0273-2289/01/91–93/0023/$13.00

Genetic Improvement of Poplar Feedstock Quality for Ethanol Production

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Abstract

Opportunities for matching chemical and physical properties of woody feedstocks to ethanol production process requirements via genetic improvement have long been recognized. Exploitation is now feasible owing to advances in trait measurement, breeding, and gene transfer technologies. Poplar genetic parameters are favorable largely for reducing lignin and increasing cellulose contents and specific gravity. Transgenic poplars with decreased lignin and increased cellulose contents, but otherwise normal growth and development, have been produced via genetic transformation. The long-standing debate on feasibility has thus become one of when, not if, designer varieties will become available.

Index Entries: Biomass feedstock quality; lignin; cellulose; wood specific gravity; selection and breeding; genetic transformation.

Introduction

The Bioenergy Feedstock Development Program, US Department of Energy, Oak Ridge National Laboratory (BFDP) is developing short-rotation poplars (*Populus* spp. and hybrids) as feedstock for ethanol production. Substantial gains in adaptability, growth, and pest/stress resistance have been achieved via classic breeding and intensified cultural practices. Given these and anticipated accomplishments, consideration is being given to fostering research and development on genetically modifying feedstock chemical and physical properties.

This article reviews opportunities for and feasibilities of improving short-rotation poplar feedstock quality via classic breeding and genetic transformation and is derived, in part, from a comprehensive feasibility study performed for BFDP *(1)*. Information was collected via analysis of poplar literature, with emphasis on findings published since 1995; personal communications with prominent scientists, breeders, and growers; and a BFDP-sponsored workshop held in December 1999. Results from research on other short-rotation hardwoods are used in the few situations in which poplar data could not be found.

Ethanol Conversion Processes and Feedstock Quality

Producing ethanol from wood involves using heat, acids, and enzymes to separate constituents and split cellulose and hemicellulose into individual sugar molecules. Simultaneously or subsequently sugars are fermented to ethanol. To improve process economics, some investigators have advocated using five-carbon hemicellulosic sugars to manufacture coproducts (e.g., biodegradable polymers), a reflection of past concerns that such sugars could not be fermented efficiently to ethanol. Recently, however, genetically engineered organisms have been developed to perform this function, and conversion is no longer considered an economic obstacle. Lignin and other residuals are used as fuel to generate process heat, steam, and electricity. Other markets for lignin have been and are likely to remain limited.

All processes likely used for conversion of woody feedstocks to ethanol are sensitive to feedstock quality, some more than others, with concentrated acid hydrolysis the least sensitive *(2)*. This process is well suited to handling difficult feedstocks (i.e., those having variable composition and containing large quantities of contaminants, e.g., extractives and ash). By contrast, enzymatic hydrolysis is sensitive not only to contaminants but also to lignin content. Lignin binds and inactivates enzymes, thereby necessitating large initial enzyme quantities and limiting enzyme recycling *(3,4)*. Lignin content, rather than composition, seems to be the major factor *(5)*. Lowering lignin content should reduce operating costs substantially, given the high costs of enzymes. All processes are sensitive to carbohydrate content. Increased carbohydrate content, especially cellulose, should therefore also rank high as a target for genetic modification. The specific gravity of wood warrants attention as well, given its generally positive correlation with cellulose content. Improving other properties seems likely to yield only incremental benefits. For example, reducing extractives, ash, and bark contents, naturally low in poplars, could have favorable effects, but far less than those of lower lignin and greater cellulose contents. Moreover, information is limited on the means for modifying such constituents. Lignin content, nonetheless, seems to be the prime target, since lignin is the least useful of all major feedstock components, and reduction equates to more cellulose per unit mass. Moreover, lowering lignin content should raise the profitability of enzymatic hydrolysis.

Some research has been done to quantify the effects of feedstock qualities on process efficiency, but analyses generally have been done via simulation models and have addressed only growing, harvesting, handling, transport, and storage costs. Few have addressed directly the effects of changes in quality, but sensitivity of enzymatic processes to enzyme costs *(6)*, and therefore lignin content, and proportionality of ethanol yield to cellulose content *(7)* have been projected. Because changes in feedstock composition can affect processes at a variety of points and in numerous ways, definitive trials with feedstocks of differing qualities are needed to determine the impacts of changes on each of the steps in individual processes. Individuals engaged in improving and growing feedstocks must know which traits to modify, how to modify them for what process, and how modification will affect the cost/benefit ratio.

Opportunities for Improving Poplar Feedstock Quality

Viewed against the aforementioned improvements in poplar productivity, the time seems ripe for undertaking the research, development, and technology transfer necessary to exploit opportunities for improving feedstock quality. Indeed, the shortened rotations made possible by past improvements mean that such opportunities can be more easily and rapidly exploited. Also, many poplars are propagated clonally, thereby permitting rapid, inexpensive multiplication of valuable variants. Poplars seem an ideal venue for testing and applying techniques to improve feedstock quality.

Feedstock Quality Assessment

Efficient genetic improvement requires that traits be measured accurately and inexpensively. Such measurements constitute one of the largest costs of improvement, and high measurement costs have impeded efforts to improve wood properties. As an illustration, volume, a typical measure of productivity, can be measured in minutes for pennies per tree. By contrast, traditional wet chemistry assays of lignin content take several days and cost $500 or more per sample.

Recognizing these difficulties, various research organizations have developed improved methods for assaying both chemical and physical properties. For chemical assays, techniques such as reflectance near infrared *(8)*, Fourier transform infrared *(9)*, and pyrolysis molecular beam mass spectrometry *(10)* offer reduced costs as well as speed and convenience. Results correlate strongly with baseline values obtained by traditional methods. Assays require only a few grams or less of wood derived from nondestructively collected increment cores and can be completed in minutes at costs many times less than those of traditional analyses.

Concerning specific gravity, various densitometric techniques have long been used in research, but recent improvements have increased utility and reduced costs *(10)*. Automated systems are now available for not only determining specific gravity but also for elucidating the impact of contributing factors (e.g., cell number and cell wall thickness) *(11)*. Such techniques rely on increment cores and can analyze thousands of samples per year. Sampling costs have been reduced by the use of hydraulically driven increment corers mounted on all-terrain vehicles.

Accurate, rapid, and inexpensive measurement techniques that rely on small, nondestructively collected samples are opening the way to improving wood properties, specifying processing conditions for different feedstocks, and compensating growers for improvements.

Classic Breeding

Feedstock quality improvement via classic breeding requires that valuable traits possess usable levels of genetic variation, meaningful degrees of genetic control, and favorable correlations with other important traits. Stability across ages and environments is also important. Quantity and quality of genetic information on wood chemical and physical properties has increased recently as a result of intensified interest in facilitating the manufacture of traditional wood and paper products, as well as the availability of convenient measurement techniques. The following discussion focuses on lignin and cellulose contents, given their importance to ethanol production. Specific gravity, given its association with cellulose content, is also discussed.

Past reviews indicated the existence of genetic variation in lignin content among and within poplar and many other hardwood species *(12)*. Most recent data generally derives from research on short-rotation *Eucalyptus* species, but similar patterns are likely to be found in most short-rotation poplars. Genetic variation within species is small but statistically significant *(13)*, often only a few percentage points but sometimes as large as nine. Heritabilities are modest to high and typically exceed those for growth. Clone-by-site interactions are sometimes significant. Information on correlations between lignin content and other traits is sparse, but some reports *(14)* suggest unfavorable, though weak, relationships with specific gravity. The high heritabilities already noted infer that lignin content can be reduced by classic breeding, but the extent and pace of reduction would be limited by the narrow range of genetic variability as well as probable unfavorable relationships with growth and interactions across environments. Variability in poplars might be expanded via hybridization of adapted species. In continuing BFDP-sponsored research, 16 interspecific poplar hybrids, derived from several species, exhibited a 9.3 percentage point range in lignin content, as measured by traditional wet chemistry methods (unpublished data). Given the variety of interfertile poplar species, interspecific hybridization is an attractive tack *(15)*.

Variation in cellulose content is roughly 7 to 15 percentage points, a range larger than those for lignin or hemicellulose. Heritabilities typically are low. Olson et al. *(16)* found significant genetic variation in cellulose contents of 75 3-yr-old *Populus deltoides* grown at a single location. Broad-sense heritability (i.e., the proportion of genetic to phenotypic variation) was 0.34, a value greater than that for growth but smaller than those for most wood properties. Both variation and heritability were considered sufficient for improvement. Correlations with growth, however, were negative, and simultaneous improvement seemed impractical at the time. Olson et al. *(16)* nevertheless recommended preserving clones having high cellulose content for possible use in later years. This recommendation recognized that correlations are imperfect and may change with age, and that trait valuations may likewise change with time. Low heritabilities imply that cellulose content may be increased, but only by mating outstanding trees and clonally propagating and planting selected offspring—practices that are well known to and frequently used by poplar breeders. The generally negative, though weak correlation with growth, however, implies that breeding for cellulose content could impede breeding for growth. Decisions to breed or not breed for increased cellulose content would be facilitated by more information on strength of trait and age-age correlations as well as stability across environments.

Data on genetic variation in and control of hemicellulose content are scarce. Early literature disclosed statistically significant variation, but too limited for genetic improvement *(12)*. More recently, assay of 17 poplar clones representing two species plus F_1 and F_2 hybrids disclosed phenotypic variation similar to that in lignin content (unpublished data). Significant variation and strong genetic control has also been found in short-rotation *Eucalyptus* species *(13)*. Further research on poplars seems warranted. Lower five-carbon and higher six-carbon sugar contents as well as fewer hemicellulose-lignin linkages would benefit the efficiency of ethanol conversion.

Although often viewed as a single trait, specific gravity is a composite of several properties, including proportions of juvenile to mature wood and early to late wood; cell types, numbers, and sizes; and cell wall thicknesses. The situation in hardwoods is especially complicated, given the wide variety of cell types. Since characteristics influencing specific gravity can vary together or independently, individual trees can have similar or different specific gravities for different reasons. Improving specific gravity therefore depends on understanding what to change and how changes, in turn, affect process and product.

The considerable literature on specific gravity indicates abundant genetic variation and moderate to strong heritabilities for most hardwood species *(17)*. Olson et al. *(16)* found a broad-sense heritability of 0.62 for specific gravity in 3-yr-old *P. deltoides* and noted a negative genetic correlation (–0.65) with growth. The correlation with cellulose content, however, was positive (0.54). They deemed simultaneous selection for the several traits unprofitable in their situation but advocated preserving trees outstanding for each trait in breeding populations. In short-rotation *Eucalyptus grandis*, specific gravity was positively related to fiber wall thickness and volume of cell wall substance, but negatively correlated with lignin content *(14)*. Concerning age-age correlations, Yanchuk et al. *(18)* found that specific gravity in *Populus tremuloides* declined gradually through the tenth annual ring, and then rose across subsequent rings. Herpka *(19)* documented a similar pattern in other poplar species and hybrids and considered values at 2 or 4 yr of age predictive of those at older ages.

As indicated, genetic variation in and heritability of specific gravity is more than adequate for improvement via classic breeding. The trait seems stable across ages and environments. Favorable correlations with cellulose content and cell wall substance are advantages. Often negative correlations with growth are inconvenient but not insurmountable problems. From tests of several poplar species and hybrids, Herpka *(19)* concluded that variabilities and heritabilities were so large that trees having increased dry wood substance production per unit area and time (i.e., combining rapid growth and high specific gravity) could be selected at early ages with ease. In the end, finding trees excelling in two or more traits is a numbers game and is decided by how much additional time and expense can be devoted to testing and selecting the extra trees needed to identify outliers.

Having achieved substantial gains in survival and growth, poplar breeding programs seem sufficiently mature to pursue broader objectives. Growth, after all, is but one element governing the costs of ethanol production; chemical and physical properties of feedstock are also important. Viewed in this more inclusive context, future breeding efforts should be designed to reduce costs and raise efficiencies at all significant leverage points in production. Accordingly, breeders must generate more and better information on genetic parameters, particularly on correlations among traits. Process specialists must document how and to what extent changes in quality traits affect process efficiency and determine economic weights associated with change. These data can then be combined in selection indices for use by breeders and growers in optimizing genetic gain per unit time. The outcome should be poplar varieties adapted to process requirements, and more likely to reduce production costs than those with only improved growth.

Genetic Transformation

Improvement via genetic transformation has several advantages, and coupling it with classic selection and breeding makes for a potent, complementary combination. Should genes for a trait not exist in the species of interest, they can be procured from other, even unrelated, species and inserted into trees selected for breeding and planting. Perhaps more important, transformation can be used to alter expression of existing genes. For example, so-called antisense gene constructs can be used to suppress expression of genes affecting lignin biosynthesis or sense constructs can be used to increase cellulose biosynthesis. By virtue of cosuppression, sense constructs can sometimes be used to reduce gene activity. Not to be overlooked is the probability that gene transfer can save time via bypassing the sexual cycle and often long generation intervals.

Ideally, traits considered for modification via transformation should have significant economic value, be modified such that change is large relative to that attainable by other means, be the product of a reasonably well-understood biochemical pathway, and be controlled by one or a small family of related genes *(20)*. Moreover, modification should not adversely affect survival and growth. Lignin content meets these criteria rather well and cellulose content comes close.

Basic features of and many enzymes in the lignin biosynthetic pathway have been known for some years *(21)*. Knowledge has increased greatly in recent years, often owing to the use of genetic transformation as a research tool *(22)*. Roughly a decade ago information on biochemistry and genetics of enzymes involved in cellulose biosynthesis was considered inadequate to support research on modification *(12)*. Advances since then have been dramatic *(23)*, and several laboratories have achieved notable transformation successes. Most modifications have been made near the end of the biosynthetic pathways, where changes in enzymatic activity are less likely to have adverse effects on other metabolic processes.

Several methods for poplar transformation are available, and numerous genes have been inserted and expressed in various species and hybrids. Indeed, poplars are often used as models in transformation research, given the ease with which many can be manipulated in and regenerated from cell and tissue cultures. Improvements in transformation efficiency and extension to wider arrays of genotypes, however, remain important research needs *(24)*.

Considerable information is available on genetic modification of enzymes involved in poplar lignin biosynthesis. Most research concerns manipulation of enzymes catalyzing synthesis and interconversion of lignin precursors. Some of the first research was conducted on phenylalanine-ammonia lyase (PAL), the enzyme at the gateway for carbon entry to lignin biosynthesis. Emphasis has since switched to enzymes functioning later in biosynthesis owing to concerns over adverse effects on other pathways. Intermediates through at least the thioester stage are known to be involved in the biosynthesis of metabolites in addition to those slated for lignification. In addition, determining which of the many forms of PAL are active when and where is difficult *(21)*.

O-methyltransferases and caffeate *O*-methyltransferases (COMTs) are thought to catalyze the ortho-methylation of caffeate to ferulate and 5-hydroxyferulate to sinapate in some plants and have also been implicated in methylation of other intermediates. van Doorsselaere et al. *(25)* suppressed COMT activity by transferring an antisense construct into hybrid poplars. Lignin content was not reduced but syringyl/guaiacyl ratios were reduced; a novel lignin monomer, 5-hydroxyguaiacyl, was produced; and xylem was rose colored. Similar results were obtained in *P. tremuloides* by Tsai et al. *(26)* via cosuppression with a COMT sense construct.

In angiosperms, 4-coumarate 3-hydroxylases (4CLs) convert 4-coumarate, caffeate, ferulate, 5-hydroxyferulate, and sinapate to their respective thioesters. Hu et al. *(27)* discovered two different 4CL genes (*Pt4CL* and *Pt4CL2*) in *P. tremuloides*. The activity of *Pt4CL* was specific to lignifying xylem tissues, implying involvement mainly in lignin biosynthesis. Transformation with antisense constructs yielded trees with a 45% decrease in lignin and 15% increase in cellulose contents *(22)*, without changes in lignin composition or structure. Hemicellulose composition was also altered; arabinose, galactose, and rhamnose contents were increased. Transgenic trees are growing faster than nontransgenics, but are morphologically and anatomically normal. This is the first clear-cut reduction in lignin content apparent in the literature. Douglas et al. *(28)* identified similar genes in *Populus trichocarpa* and its hybrid with *P. deltoides* and are using antisense constructs to explore the effects of suppression.

Caffeoyl CoA *O*-methyltransferase (CCoAOMT) is considered responsible for converting caffeoyl-CoA to feruloyl-CoA and, perhaps, 5-hydroxyferuloyl-CoA to sinapoyl-CoA. Several European laboratories are contemplating *(29)* or initiating *(30)* manipulation of CCoAOMT activity.

The enzyme cinnamoyl-CoA reductase (CCR) converts thioester forms of lignin precursors to the corresponding aldehydes, e.g., feruloyl CoA to coniferyl aldehyde. Transgenic poplars with modified CCR activities are being evaluated *(31)*.

Cinnamyl alcohol dehydrogenase (CAD) catalyzes the last step in biosynthesis of lignin precursors, conversion of aldehydes to alcohols. Transformation of poplars with antisense CAD constructs had little or no effect on lignin content but caused changes in lignin composition (i.e., incorporation of aldehydes into lignin) and red-brown xylem *(29)*. Baucher et al. *(30,32)* confirmed these findings and also observed vanillin and syringaldehyde accumulations in cell walls. In related research, Lapierre et al. *(33)* reported that lignin contents of 2-yr-old transgenic poplars were only slightly lower than that of nontransformed controls, but that frequency of free phenolic groups was greater and that coloration was caused by syringaldehyde accumulation. In a somewhat more positive vein, Pilate et al. *(34)* inserted a CAD antisense construct into a poplar different than that used by Lapierre et al. *(33)*. CAD activity was suppressed to a greater extent; outcomes were a 10–15% reduction in lignin content, lower syringyl/guaiacyl ratios, and atypical compounds in lignin.

Information on biochemistry and genetics of enzymes involved in transport, storage, and polymerization of lignin precursors is not nearly as abundant as that on their biosynthesis. Data nevertheless are accumulating rapidly, and various laboratories are attempting improvement via gene transfer. Dinus *(1)* summarized recent research on glucosidases, glucosyltransferases, peroxidases, laccases, and an alcohol oxidase. Modifying functions this far into lignin biosynthesis seems attractive, since intervention should have minimal effects on other metabolic processes.

Concerning improvement in cellulose biosynthesis, Loopstra et al. *(35)* have isolated and sequenced a family of cellulose synthase (celA) genes from poplar. Transgenic *P. tremuloides* containing a celA sense construct have been produced at Michigan Technological University (Chiang, V. L., personal communication). The trees are morphologically normal but are growing faster than nontransformed controls. Transformation of hybrid poplar with a sense construct for a UDP-pyrophosphorylase gene is being attempted (Ellis, D. D., personal communication). This undertaking follows earlier research in which transformation of tobacco *(Nicotiana tabacum)* with a similar construct increased enzyme activity, cellulose synthesis, and biomass *(36)*. Prospects for increasing cellulose content via genetic transformation look almost as good as those for reducing lignin content.

Much transformation research has been done with constitutive promoters, e.g., the CV35S promoter or a strengthened version thereof. This is useful in early stages of research, since the approach enhances probabilities of observable expression. Practical utility, however, demands that research progress beyond this proof-of-principle phase to ensure that transgenes are expressed in appropriate tissues and developmental stages. A few investigators are using sense/antisense constructs fused to xylem-specific promoters. Even greater specificity, nevertheless, seems necessary. As an example, reducing lignin content of secondary cell walls in fibrous cells seems quite useful. Reduction in vessel elements, on the other hand, could disrupt water transport and support functions. Accelerated research on tissue and developmental stage specific promoters *(21)* remains imperative.

An obstacle to commercialization of transgenic trees is public concern over the possibility that widespread planting will spread so-called foreign genes to natural populations *(37)*. Rendering transgenic trees sexually sterile is one means of minimizing, if not avoiding, this risk. Genetic constructs for sterility in poplar should be available in 5–10 yr. Useful side effects may also accrue; eliminating reproductive structures could channel more energy, water, and nutrients into wood production. Concern also might be eased by the availability of genetic markers to monitor potential for gene flow. In addition, confidence and acceptance can be built by responsible, transparent testing along with clear, consistent communication of risks and benefits. Sterile, transgenic trees, after all, can be expected to lessen the environmental impacts of growing and harvesting.

Conclusion

Poplar breeders and growers have achieved substantial improvements in adaptability, growth, and pest/stress resistance. Over the long run, however, productivity is just one element in the matrix needed to enhance the efficiency of ethanol production. Chemical and physical properties of wood also govern cost and yield. Viewed in this more exclusive context, bioenergy projects would be well served by enlarging research, development, and technology transfer efforts to include genetic improvement of important feedstock qualities. Improvements can be made by both classic breeding and genetic transformation. Pursuit of these approaches will be eased by newly available, convenient, and inexpensive trait measurement methods. Progress will be slow, however, until the costs and benefits of modification are clarified, and economic weights are translated into guidelines for breeding, transformation, and compensating feedstock producers.

Most feedstock quality traits can be improved via classic breeding. Lignin, the least valued of feedstock chemical components, can be reduced, but limited variability will limit progress. Even small changes, however, could improve efficiency of conversion processes, especially that of enzymatic hydrolysis. Raising cellulose content could prove more difficult, given its rather modest heritability and negative association with growth. Even so, useful changes in these traits seem obtainable in perhaps two or three generations of breeding and testing. As an alternative, breeding might be better focused on traits likely to decrease lignin and increase cellulose contents indirectly. For example, simultaneously improving specific gravity and growth (i.e., increased wood substance production per unit area and time) should positively affect productivity, harvesting, and transportation, as well as processing. This tack seems particularly desirable in that the wood would be attractive to a variety of customers. Regardless of strategy, continued research is needed to acquire better genetic information and more economic data for construction of selection indices.

The considerable research done in recent years on genetic transformation has produced transgenic trees with modified lignin content and composition as well as increased cellulose contents. Antisense suppression of most enzymes involved in lignin precursor biosynthesis has changed only composition. Suppression of a few (e.g., CAD) has not only altered composition but also somewhat reduced quantity. Compositional changes noted to date involve incorporation of atypical precursors in lignin, deposition of free compounds in cell walls, and coloration of xylem. Most such changes, unfortunately, are likely to have detrimental effects under the acidic conditions of ethanol conversion; that is, they could raise contaminant levels in process sugar streams, thereby complicating capture of residuals and increasing both effluent quantity and color. That compositional changes have been induced, however, indicates that lignin biosynthesis is quite plastic and suggests that lignin could be bioengineered to favor ethanol conversion. Suppression of only one enzyme, 4CL, gave a 45% reduction in lignin content, without any adverse effects on composition or growth and development. Several laboratories have also increased cellulose content with sense contructs. Modifying lignin and cellulose contents simultaneously could yield commercial varieties with significantly enhanced conversion efficiency. Transformation works, and commercialization is only a matter of time, perhaps 5–10 yr. Ensuring that dividends from this exciting technology are realized in a reasonable time frame nevertheless requires continued, balanced research on transformation methods; biochemistry and genetics of lignin, cellulose, and hemicellulose biosynthesis; gene regulation and control; sexual sterility; and detection and resolution of any adverse side effects.

Poplar feedstock bred for rapid growth and high wood substance production and genetically transformed to have decreased lignin and increased cellulose contents stands to have tremendous beneficial effects on ethanol production.

Acknowledgments

I wish to thank Vincent Chiang, Linda Belles Dinus, Peggy Payne, Vincent Sewalt, Mitchell Sewell, Jerry Tuskan, Lynn Wright, and two anonymous reviewers for their thoughtful contributions. I also acknowledge the US Department of Energy, Bioenergy Feedstock Development Program, Oak Ridge National Laboratory, for funding to analyze literature, collect information via personal communications, conduct a review workshop, and prepare and present this manuscript.

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