

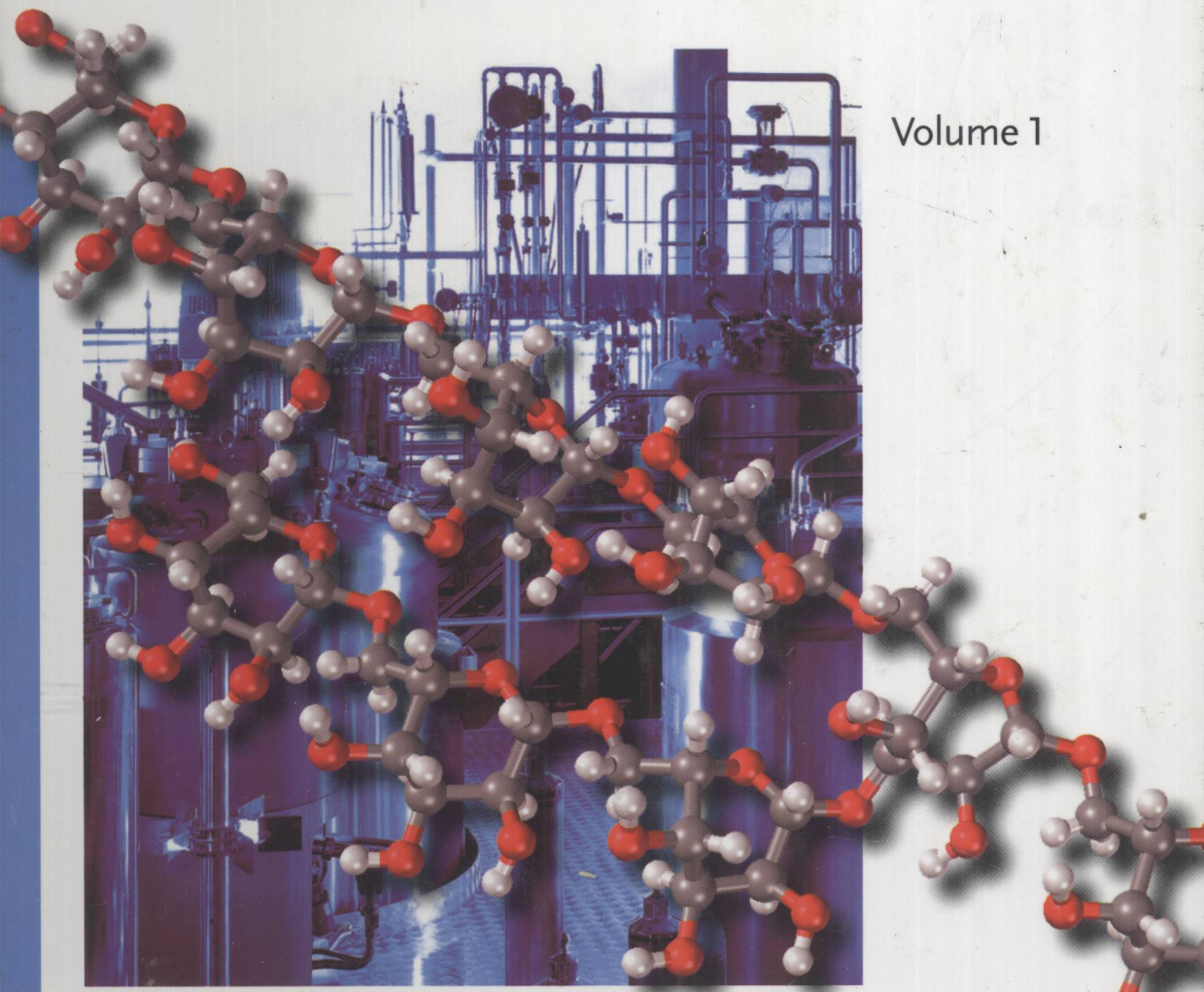
Edited by Alexander Steinbüchel
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Biotechnology of Biopolymers

From Synthesis to Patents

Volume 1



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Biotechnology of Biopolymers

From Synthesis to Patents

Edited by A. Steinbüchel and Y. Doi

Volume 1

Lignin, Coal, Polyisoprenoids, Polyesters and Polysaccharides



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Cover Illustration:

The dextran molecule is a fine example for a biotechnologically produced biopolymer. Scaling up biotechnical production from the detail to the plant goes hand in hand with economic success.

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Preface

Biopolymers and their derivatives are diverse, abundant, and important for life, exhibit fascinating properties and are of increasing importance for various applications. Living matter is able to synthesize an overwhelming variety of polymers, which can be divided into eight major classes according to their chemical structure: (i) nucleic acids, such as ribonucleic acids and deoxyribonucleic acids, (ii) polyamides, such as proteins and poly(amino acids), (iii) polysaccharides, such as cellulose, starch and xanthan, (iv) organic polyoxoesters, such as poly(hydroxyalkanoic acids), poly(malic acid) and cutin, (v) polythioesters, which were reported only recently, (vi) inorganic polyesters with polyphosphate as the only example, (vii) polyisoprenoides, such as natural rubber or Gutta Percha and (viii) polyphenols, such as lignin or humic acids. Biopolymers may occur in any organism, and contribute in most of them by far to the major fraction of the cellular dry matter. Biopolymers fulfill a wide range of quite different essential or beneficial functions for these organisms, including conservation and expression of genetic information, catalysis of reactions, storage of carbon, nitrogen, phosphorus and other nutrients and of energy, defense and protection against attacks by other cells or hazardous environmental or intrinsic factors, sensors of biotic and abiotic factors, communication with the environment and other organisms, mediators of the adhesion to surfaces of other organisms or of non-living matter, and many more. In addition, many of them are structural components of cells, tissues and entire organisms.

To fulfill all these different functions, biopolymers need to exhibit rather diverse properties. They must very specifically interact with a large variety of different substances, components and materials, and often they need to have extraordinary high affinities to them. Finally, they must have a high degree of strength. Some of these properties are utilized directly or indirectly for various applications. This and the possibility to produce them from renewable resources, as living matter mostly does, make biopolymers interesting candidates for industry.

Basic and applied research has already contributed much knowledge about the enzyme systems catalyzing the biosynthesis, degradation and modification of biopolymers, as well as about the properties of biopolymers. This has also resulted in an increased interest in biopolymers for various applications in industry, medicine, pharmacy, agriculture, electronics and in many other areas. However, taking into account the developments over the last two decades and a review of the literature shows that our knowledge is still scarce. The genes for the biosynthesis pathways of many biopolymers are still not available or were

identified only recently, many new biopolymers have been described only recently, and the biological, chemical, physical and material properties have only been investigated for a minor fraction of the biopolymers. Often promising biopolymers are not available in sufficient amounts for this purpose. Nevertheless, polymer chemists, engineers and material scientists in academia and industry have discovered biopolymers as chemicals and materials for many new applications, or are considering biopolymers as model compounds to design novel synthetic polymers.

The complexity and relevance of biopolymers initiated the publication of the ten-volume handbook "Biopolymers", which comprehensively reviews and compiles information regarding (i) occurrence, synthesis, isolation and production, (ii) properties and applications, (iii) biodegradation and modification not only of natural but also of synthetic polymers, and (iv) the relevant analysis methods to reveal the structures and properties. This book series was published between 2001 and 2003. We are very grateful to the publisher Wiley-VCH for recognizing the demand for such a book series and to undertake this large, new project. Publication of the "Biopolymers" was very successful, and the entire book series has even recently been translated into Chinese. Special thanks are due to Karin Dembowski and Dr. Andreas Sendtko and many others at Wiley-VCH for their initiatives, constant efforts, helpful suggestions, constructive criticism and wonderful ideas.

From this book series we selected 39 chapters, which deal with biotechnologically relevant aspects of biopolymers, and which are now published in this separate, new book. By doing this, interested readers from academia and industry will gain access to a very comprehensive book covering the most important applied aspects of biopolymers and their production. We are very grateful to the authors of the selected chapters for allowing the contents of their "Biopolymers" contributions to be included in this new title.

Münster and Saitama
September 2004

Alexander Steinbüchel
Yoshiharu Doi

Contents

Volume 1

Preface	V
I Lignin and Coal	1
1 Synthesis of Lignin in Transgenic and Mutant Plants <i>Jeffrey F. D. Dean</i>	3
2 Biotechnological Applications of Lignin-Degrading Fungi (White-Rot Fungi) <i>Gary M. Scott, Masood Akhtar</i>	27
3 Biotechnological Conversion of Coals into Upgraded Products <i>Horst Meyrahn, Alexander Steinbüchel</i>	55
II Polyisoprenoids	71
4 Biochemistry of Natural Rubber and Structure of Latex <i>Dhirayos Wititsuwannakul, Rapepun Wititsuwannakul</i>	73
5 Biotechnological Processes for Recycling of Rubber Products <i>Katarina Bredberg, Magdalena Christiansson, Bengt Stenberg, Olle Holst</i>	125
6 Biotechnological Processes for Desulfurization of Rubber Products <i>Katarina Bredberg, Magdalena Christiansson, Bengt Stenberg, Olle Holst</i>	141
III Polyesters	159
7 Metabolic Pathways and Engineering of PHA Biosynthesis <i>Kazunori Taguchi, Seiichi Taguchi, Kumar Sudesh, Akira Maehara, Takeharu Tsuge, Yoshiharu Doi</i>	161
8 Metabolic Flux Analysis on the Production of Poly(3-hydroxybutyrate) <i>Sang Yup Lee, Soon Ho Hong, Si Jae Park, Richard van Wegen, Anton P. J. Middelberg</i>	193
9 Fermentative Production of Short-chain-length-PHAs <i>Sang Yup Lee, Si Jae Park</i>	207
10 Fermentative Production of Medium-chain-length Poly(3-hydroxyalkanoate) <i>Ruud A. Weusthuis, Birgit Kessler, Marcia P. M. Dielissen, Bernard Witholt, Gerrit Eggink</i>	235

11	Biosynthesis and Fermentative Production of Short-chain-length Medium-chain-length-PHAs	261
	<i>Sang Yup Lee, Si Jae Park</i>	
12	Production of Polyhydroxyalkanoates in Transgenic Plants	281
	<i>Yves Poirier, Kenneth J. Gruys</i>	
13	Fermentative Production of Building Blocks for Chemical Synthesis of Polyesters	317
	<i>Sang Yup Lee, Sang Hyun Park, Soon Ho Hong, Young Lee, Seung Hwan Lee</i>	
IV	Polysaccharides	379
14	Bacterial Cellulose	381
	<i>Stanislaw Bielecki, Alina Krystynowicz, Marianna Turkiewicz, Halina Kalinowska</i>	
15	Bioemulsans: Surface-active Polysaccharide-containing Complexes	435
	<i>Eugene Rosenberg, Eliora Z. Ron</i>	
16	Curdlan	457
	<i>In-Young Lee</i>	
17	Succinoglycan	481
	<i>Miroslav Stredansky</i>	
18	Alginates from Bacteria	501
	<i>Bernd H. A. Rehm</i>	
19	Xanthan	535
	<i>Karin Born, Virginie Langendorff, Patrick Boulenguer</i>	
20	Dextran	575
	<i>Timothy D. Leathers</i>	
21	Levan	599
	<i>Sang-Ki Rhee, Ki-Bang Song, Chul-Ho Kim, Buem-Seek Park, Eun-Kyung Jang, Ki-Hyo Jang</i>	
22	Hyaluronan	627
	<i>Peter Prehm</i>	
23	Exopolysaccharides of Lactic Acid Bacteria	655
	<i>Isabel Hallemeersch, Sophie De Baets, Erick J. Vandamme</i>	
24	Scleroglucan	679
	<i>Ioannis Giavasis, Linda M. Harvey, Brian McNeil</i>	
25	Schizophyllan	703
	<i>Udo Rau</i>	
26	Alginates from Algae	735
	<i>Kurt Ingar Draget, Olav Smidsrød, Gudmund Skjåk-Bræk</i>	

Volume 2

V Polyamides and Complex Proteinaceous Materials	765
27 Cyanophycin	767
<i>Fred Bernd Oppermann-Sanio, Alexander Steinbüchel</i>	
28 Poly- γ -glutamic Acid	791
<i>Makoto Ashiuchi, Haruo Misono</i>	
29 Modifications of Proteins and Poly(amino acids) by Enzymatic and Chemical Methods	843
<i>Kousaku Ohkawa, Hiroyuki Yamamoto</i>	
30 Biology and Technology of Silk Production	873
<i>Fritz Vollrath, David Knight</i>	
31 Fibrous Proteins from Recombinant Microorganisms	895
<i>Stephen R. Fahnestock</i>	
32 Spider Silk Proteins from Transgenic Plants	929
<i>Jürgen Scheller, Udo Conrad</i>	
33 High-toughness Spider Silk Fibers Spun from Soluble Recombinant Silk Produced in Mammalian Cells	945
<i>Costas N. Karatzas, Nathalie Chretien, François Duguay, Annie Bellemare, Jiang Feng Zhou, Andrew Rodenhiser, Shafiul A. Islam, Carl Turcotte, Yue Huang, Anthoula Lazaris</i>	
34 Structure, Function, and Evolution of Vicilin and Legumin Seed Storage Proteins	967
<i>James Martin Dunwell</i>	
35 Role of Nucleic Acid and Protein Manipulation Technologies in High-throughput Structural Biology Efforts	999
<i>David J. Aceti, Paul G. Blommel, Yaeta Endo, Brian G. Fox, Ronnie O. Frederick, Adrian D. Hegeman, Won Bae Jeon, Todd L. Kimball, Jason M. Lee, Craig S. Newman, Francis C. Peterson, Tatsuya Sawasaki, Kory D. Seder, Michael R. Sussman, Eldon L. Ulrich, Russell L. Wrobel, Sandy Thao, Dmitriy A. Vinarov, Brian F. Volkman, Qin Zhao</i>	
VI Miscellaneous Polymers and General Aspects	1027
36 Inorganic Polyphosphates	1029
<i>Gerard J. J. Kortstee, Mark C. M. van Loosdrecht</i>	
37 Polythioesters	1063
<i>Tina Lütke-Eversloh, Alexander Steinbüchel</i>	
38 Biotechnological Processes for the Production of Monomers for Subsequent Chemical Polymer Synthesis	1081
<i>Sang Yup Lee, Si Jae Park, Young Lee, Seung Hwan Lee</i>	
39 Economic Aspects of Biopolymer Production	1107
<i>Sang Yup Lee, Si Jae Park, Jong Pil Park, Young Lee, Seung Hwan Lee</i>	
40 Index	1139

I

Lignin and Coal

1

Synthesis of Lignin in Transgenic and Mutant Plants

Dr. Jeffrey F. D. Dean

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1	Introduction	4
1.1	Potential Applications	6
1.1.1	Pulp and Paper	6
1.1.2	Solid Wood Products	7
1.1.3	Textile Fibers	7
1.1.4	Biomass	8
1.1.5	Novel Products	8
1.2	Challenges	8
2	Historical Outline	8
2.1	Chemistry	8
2.2	Biology	9
3	Lignin Mutants	9
3.1	Brown Midrib Monocots	9
3.2	Loblolly Pine	10
3.3	<i>Arabidopsis</i>	11
4	Lignin Transgenics	11
4.1	The Shikimate Pathway and Phenylalanine Ammonia Lyase	11
4.2	Cinnamate 4-Hydroxylase and 4-Coumarate-3-Hydroxylase	13
4.3	4-Coumarate:CoA Ligase	13
4.4	O-Methyltransferases	15
4.5	Cinnamoyl-CoA Reductase	17
4.6	Ferulate 5-Hydroxylase	17
4.7	Cinnamyl Alcohol Dehydrogenase	17
4.8	Coniferin β -Glucosidase	19
4.9	Peroxidase and Other Oxidases	19

4.10	Transcription Factors and Other Targets	20
5	Outlook and Perspectives	20
6	Patents	20
7	References	21

4-CL	4-coumarate, CoA ligase
AldOMT	5-hydroxyconiferyl aldehyde O-methyltransferase
<i>bm</i>	<i>brown midrib</i>
C3H	4-coumarate 3-hydroxylase
C4H	cinnamate 4-hydroxylase
CAD	cinnamyl alcohol dehydrogenase
CAGT	5'-diphosphoglucose:coniferyl alcohol glucosyltransferase
CAld5H	coniferyl aldehyde 5-hydroxylase
CBG	coniferin- β -glucosidase
CCoA3H	4-coumaryl:CoA hydroxylase
CCoAOMT	caffeoyl-CoA 3-O-methyltransferase
CCR	cinnamoyl-CoA reductase
CoA	coenzyme A
COMT	caffeate O-methyltransferase
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
F5H	ferulate 5-hydroxylase
<i>fah</i>	<i>ferulic acid hydroxylase</i>
IAA	indole acetic acid
LAC	laccase
MYB	transcription factor related to avian myeloblastosis transforming gene
NMR	nuclear magnetic resonance
PAL	phenylalanine ammonia lyase
<i>rol</i>	<i>Agrobacterium tumefaciens</i> R ₁ -plasmid localized root locus oncogenes
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine

1

Introduction

These are exciting and contentious times for investigators working to understand the structure and biosynthesis of lignin. Technological advances are enabling us to discern with high resolution under nearly in situ conditions previously unappreciated structures and linkages within native lignin

polymers (Dean, 1997; Argyropoulos, 1999). Meanwhile, mutants blocked at specific points in the lignin biosynthetic pathway are being identified in natural populations, or are being created in the laboratory through genetic engineering (MacKay et al., 1997; Provan et al., 1997; Baucher et al., 1998a). At the confluence of these advances there again rages a debate as to the very nature of what does and does not constitute lignin, and

recent findings suggest that the possibilities for manipulating lignin characteristics *in vivo* are far greater than could have been imagined even a few years ago (Lewis, 1999; Ralph et al., 1999a). Lignin researchers appear cursed to live in interesting times, indeed.

In the most general of terms, lignin is a chemically recalcitrant polymer of phenylpropanoid units linked together in a complex and irregular pattern which varies from species to species, tissue to tissue, and cell to cell. Vascular plants use lignin to line their conductive tissues as a barrier to water loss; thus, lignin was instrumental in the spread of these plants throughout the terrestrial landscape. Plants have subsequently harnessed lignin to bind cells together, rigidify their lamellate cell walls into microcomposite structures of remarkable strength, and provide a physical barrier against invading microorganisms. Such chemical characteristics as hydrophobicity and stable, irregular cross-links make lignin an ideal material to limit water loss and stifle pathogen invasion. At the same time, these characteristics have only begrudgingly yielded to chemical analyses and structural dissection, and rapid advancement in the area of structural analysis has really only begun to accelerate in the past decade.

Lignin is most abundant in wood, where it comprises 20–30% of the total dry weight, and constitutes the principal barrier to the production of pulp and paper. Historically, efforts to manufacture paper more economically have provided the principal impetus for studies of lignin structure and biosynthesis. Consequently, the accumulated information about lignin produced in arborescent plants tends to skew our perception of what lignin is and does. Thus, Freudenberg (1968) defined lignin as the heteropolymer resulting from the dehydrogenation of a mixture of three *p*-hydroxycinnamyl alcohols – *p*-coumaryl, coniferyl, and sinapyl alcohols – best exemplified by spruce milled-wood lignin. This definition

was drawn specifically to delineate a perceived difference between the polymer isolated from trees and other higher-order vascular plants and the aromatic polymers that can be isolated from various bryophytes and pteridophytes.

However, studies of lignin mutants in both herbaceous and woody plants have more recently provided us with a greater appreciation of the true diversity of function to which plants have adapted this polymer. Consequently, Freudenberg's definition of what constitutes lignin seems increasingly restrictive. No doubt further compositional and structural surprises await those who will extend the latest techniques for micro-scale and subcellular lignin analysis (multi-dimensional NMR, pyrolysis-mass spectrometry, UV microspectrometry, etc.) to lower plants in order to address lingering questions pertinent to the evolution of lignin. Specifically, what is the true chemical nature of the polyphenolic materials identified in mosses and algae (Siegel, 1969; Miksche and Yasuda, 1978; Delwiche et al., 1989), and is it possible that these compounds and their biosynthetic pathways share a common ancestor with angiosperm and gymnosperm lignin? These are questions that need to be addressed at the level of individual cells (e.g., hydroid cells in hornworts), and the answers have great potential to extend further our ideas as to how lignin structure and composition might be modified through genetic manipulation of existing metabolic pathways in plants. Given the observations of Ralph and co-workers (1999a) that plants can incorporate a much wider variety of phenolic precursors into lignin than would be anticipated from Freudenberg's definition, it is tempting to speculate that lignin composition and structure could be changed even more drastically by drawing on our expanding knowledge of developmental processes that parallel lignification in other organisms, e.g., sclerotiza-

tion of insect cuticle (Andersen et al., 1996) and melanization of fungal rhizomorphs (Butler and Day, 1998). The introduction of new metabolic pathways from these organisms into woody plants could lead to materials having unique and valuable properties.

1.1

Potential Applications

1.1.1

Pulp and Paper

Based on value of shipments, the pulp and paper industry ranks eighth among all U.S. manufacturing industries, and pulp, paper, and paperboard mills account for about 12% of total manufacturing energy use in the U.S. (Nilsson et al., 1995). Although the industry produces more than half of all the energy it consumes, energy still constitutes about 17% of total costs to the industry (this number does not account for the value of co-generated energy from burning wastes and residues). Since the production of pulp from wood is almost entirely a matter of disrupting the lignin matrix between and within fibers, it would seem that modifications to lignin would have the potential for providing this industry with substantial energy savings. However, with respect to lignin modification, it is important to note that there are two very different pulping processes – mechanical and chemical (e.g. Kraft) – and the energy demands for these two processes are radically different.

In the case of mechanical pulping, physical grinding is used to disrupt the interfiber lignin matrix and separate the wood fibers, but little or no lignin is removed from the pulp. Mechanical pulp is used primarily for the production of newsprint and other low-grade papers, and its manufacture consumes enormous amounts of energy. For example, a medium-sized mechanical pulp mill having the capacity to produce sufficient newsprint for just five or six metropolitan newspapers

can consume enough energy to power a residential suburb of 250,000 people. Although mechanical pulp only represents about 20% of the pulp produced in the U.S., it is by far the most energy-consuming product manufactured in this sector of the economy. Studies have shown that introduction of ionizable groups in lignin, such as occurs during sulfite treatment of wood chips prior to chemimechanical pulping, reduces energy consumption and improves the efficiency of the chip refining process (Htun and Salmen, 1996). This suggests that genetic manipulations resulting in trees which incorporate phenolic acids into their lignin, similar to the situation noted for a loblolly pine mutant lacking cinnamyl alcohol dehydrogenase activity (MacKay et al., 1997), could be used to improve trees for specific use in mechanical pulping. Depending upon the economics, it might also be advantageous to reduce the total amount of lignin in trees destined for mechanical pulping. However, it must be recognized when proposing such modifications that mechanical pulping is a high-volume, high-yield process with low profit margins; thus, it might be difficult to justify the cost of genetically engineering trees specifically for mechanical pulping.

In contrast to mechanical pulp, more than 80% of all paper is produced from chemical or Kraft pulp, and the Kraft process, overall, generates more energy than it consumes. In the Kraft process, lignin extracted at high temperature under highly alkaline conditions ($\text{pH} > 11.0$) is burned so as to recover the caustic chemicals, while at the same time generating enough electrical power to run the process, as well as excess power that can be sold to local utilities. Thus, trees modified specifically to suit the needs of the Kraft pulp industry should contain at least as much lignin as normal trees, but that lignin should be more easily and thoroughly extracted under the initial pulping conditions. In the

Kraft pulping process, as much as 90% of the starting lignin is removed during the initial cook. The remaining 10% *residual* lignin imparts a brown color to the pulp, necessitating bleaching treatments if the pulp is destined for the production of white paper. Some of the residual lignin is covalently attached to fibers as a result of chemical reactions occurring during the pulping process, but there is evidence that some covalent linkages are formed during lignin biosynthesis (Helm, 2000). As pulp bleaching processes are expensive and usually have a negative environmental impact, genetic manipulations to reduce the naturally occurring covalent cross-links between lignin and the other fiber polymers would be of significant commercial interest. As Kraft pulps generally command higher prices than mechanical pulps, the economics for modifying lignin by genetic engineering might be easier to justify in trees destined for Kraft pulping.

1.1.2

Solid Wood Products

The properties of wood most relevant to structural use are strength, dimensional stability, and resistance to decay (Whetten and Sederoff, 1991). Although each of these characteristics is impacted by the lignin composition and content of wood, there are relatively few studies which directly relate lignin and structural wood qualities in ways that would suggest what changes to lignin might be desirable for solid wood products. However, the need for such studies is growing as solid wood industries come to rely increasingly on trees from intensively managed plantations, as well as second-growth, rather than old-growth, forests. The juvenile wood from these sources is generally considered inferior for solid wood products, in part because it has a lower lignin content and lower strength, coupled with higher microfibril angles (Kennedy, 1995). The latter is

particularly problematic for solid wood products, as high microfibril angle causes directional shrinkage which is manifested as warping and twisting in structural lumber. Studies, such as those by Tjeerdsmā et al. (1998) showing how heat treatment of lumber can bring about autocondensation of lignin and thereby improve dimensional stability, may suggest what types of chemical alterations would be desirable in lignin. However, even with such information it will still be necessary to identify and introduce into the trees genes capable of bringing about the correct modifications to lignin precursors.

1.1.3

Textile Fibers

Industrial interest in bast fibers, such as those produced from flax, kenaf, ramie, hemp, or jute, has increased in recent years (Smeder and Liljedahl, 1996). Lignin has both positive and negative effects on the quality of bast fibers used in the production of textiles and cordage. Individual fiber cells are stiffened by the lignin within in their secondary walls, while bundles of these fiber cells, which constitute the material most often used in woven materials, are strengthened by the inter-fiber lignin matrix (see for example, Angelini et al., 2000). Lignin also contributes to the coloration of these fibers, necessitating bleaching processes to produce white cloth (Amin et al., 1998). Although techniques are available for the genetic engineering of flax (Dong and McHughen, 1993) and ramie (Dusi et al., 1993), there is as yet insufficient information relating lignin localization, composition and content to fiber characteristics to allow for predictions as to how lignin biosynthesis might best be modified to improve these fibers. On the other hand, it may be useful to consider the commercial potential for naturally red-colored fibers that could be developed by manipulating the expression of certain enzymes involved in monolignol