methods of strain improvement involving mutagenesis may not yield organisms that are sufficiently robust for solid substrate fermentation conditions. The prospects with strains obtained using genetic crosses and/or site directed mutagenesis may be better.
9. The development of particular organisms or of ways of producing enzymes in high yield are only the start of the development of industrial processes. There are many aspects of the problem which will require substantial inputs from microbial physiologists and chemical engineers, in particular, before one can arrive at viable processes.
10. The use of whole organisms would have the advantage of low price compared with the use of enzymes, but the problems of survival and release of whole organisms must be addressed, especially if *in vitro* genetic manipulation techniques are used to construct such organisms.

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