5 Development of Alfalfa (*Medicago sativa* L.) as a Feedstock for Production of Ethanol and Other Bioproducts

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Alfalfa (*Medicago sativa* L.) has considerable potential as a feedstock for production of fuels, feed, and industrial materials. However, unlike other major field crops such as corn and soybeans, which are commonly refined for production of fuel and industrial materials, refining of alfalfa remains undeveloped. Instead, alfalfa is primarily processed and used on-farm in the form of dried hay, silage, and fresh forage known as "greenchop," or is grazed by animals in pastures. In

many countries, including the United States, alfalfa is used as a basic component in feeding programs for dairy cattle and is an important feed for beef cattle, horses, sheep, and other livestock. Known as the "Queen of the Forages," alfalfa provides highly nutritious forage in terms of protein, fiber, vitamins, and minerals for ruminant animals. If alfalfa is developed to its full potential as a feedstock for biorefining, a major shift may occur in the manner in which alfalfa is produced and used for feeding farm animals.

CURRENT ALFALFA CULTIVATION AND UTILIZATION

A number of attributes make alfalfa an attractive crop for production of biofuels and for biorefining. Alfalfa has a long history of cultivation around the world. It was introduced several times into North America during the 1700s and 1800s and is currently grown across the continent (Russelle, 2001). In the United States, alfalfa is the fourth most widely grown crop with over 9.3 million hectares of alfalfa harvested in 2003 (USDA-NASS, 2004). It is a perennial plant that is typically harvested for four years (an establishment year plus three subsequent years). Depending on location, alfalfa is harvested three or more times each year by cutting the stems near ground level. On average across the United States, alfalfa yields 7.8 Mg of dry matter (DM) per hectare each year, although yields can vary by location from 3.4 (North Dakota) to 18.4 (Arizona) Mg ha^{-1} (USDA-NASS, 2004). In 2003 the national harvest of alfalfa was over 69 million metric tons (USDA-NASS, 2004). The technology for cultivation, harvesting, and storing alfalfa is well established, machinery for harvesting alfalfa is widely available, and farmers are familiar with alfalfa production. There is a well-developed industry for alfalfa cultivar development, seed production, processing, and distribution. Alfalfa breeders have utilized the extensive germplasm resources of alfalfa to introduce disease and insect resistance, expand environmental adaptation, and improve forage quality. Nonetheless, alfalfa cultivation requires fertile, deep, well-drained soils of near neutral pH and is limited to humid areas with adequate rainfall. In arid or semi-arid areas, irrigation is essential for crop production. Despite breeding efforts that have increased disease and pest resistance, alfalfa yields have not increased substantially over the past 25 years (Brummer, 1999).

The high biomass potential of alfalfa is based on underground, typically unobserved traits. Alfalfa develops an extensive, well-branched root system that is capable of penetrating deep into the soil. Root growth rates of 1.8 m a year are typical in loose soils (Johnson et al., 1996) and metabolically active alfalfa roots have been found 18 m or more below ground level (Kiesselbach et al., 1929). This deep root system allows alfalfa plants to access water and nutrients that are not available to more shallowly rooted annual plants, which enables established alfalfa plants to produce adequate yields under less than optimal rainfall conditions. Alfalfa roots engage in a symbiotic relationship with the soil bacterium *Sinorhizobium meliloti*. This partnership between the plant and

bacterium results in the formation of a unique organ, the root nodule, in which the bacterium is localized. The bacteria in root nodules take up nitrogen gas (N_2) and "fix" it into ammonia. The ammonia is assimilated through the action of plant enzymes to form glutamine and glutamate. The nitrogen-containing amide group is subsequently transferred to aspartate and asparagine for transport throughout the plant. On average, alfalfa fixes approximately 152 kg N_2 ha^{-1} on an annual basis as a result of biological nitrogen fixation, which eliminates the need for applied nitrogen fertilizers (Russelle and Birr, 2004). Although a significant proportion of the fixed nitrogen is removed by forage harvest, fixed nitrogen is also returned to the soil for use by subsequent crops. This attribute of increasing soil fertility has made alfalfa and other plants in the legume family crucial components of agricultural systems worldwide. Cultivation of alfalfa has also been shown to improve soil quality, increase organic matter, and promote water penetration into soil.

Responsible stewardship of agricultural lands has never been more important. Utilization of alfalfa as a biomass crop has numerous environmental advantages. There is an urgent need to increase the use of perennials in agricultural systems to decrease erosion and water contamination. Annual row crop production has been shown to be a major source of sediment, nutrient (nitrogen and phosphorus), and pesticide contamination of surface and ground water. Perennial crops such as alfalfa can reduce the nitrate concentrations in soil and drainage water, and prevent soil erosion (Huggins et al., 2001). In addition, energy costs associated with production of alfalfa are low. A recent study shows that energy inputs for production of alfalfa are far lower than for production of corn and soybean, and very similar to switchgrass (Kim and Dale, 2004), primarily because alfalfa does not require nitrogen fertilizer. Biorefining could increase the return on alfalfa production so that cultivation of the crop is more economically attractive, as well as environmentally beneficial.

An additional advantage of using alfalfa for biofuel production compared to other crops is the ability to easily separate leaves and stems to produce co-products. In fact, alfalfa herbage can almost be considered two separate crops because leaves and stems differ so dramatically in composition. On a dry weight basis, total alfalfa herbage contains 18–22% protein with leaves containing 26–30% protein and stems only 10–12% (Arinze et al., 2003). In some analyses, alfalfa protein has been valued highly, theoretically greatly reducing the cost of the lignocelluose fraction (Dale, 1983). Several different integrated processes for refining alfalfa have been proposed based primarily on the method of refining the protein fraction. From field-dried hay, leaves may be separated from stem material mechanically (see "Protein and Fiber Separation" below). The leaf meal could be used as a high-protein feed with the stems utilized for gasification and conversion to electricity (Downing et al., 2005) or fermentation to ethanol (Dale, 1983). Alternatively, protein could be extracted from total ground material and the residue used for fermentation. Fresh forage can be "juiced" to remove protein and the residue fermented to ethanol or other products (Koegel et al., 1999; Sreenath et al., 2001; Weimer et al., 2005). An economic analysis of these

alternatives is beyond the scope of this chapter. However, a comparison of the potential costs and revenues of different biobased feedstocks to produce ethanol and other products is clearly needed to advance biomass refining from the theoretical to practical stages.

DEVELOPMENT AND CULTIVATION OF ALFALFA FOR BIOMASS

Genetic modification to improve alfalfa over the past century has increased resistance to several diseases and pests and widened the range of environmental adaptation of the crop by producing varieties that differ in fall dormancy and winter hardiness. Most improvements in forage quality of alfalfa have occurred through changes in harvest management and production practices. Alfalfa produced as feed for ruminant livestock is harvested frequently at early maturity when the leaf to stem ratio is high, producing hay that is high in protein and easily digested. Maximum forage yield, which occurs at later maturity stages in alfalfa, is usually sacrificed in order to produce high-quality hay. For competitive use of alfalfa as a biofuel feedstock, research is needed to develop alfalfa germplasm and management strategies that yield more biomass (both leaf and stem) with minimal production costs.

Marquez-Ortiz et al. (1999) reported that individual stem diameter was heritable and controlled by additive genetic effects and suggested that selection for larger stems in alfalfa was feasible. Volenec et al. (1987) found that selection for high yield per stem may be an effective means to increase forage yield, but plants may have less digestible, larger stems. Germplasms from southern Europe referred to as Flemish types are a genetic source for large stem size and resistance to foliar diseases in alfalfa, but display early maturity, lack winter hardiness, and are susceptible to root and crown diseases (Barnes et al., 1977).

The effects of plant population or density on stem, leaf and total forage yield have been well documented in alfalfa. As alfalfa plant densities increase, annual forage yield per land area unit increases, but yield of individual alfalfa stems and number of stems per plant decreases (Cowett and Sprague, 1962; Rumbaugh 1963). Hansen and Krueger (1973) reported that higher plant densities produced finer stems, decreased root and crown weights and increased leaf drop due to shading. Volenec et al. (1987) stated that stem diameter and nodes per stem decreased as plant density increased and that shoot weight was an important component of plant weight, especially at high plant densities. Decreasing plant density to approximately 45% (180 plants m^{-2}) of that conventionally used in alfalfa hay production stands (450 plants m^{-2}) and delaying harvest until the green pod stage maximized leaf and stem yield in four unrelated alfalfa germplasms (Figure 5.1). The reduced plant density decreased plant-to-plant competition for light, water, and nutrients, which minimized leaf drop caused by shading. Delaying harvest until late flower to green pod maturity stages increased stem yield and maximized total forage yield (Lamb et al., 2003).

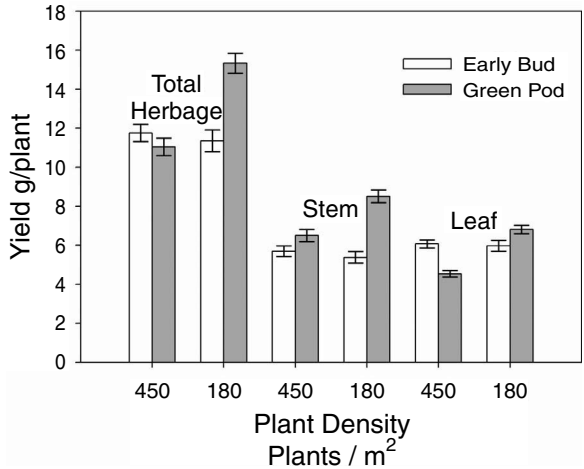

FIGURE 5.1 Mean (±1 SE) for alfalfa total herbage, stem, and leaf yield for each plant density and maturity stage combination.

CHEMICAL COMPOSITION OF ALFALFA

The utility of any biomass crop as a feedstock for ethanol production will depend in large part on its chemical composition, both in terms of the amount of potentially fermentable carbohydrates and the presence of compounds that may limit the yield of these carbohydrates. Current commercial yeast strains only utilize glucose as a substrate for ethanol production. Glucose can be derived from cellulose in the cell walls of biomass species. Therefore cellulose is of greater value than hemicellulose or pectin, polysaccharides composed of numerous sugars other than glucose. However, genetically modified yeast strains and other microorganisms are under study and under development that will use a wider diversity of hexose and pentose sugars. Reduced concentrations of hemicellulose and lignin, a phenolic polymer in the cell wall, would provide benefits to an ethanol conversion system by reducing pretreatment process inputs of heat and acid prior to cellulose addition. Also, reduced lignin content of biomass should result in high concentrations of the cell wall polysaccharides, thereby increasing the potential amount of fermentable sugars. Unfortunately, composition of biomass crops is very diverse and varies due to species, genetics, maturity, and growth environment.

A survey of 190 alfalfa plant introductions in the U.S. germplasm collection found that leaves averaged 283 g crude protein (CP) kg^{-1} dry matter (DM) compared to only 93 g CP kg^{-1} DM in stem material (Jung et al., 1997). In contrast, the neutral detergent fiber (NDF) concentration of stems far exceeded that of leaves (658 and 235 g NDF kg^{-1} DM, respectively). These differences are reflective of the role of stems in providing an upright growth form and supporting the leaf mass. Stems of alfalfa develop extensive xylem tissue (wood) with thick

TABLE 5.1
Composition of Immature (Bud Stage) and Mature (Full Flower) Alfalfa Stem Material

Component	Immature	Mature
	g kg^{-1} dry matter	
Protein	127	88
Lipid	9	7
Ash	81	58
Soluble carbohydrates	55	49
Starch	3	2
Cellulose	275	306
Hemicellulose	105	122
Pectin	125	119
Lignin	158	175

Source: Dien, B.S., Jung, H.G., Vogel, K.P., et al., *Biomass Bioenergy*, preprint [submitted].

cell walls comprised of cellulose, hemicellulose, pectin, and lignin (Theander and Westerlund, 1993; Wilson, 1993). Because leaves are the site of most photosynthetic activity in alfalfa, the leaves have high concentrations of enzymes and thin cell walls to facilitate light absorption and gas exchange. Representative composition of alfalfa stem material is shown in Table 5.1. Both leaves and stems have low concentrations of simple sugars and starch (Raguse and Smith, 1966), although alfalfa roots store substantial quantities of starch (150 to 350 g kg^{-1} DM) (Dhont et al., 2002). Lipid content of alfalfa is quite low (~20 g kg^{-1} DM) (Hatfield et al., 2005).

Because alfalfa is indeterminate in its growth habit, the plants increase in size and mass until harvested or a killing frost occurs. Alfalfa leaf mass increases during maturation, but at a lower rate than the increase in stem mass (Sheaffer et al., 2000). This results in a decline in leaf percentage in the total herbage harvested that can range from more than 70% leaf during early vegetative stages to less than 20% leaf when ripe seed is present (Nordkvist and Aman, 1986). During plant maturation, alfalfa leaves change very little in CP or NDF concentration whereas stem CP declines and NDF content increases dramatically (Sheaffer et al., 2000). The reason for the increase in NDF content of alfalfa stems during maturation is the addition of xylem tissue due to cambial activity (Jung and Engels, 2002). This xylem tissue has thick secondary walls and stem xylem accounts for most cell wall material when the crop is harvested.

Cell walls of alfalfa differ from grass cell wall material because of the greater pectin content of alfalfa cell walls. In very immature alfalfa stem internodes that are growing in size, pectins can account for up to 450 g kg^{-1} of the cell wall. Cellulose and hemicellulose contribute 340 and 120 g kg^{-1}, respectively, to the

total cell wall, with lignin accounting for the remaining wall material, in such young internodes (Jung and Engels, 2002). At this developmental stage, all of the lignin is localized in the protoxylem vessel cells and no other tissues are lignified. Once alfalfa internodes complete their growth in length, cambium meristematic activity begins to add new xylem fiber and vessel cells that lignify almost immediately. The predominant cell wall component in these tissues is cellulose (400 g kg^{-1} cell wall) with the rest of the cell wall material being equally divided among hemicellulose, pectin, and lignin (Jung and Engels, 2002). Phloem fiber cells also develop thickened secondary cell walls as the plant matures; however, this secondary wall is especially rich in cellulose and does not contain lignin (Engels and Jung, 1998). Lignin is deposited in a unique ring structure in the primary wall region of phloem fiber cells. With the exception of pith parenchyma cells, all of the other tissues in alfalfa (chlorenchyma, collenchyma, epidermis, cambium, secondary phloem, and protoxylem parenchyma) do not lignify no matter how mature the stem becomes (Engels and Jung, 1998). These tissues retain only primary cell walls that are rich in pectin. The pith parenchyma will ultimately lignify, although with only marginal secondary wall development, but usually pith parenchyma cells senesce, leaving a hollow stem cavity (Jung and Engels, 2002).

The composition of the major cell wall polysaccharides and lignin also change during maturation. Hemicellulose composition shifts from slightly more than 50% xylose residues, with the remainder being primarily to mannose, in very immature elongating stem internodes to 80% xylose residues in very mature internodes (Jung and Engels, 2002). The composition of the pectin fraction shifts less dramatically, with uronic acids increasing from 60% of the pectin to 67% with decreases in galactose and arabinose content, but no change in rhamnose concentration. The largest shift in cell wall composition due to maturity is in monolignol components of lignin. The syringyl-to-guaiacyl ratio increases from 0.29 to 1.01 as alfalfa stem internodes mature (Jung and Engels, 2002).

While maturity is the single most important factor that impacts composition of alfalfa, growth environment causes some additional shifts in composition. Unfortunately these environmental impacts are complex and difficult to predict. In a study by Sanderson and Wedin (1988), alfalfa herbage from a summer regrowth harvest in one year had a substantially higher NDF concentration than observed for that year's spring harvest (538 and 476 g NDF kg^{-1} DM, respectively); however, the same plots harvested in the following year showed a small difference between summer and spring harvests (588 and 546 g NDF kg^{-1} DM, respectively). Acid detergent lignin (ADL) concentration of the NDF fraction was greater for summer-harvested alfalfa in both years. During the spring growth period of the second year, air temperatures were warmer and there was less rainfall than in the first year of the study (Sanderson and Wedin, 1988). Vegetatively propagated clones of individual alfalfa plants divergently selected for stem cell wall quality traits showed environmental variability when evaluated over twelve cuttings (two locations, over two years, with three harvests per year). One clone averaged 233 g kg^{-1} for stem Klason lignin concentration but varied in response

from 198 to 261 g kg^{-1} over the environments tested. Another clone selected for stem cellulose concentration ranged from 396 to 467 g kg^{-1} for the twelve samples (Lamb and Jung, unpublished data).

In the previous study, the impacts of temperature and moisture cannot be evaluated separately. When these two environmental factors have been evaluated independently, the major effect of moisture stress alone appeared to be on amount of cell wall accumulated by alfalfa plants as opposed to changes in cell wall composition. When rainfall was eliminated using a moveable shelter and alfalfa plots were irrigated to three field capacities (65, 88, and 112% saturation), stem cell wall concentration was reduced when the alfalfa was grown under water-deficit conditions (Deetz et al., 1994). Klason lignin concentration of the cell walls was not altered due to water-deficit and concentrations of xylose, galactose, and rhamnose in the cell wall were marginally increased and glucose was decreased, under the 65% field capacity treatment. In contrast to the impact of moisture, temperature was found not to alter cell wall concentration, but did apparently influence cell wall composition. A greenhouse study where alfalfa was grown under adequate moisture conditions indicated that higher temperatures (32°C and 26°C, day and night respectively) resulted in no changes in leaf or stem NDF concentration compared to cooler growth conditions (22°C and 16°C, day and night respectively), but ADL content of the NDF was increased by the higher temperatures (Wilson et al., 1991). However, these temperature effects should be viewed with some caution because both the NDF and ADL concentrations observed for the greenhouse-grown alfalfa in this study were much lower than normally observed for field grown plants.

GENETIC IMPACTS ON COMPOSITION

Genetic differences in chemical composition among alfalfa plant introductions, varieties, and individual genotypes have been reported. Leaf and stem CP differed among a group of 61 plant introductions, although the ranges were small, from 272 to 295 and 88 to 99 g CP kg^{-1} DM, respectively (Jung et al., 1997). Leaf NDF concentration (235 g kg^{-1} DM) did not differ significantly among these plant introductions, but stem NDF ranged from 636 to 670 g NDF kg^{-1} DM. Similar variation was observed among a group of five commercial alfalfa varieties with CP and NDF differences being noted for leaves and stems, as well as whole herbage (Sheaffer et al., 2000). Differences in stem cell wall concentration and composition were observed among a set of four alfalfa genotypes selected for divergence in whole herbage ADL and in vitro ruminal DM disappearance (IVDMD) (Jung et al., 1994) and a group of three genotypes selected for divergent IVDMD (Jung and Engels, 2002). More recently, alfalfa genotypes selected for divergent cell wall Klason lignin, cellulose, and xylan were shown to differ genetically for these cell wall components when grown across a series of environments (Lamb and Jung, 2004). While the reported genetic variation among alfalfa germplasm sources is not large, the potential for modifying cell wall

composition has not been seriously explored, because recurrent selection for these traits has not been done.

Significant genotype x environment (G × E) interactions have generally not been observed for chemical composition of alfalfa varieties. Among 61 plant introductions, no measures of cell wall concentration or composition were found to have significant G × E interactions for leaf or stem material (Jung et al., 1997). Only differences in magnitude, not rank, for composition due to G × E interactions were noted by Sheaffer et al. (2000) among five alfalfa varieties. These results mirror the conclusion of Buxton and Casler (1993) that forage quality traits generally have small G × E interaction effects compared to the impact on yield. However, in recent work with alfalfa clones selected for specific cell wall traits, it was found that G × E interactions were significant among plants selected for low and high pectin and xylan concentrations, whereas no G × E interactions were noted among clones selected for Klason lignin or cellulose (Lamb and Jung, 2004).

ALFALFA LEAF MEAL

Because alfalfa leaves contain approximately 300 g CP kg^{-1} DM, this portion of the crop has greater value as an animal feedstuff than for conversion to ethanol. Based simply on its protein concentration, alfalfa leaf meal was estimated to have a value of \$138 Mg^{-1} (Linn and Jung, unpublished). This price far exceeds the target feedstock value of \$33 Mg^{-1} assumed in a functioning corn stover-to-ethanol production system (Aden et al., 2002). In an extensive series of studies involving lactating dairy cows and fattening beef cattle, alfalfa leaf meal was shown to be an acceptable protein feed supplement in place of soybean meal (DiCostanzo et al., 1999). Besides providing protein for beef steer growth, alfalfa leaf meal also reduced the incidence of liver abscesses at slaughter, thereby increasing the market value of the cattle. Furthermore, alfalfa leaf meal could replace alfalfa hay in the diet of lactating dairy cows as a source of both protein and fiber to support normal milk production (Akayezu et al., 1997). Suckling beef calves actually gained weight more rapidly when fed alfalfa leaf meal in a supplemental creep feed than observed with a soybean meal-based supplement (DiCostanzo et al., 1999). From these results, it is clear that alfalfa leaf meal could provide a valuable coproduct for an alfalfa-to-ethanol production system.

PROTEIN AND FIBER SEPARATION

Two methods have been developed for capturing the protein-rich fraction from alfalfa and separating it from the more fiber-rich fraction. From whole field-dried plant material, leaves can be separated from denser stems using shaking screens (Arinze et al., 2003; Downing et al., 2005). Fresh material can be dried using a rotary drum drier and leaves separated aerodynamically due to their lower mass and faster drying time than that of stems (Arinz et al., 2003). Wet fractionation

involves mechanical maceration of fresh total herbage followed by the expression of protein-rich juice (Jorgensen and Koegel, 1988; Koegel and Straub, 1996). Approximately 20–30% of the herbage DM can be captured in the juice (Koegel and Straub, 1996). The proportion of DM that was captured in the juice was shown to decrease with increasing maturity of the herbage (Koegel and Straub, 1996). The juice contains both particulate and soluble proteins. The soluble proteins, which may have greater value, can be separated from particulate proteins by heating and centrifugation (Jorgensen and Koegel, 1988). Wet fractionation has been used successfully in small-scale experiments (see "Pretreatment of Alfalfa Fiber" below) to refine alfalfa into a high-value protein fraction and a fiber fraction that was further refined and fermented to produce ethanol (Koegel et al., 1999; Sreenath et al., 2001), lactic acid (Koegel et al., 1999), and wood adhesive (Weimer et al., 2005). Fiber can also be processed into animal feed. The deproteinized juice is a source for extracting xanthophyll and can also be used as a fertilizer (Koegel and Straub, 1996). Wet fractionation has the advantage of minimizing leaf loss and is less weather dependent than field drying. Dried material has the advantage of being lighter to transport and is easily stored for later processing and refining. The nature of the protein product will clearly impact the method of herbage harvest and processing.

In addition to protein, alfalfa also contains numerous secondary metabolites that are of interest in human nutrition and food production. In particular, alfalfa is a rich source of flavonoid antioxidants and phytoestrogens including luteolin, coumestrol, and apigenin (Hwang et al., 2001; Stochmal et al., 2001) that have possible health-promoting activities. Alfalfa foliage also contains high amounts of xanthophylls, which are added to chicken feed to pigment egg yolks and broiler skin (Koegel and Straub 1996). Thus, in a biorefinery model for alfalfa processing, ethanol would be one of several products produced with the protein component possibly the more valuable and economically important product.

PRETREATMENT OF ALFALFA FIBER

Ethanol production depends on fermentation of simple sugars by microorganisms. The yield of potentially fermentable sugars from the conversion process is the critical response variable in assessing the value of alfalfa as an ethanol production feedstock. Potentially fermentable sugar yield is a function of both carbohydrate composition and concentration (discussed earlier), and the efficiency with which the cell wall polysaccharides are converted to simple sugars through processing. The results of two pretreatment methods have been reported previously. Ferrer et al. (2002) described parameters of ammonia processing of whole dried alfalfa hay that influenced the susceptibility of the fiber to subsequent enzymatic hydrolysis. The ammonia loading, moisture, time and temperature of treatment were varied and then the treated material digested with a mixture of cellulase, cellobiase, and xylanase. Conditions of 2 g ammonia g^{-1} DM, with 30% moisture and processing at 85°C for five minutes was shown to convert 76% of the theoretical yield of reducing sugars in the fiber. Approximately 200 mg sugars g^{-1} DM was

obtained (Ferrer et al., 2002); however, the yield of ethanol produced from this material remains to be determined.

Liquid hot water (LHW) pretreatments of the fiber fraction obtained after wet fractionation of alfalfa have been optimized for maximum sugar conversion (Sreenath et al., 1999) and ethanol production (Sreenath et al., 2001). The LHW pretreatment was found to solubilize hemicellulose, and the resulting extract contained significant amounts of acetic acid and formic acid (Sreenath et al., 1999). The remaining fiber fraction (raffinate) when treated with cellulase released 59 g of reducing sugars from 100 g of substrate. Addition of dilute acid (0.07% sulfuric acid) to the LHW decreased the amount of reducing sugars released by cellulase treatment to 24 g 100 g^{-1} substrate (Sreenath et al., 1999). Fermentation of the raffinate fraction after LHW pretreatment was tested with two strains of *Candida shehatae* in a simultaneous saccharification and fermentation (SSF) process as well as a separate hydrolysis and fermentation (SHF) process (Sreenath et al., 2001). The yield of ethanol was 0.45 g ethanol g^{-1} sugar with SSF and 0.47g ethanol g^{-1} sugar with SHF. The extract from the LHW pretreatment was also used in fermentation experiments and was poorly fermented, most likely due to the presence of organic acids. Addition of dilute acid to the LHW treatment resulted in fractions that were poorly fermented. Although untreated fiber substrate was shown to yield 51 g reducing sugars from 100 g of substrate (Sreenath et al., 1999), the yield of ethanol by SHF and SSF was 0.25 and 0.16 g ethanol g^{-1} sugar, respectively (Sreenath et al., 2001). These experiments demonstrate the impact of pretreatment on saccharification and ethanol production as well as the requirement to optimize processes for each lignocellulosic feedstock.

CONVERSION RESPONSE AFTER DILUTE ACID PRETREATMENT

For the purposes of this chapter, the high temperature, dilute acid pretreatment and subsequent enzymatic saccharification method will be examined in more detail as a conversion technology for ethanol production from alfalfa stem fractions. The high temperature, dilute acid pretreatment is designed to remove noncellulosic cell wall polysaccharides and lignin, because these constituents will interfere with the cellulase enzyme cocktails used for hydrolysis of the cellulose. One design goal of this pretreatment is to reduce the pH of the feedstock reaction mixture to 1.3–1.5 prior to heating (National Renewal Energy Laboratory, Golden, CO; Laboratory Analytical Procedure-007, May 17, 1995). The amount of sulfuric acid required to reach this pH target for alfalfa stems was 8.1 mmol g^{-1} biomass DM in a 1% solids slurry, compared to 6.4 mmol for switchgrass and corn stover (Jung, unpublished). Maturity of alfalfa stems and switchgrass did not influence the acid requirement. Dien et al. (2005) observed that the sulfuric acid loading required to maximize release of nonglucose sugars from alfalfa stems when heated at 121°C for 1 h was 2.5% (wt/vol), whereas 1.5% was sufficient for switchgrass. The higher acid requirement for alfalfa stems is most likely due to the greater pectin content of alfalfa cell

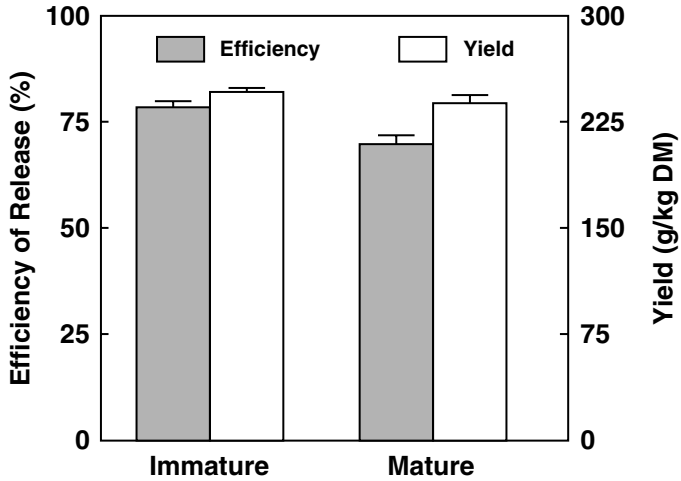

FIGURE 5.2 Efficiency of conversion and total yield of glucose from alfalfa stems when pretreated with dilute sulfuric acid at 150°C and subsequently saccharified using cellulase. (Dien et al., 2005).

walls compared to grasses; however, the hemicellulose content is lower and lignin content is similar in alfalfa stems compared to the grasses (Dien et al., 2005). Torget et al. (1990, 1992) also observed that legume feedstocks are more recalcitrant to acid pretreatment than grasses.

The efficiency of glucose release by acid pretreatment, followed by enzymatic saccharification from cell wall polysaccharides (cellulose and xyloglucans), declined as alfalfa stems became more mature (Figure 5.2). While efficiency of glucose conversion declined with maturity, the total yield of glucose was not altered (Figure 5.2), because cellulose content increased in more mature alfalfa stems. Similar declines in efficiency with maturity were observed for switchgrass and reed canary grass (Dien et al., 2005), but the efficiency of glucose release from the grasses was greater than from alfalfa stems. This may reflect the higher concentration of lignin in the alfalfa stems because across all three species, efficiency of glucose release was negatively correlated with lignin content of the feedstock. Increasing the temperature of the acid pretreatment resulted in improved efficiency of glucose release from alfalfa stems (Dien, personal communication). While efficiency of glucose release was lower for alfalfa than for grasses, total yield of glucose was very similar between the feedstocks. This again reflects the interaction of efficiency with glucose content of the feedstocks.

ALFALFA BIOTECHNOLOGY AND GENOMICS

An additional characteristic of alfalfa that makes it attractive for biorefinement is that it is amenable to genetic transformation. Rapid and efficient methods for transformation using *Agrobacterium tumefaciens* have been developed and gene

TABLE 5.2
Transgenic Alfalfa Producing Commercial Enzymes and Polymers

Enzyme	Gene	Source	Amount of Product	Citation
Phytase	phyA	*Aspergillus ficuum*	0.85–1.8% of soluble protein	Austin-Phillips and Ziegelhoffer, 2001 Ullah et al., 2002
Manganese-dependent lignin peroxidase	Mn-P	*Phanerochaete chrysoporium*	0.01–0.5% of soluble protein	Austin et al., 1995
α-amylase	α-amylase	*Bacillus licheniformis*	0.001–0.01% of soluble protein	Austin et al., 1995
Endo-glucanase	E2	*Thermomonospora fusca*	0.01% of soluble protein	Ziegelhoffer et al., 1999
Cellobiohydrolase	E3	*Thermomonospora fusca*	0.001–0.002% of soluble protein	Ziegelhoffer et al., 1999
β-ketothiolase	phbA	*Ralstonia eutropha*	0.025–1.8 g PHB/kg dry leaves	Saruul et al., 2002
Acetoacetyl-CoA reductase	phbB			
PHB synthase	phbC			

promoters identified for high constitutive expression and for tissue-specific expression (reviewed by Samac and Temple, 2004; Somers et al., 2003). Transformation has been used to alter alfalfa for production of valuable coproducts (Table 5.2) and for improving digestion of alfalfa fiber. Transgenic alfalfa has been shown to be capable of producing high levels of phytase (Austin-Phillips and Ziegelhoffer, 2001; Ullah et al., 2002), a feed enzyme that degrades phytic acid and makes phosphorus in vegetable feeds available to monogastric animals such as swine. Adding phytase to feeds reduces the need to add supplemental phosphorus to feed and reduces the amount of phosphorus excreted by animals. In field studies, juice from wet-fractionated alfalfa plants contained 1–1.5% phytase. Phytase activity in juice was stable over two weeks at a temperature of 37°C. Activity is also stable in dried leaf meal. Both juice and dried leaf meal added to feed were as effective in feeding trials as phytase from microbial sources. The value of the enzyme and xanthophyll in the juice was estimated at $1900/acre (Austin-Phillips and Ziegelhoffer, 2001). A wide range of feed enzymes is used to enhance digestion of feed and improve animal performance. Use of feed enzymes in monogastric and ruminant animals in expected to increase worldwide (Sheppy, 2001). Production of feed enzymes in transgenic plants, particularly in plants used as animal feed, would be an opportunity to increase feed utilization as well as value of the feed.

Transgenic alfalfa has also been used to produce several industrial enzymes. A manganese-dependent lignin peroxidase, which can be used for lignin degradation and biopulping in the manufacture of paper, was expressed in alfalfa. However, high levels of production of this enzyme appeared to be detrimental to plants (Austin et al., 1995). In the same study, α-amylase was produced at a level of approximately 0.01% of soluble protein without having a negative effect on plant development. Two cellulases, an endogluconase and a cellobiohydrolase, have been expressed at low levels in alfalfa (Ziegelhoffer et al., 1999). These enzymes were stable in dried leaf meal. Expression of cellulose degrading enzymes in biomass plants is one strategy to decrease the costs of saccharification that precedes ethanol fermentation. Alfalfa plants have also been shown to be an excellent "factory" for the production of chitinase (Samac et al., 2004). Chitin, found in shells of crustaceans, is the second most abundant carbohydrate after cellulose, and a potential feedstock in a biorefinery.

In addition to production of proteins, the use of transgenic alfalfa to produce other industrial feed stocks has been explored. Polyhydroxyalkanoates (PHAs) are produced by many species of bacteria and some PHA polymers are commercially valuable as biodegradable plastics. PHA synthesis in plants is seen as a more economically viable means of producing large quantities of these polymers (Poirier, 1999; Slater et al., 1999). Alfalfa was engineered to constitutively express three bacterial genes for the production of poly-β-hydroxybutyrate (PHB) (Saruul et al., 2002). Granules of PHB were shown to accumulate in chloroplasts without any negative impact on plant growth. Yield of PHB by chemical extraction was relatively low (1.8 g kg^{-1} DM), but may be improved by optimizing extraction methods or by utilizing stronger gene promoters.

A major limitation to use of biomass in the production of ethanol is the recalcitrance of the material to saccharification. Cross-linking of lignin with cell-wall polysaccharides interferes with enzymatic degradation of cellulose and can severely limit the conversion of herbaceous plant material into ethanol. Lignin in alfalfa stems also limits digestion of feed by ruminant animals. In experiments aimed at increasing feed digestion by ruminants, transgenic alfalfa was produced that had decreased expression of caffeoyl coenzyme A 3-*O*-methyltransferase, an enzyme involved in synthesis of lignin precursors. These plants were shown to have approximately 20% less lignin and 10% additional cellulose than the controls (Marita et al., 2003). The rate of digestion of the transgenic material was determined by *in vitro* rumen digestibility assays. In the transgenic material, a 2.8–6.0% increase in the rate of digestion was observed (Guo et al., 2001). This material could have a very significant impact on both animal nutrition and alfalfa biorefining. Casler and Vogel (1999) determined that a 1% increase in forage digestibility would lead to a 3.2% increase in average daily live-weight gain by beef steers. Although this material has not yet been tested with different pretreatment methods or used in saccharification or fermentation studies, based on chemical analyses, it may also have improved qualities as a feedstock for bioethanol production.

During the past several years, barrel medic (*Medicago truncatula*) has been the object of a broad range of research efforts worldwide. This annual plant, which is closely related to alfalfa, is a model plant for study of plant-microbe interactions and plant development (Cook, 1999). Chromosome mapping has shown that there is a high degree of gene synteny between the two species as well as a high degree of DNA sequence homology (Choi et al., 2004). Numerous genomic tools have been developed for *M. truncatula* including isolation of over 189,000 expressed sequence tags (ESTs), identification and sequencing of more than 36,000 unique genes (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago), extensive genetic and physical mapping (Choi et al. 2004), development of microarrays for transcript profiling, and a genome sequencing project is currently underway (http://www.medicago.org). In particular, microarrays are valuable tools for identifying genes involved in important agricultural processes as they enable researchers to measure expression of thousands of genes simultaneously. More than 100 genes are involved in cell-wall biosynthesis in plants and little is known about regulation of their expression. EST resources may be useful both as markers for selecting plants with favorable characteristics in bioconversion and in modifying gene expression in transgenic plants for enhancing the efficiency of ethanol production or enhancing yields of valuable coproducts.

CONCLUSIONS

Although commercial biorefining of alfalfa remains undeveloped, alfalfa has tremendous potential as a feedstock for production of ethanol and other products. Alfalfa is widely adapted and produces large amounts of biomass over the course of four or more years. The production costs of alfalfa are low and cultivation of the crop has numerous environmental benefits. Importantly, alfalfa leaves contain the majority of the protein in the plant and are easily separated from stems through processing. Leaf meal is a valuable coproduct in its own right as animal feed, as well as a potential source for human nutritional supplements and products derived from transgene expression. The stem fraction of alfalfa is rich in cell wall polysaccharides that can be used as a source of fermentable sugars to produce ethanol and other bioproducts. A biomass-type of alfalfa is being developed that is more upright in growth habit and performs well in a reduced frequency harvest management system, maximizing the yield of both leaf and stem fractions while lowering production costs. Incorporation of enhanced compositional traits such as more cellulose, less lignin and valuable transgenic protein products into this alfalfa biomass type through traditional breeding and using the tools of biotechnology will add to the value alfalfa brings to biofuels and bioproduct systems.

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