**Optimizing Feedstock Selection for Biofuel Production in Hawaii: CuO Oxidative Lignin
Products in C4 Grasses**

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Abstract

Global interest in renewable fuels is rapidly growing with particular emphasis on local energy growth and creating new energy feedstocks, specifically liquid fuel sources. However, the interactive effect of plant species/variety and growth environment on plant structural components, which may influence conversion efficiency and thus play an important role in optimizing the production of biofuels, is not fully understood. In this study cupric oxide (CuO) extractable lignin, which extracts and quantifies lignin-derived monomers, was determined for 25 cultivated and naturalized tropical perennial C₄ grass varieties of napiergrass and Guinea grass that were under assessment for suitability as feedstocks for liquid fuel generation. Principal component analysis of CuO extractable lignin-derived monomers showed differences in composition between napiergrass and Guinea grass, as well as environmental differences within many, but not all napiergrass varieties. Among the samples tested, the greatest differences in lignin composition occurred specifically in vanillyl lignin structures. A wide range (1-2.5) in the ratio of cinnamyl to vanillyl structures (C/V), which often relates to enzymatic degradability in natural systems, was also found. It is expected that the observed CuO lignin differences representing the structure and bonding of lignin will relate to ease of chemical and enzymatic conversion and the effectiveness of pretreatment and conversion in biofuel application. We hypothesize that the C/V ratio of feedstock will positively relate to conversion efficiency and if supported, then compositional lignin metrics such as the C/V ratio could be a predictor to select for more easily degradable biomass for biofuel production.

Keywords

biofuels, conversion, cupric oxide (CuO), lignin, lignocellulosic, thermochemical

50 **Abbreviations:**

51 C – cinnamyl

52 CuO – cupric oxide

53 C/V – cinnamyl to vanillyl ratio

54 PCA – principle component analysis

55 PM – permanganate

56 S – syringyl

57 SVC – the addition of syringyl, vanillyl, and cinnamyl lignin types

58 S/V – syringyl to vanillyl ratio

59 V – vanillyl

60

61 **1. Introduction**

62 Alternative fuel sources are entering an era of greater importance as demand for local, secure, and
 63 sustainable energy grows. Hawaii, as the most geographically isolated islands on the globe and fossil fuel
 64 dependent state in the United States, is particularly determined to reduce imported energy dependence[1].
 65 To this end, through the Hawaii Clean Energy Initiative, the state has laid out an aggressive goal of 70%
 66 clean energy by 2030 with 40% of that made up of local renewable sources[2]. Hawaii's remote location
 67 and dependence on commercial and military transport will also necessitate liquid fuels well into the
 68 foreseeable future. To meet this need, the Office of Naval Research and the United State Department of
 69 Agriculture created the national "Farm to Fleet" initiative[3] alongside state legislative mandates to
 70 expand biofuel production in Hawaii[4].

71 Biofuel feedstock selection and conversion pathway optimization will be of great importance as
 72 biofuel research, development, and production mature to meet Hawaii's energy targets. However, the
 73 landscape, soils, and climate in Hawaii are often characterized by sharp gradients[5] creating
 74 environmental challenges for feedstock production systems and limiting available area for large-scale
 75 agriculture. The interactive effect of varied growth environment and feedstock choice on plant structural

components[6] thus may have large influences on conversion efficiency, illustrating the importance of site and feedstock choice. Site specific variables such as soil can affect lignin expression, feedstock quality, and possibly biomass recalcitrance in biofuel conversion[7], further indicating that identification and selection of site-species combinations best suited to the local environment will be important considerations for final feedstock and conversion choice.

Previous research on plant digestibility has been conducted in many fields from feed utilization studies[8] to soil biogeochemistry research on lignin degradation in soils[9] and metabolic engineering[7, 10-13]. However, there have been mixed results relating a feedstock's degradability to its lignin, hemicellulose, and cellulose content. Li, et al.[11] report that the lignin composition is important for feedstock conversion, while Grabber, et al.[8] and Mansfield, et al.[12] report that total lignin content in biomass is the most important consideration. Differences in findings may arise from extremely variable polymerization and structure of lignin and its role in protecting cellulose and hemicellulose structures in plant material[10, 14]. Detailed lignin analysis is thus essential to further identify types of lignin structures and bonding that relate to ease of conversion.

The CuO oxidation extraction, originally developed for identification of terrestrial plant inputs into soils and sediments[15-17] and used recently in the study of plant decay and soil organic matter[18-21], was chosen as a lignin compositional metric. It is thought that degradation parameters extensively explored in the breakdown of plant materials in soils, such as the C/V ratio as a proxy of soil input quality and subsequent enzymatic and microbial decay[9], can be extended to investigate the similar enzymatic and oxidative bond cleavage of biomass in biofuel pretreatment and conversion applications. In this study, as part of a much larger ongoing bioenergy project, lignin concentration and composition of 25 potential tropical perennial C₄ grasses were initially investigated under contrasting environmental conditions to measure environmental effects on lignin composition and feedstock suitability in biochemical or thermochemical processing into biofuels.

2. Methods

2.1. Experimental design

Cultivated and naturalized tropical perennial C_4 grass varieties of napiergrass and Guinea grass were collected from across the Hawaiian Islands in the summer of 2009 to be studied for suitability as bioenergy feedstocks. Of the collected varieties, 17 napiergrass, 7 Guinea grass, and 1 hybrid were planted in a randomized complete block design at two separate sites. One trial was planted on Oahu at the University of Hawaii's Waimanalo Research Station (Waimanalo). Located at 24 meters above sea level, the Waimanalo field trials contained 4 replicate plots grown in Waialua series Mollisol (very-fine, mixed, superactive, isohyperthermic Pachic Haplustoll). Waimanalo received a mean annual rainfall of $1,151.9 \pm 1.7$ mm[22], and experienced a mean annual temperature of 23.4°C [23]. The grasses were planted November 10, 2009 and were irrigated to prevent stress. The second site was on Maui at the University of Hawaii's Maui Agricultural Research Center, also known as Kula Experiment Station (Kula). Located at an elevation of 1,005 meters, the Maui trials were grown in Kula series Andisol (medial, amorphic, isothermic Humic Haplustand). Kula received a mean annual rainfall of 626.6 ± 1.0 mm[22], and experienced a mean annual temperature 17.8°C [23]. The experimental design was the same as Waimanalo except only three replicates were used and plots were established April 6, 2010.

Total aboveground biomass was measured at harvest every 8 months under a ratoon (zero-tillage) management schedule. The Kula trials showed that 1,005 meters was outside the habitat range of Guinea grass, which did not survive initial planting, thus data for Guinea grass is only available from Waimanalo. As for napiergrass, several species were found to perform particularly well based on yield at both locations, with PMxD, Bana, Green, and Purple varieties targeted in this paper as top potential feedstocks.

2.2. Above Ground Biomass Yield and Total Lignin

Above ground biomass from the 3rd ratoon was harvested July 2011 with the middle two rows cut 20 cm from the soil. Fresh biomass weight was recorded and three stalks were randomly selected. Stalks were chopped, individually weighed, dried at 70°C to constant weight in a forced draft oven, and weighed

again for moisture determination. Dried stalks were ground to pass through a 2 mm sieve (Retsch model no. SM200, Haan, Germany) for forage analysis at the Agricultural Diagnostic Service Center at The University of Hawaii at Manoa. Digestive gravimetric quantification of lignin using permanganate was performed[24], referred to henceforth as PM lignin. Means and standard error were calculated for the replicated plots for yield and PM lignin. Significant differences among means were determined by two-way ANOVA (variety x site) and Tukey's HSD test (R); significance level was set at $p < 0.05$.

2.3. Lignin composition and interpretation

Subsamples from the replicates of each variety and site were composited and ground to pass through a 150 micron sieve for CuO oxidation and extraction, performed following the method of Hedges and Ertel (1982) with modifications described by Dalzell et al. (2005). Samples were extracted in analytical duplicate on composited sample of each variety and site. Following ethyl acetate extraction, CuO extractable lignin monomers were silanized before analysis on a QP-2010 Ultra GC/MS (Shimadzu, Columbia, MD). Identification and quantification of 11 different lignin structural phenols[15] was accomplished by comparison of mass spectral sample response to an internal ethyl vanillin standard and an external calibration curve of authentic lignin phenolic compounds. The concentration of CuO lignin monomers relativized to the total of each variety were evaluated by principal components analysis (PCA)[20] to identify broad differences in lignin composition between and within species and sites. Lignin monomers extracted using the CuO oxidation method belong to four classes depending on structural side chain differences: syringyl (S; syringaldehyde, acetosyringone, syringic acid), vanillyl (V; vanillin, acetovanillone, and vanillic acid), cinnamyl (C; ferulic acid and hydroxycinnamic acid), and p-hydroxys (p-hydroxybenzaldehyde, p-hydroxyacetophenone, and p-hydroxybenzoic acid). Commonly cited lignin structural class ratios in soil and sediment literature (e.g., S/V and C/V) were also evaluated[9, 16-18, 21]

3. Results and Discussion

3.1. Lignin Composition

The lignin structural chemistry of the aboveground tissue differed substantially by species and, within napiergrass some varieties differed by site whereas others showed site insensitivity (Fig 1.). Axis 1 of the PCA, which explains 48.3% of variance within the dataset, shows a strong spread of the species and site based on the relative abundance of the V lignin phenols, with lesser variance also explained by p-hydroxycinnamic acid, p-hydroxybenzaldehyde, and acetosyringone concentrations. Axis 2, which explains a further 18.4% of the variance in the data, shows a spread within species based on differing concentrations of ferulic acid and syringic acid. Site sensitivity of the lignin structural chemistry emerges within the napiergrass varieties that are positively correlated with Axis 2 (i.e., with high concentrations of syringic acid and low concentrations of ferulic acid). As shown in several other studies, changes in V type lignin (a measured subset of guaiacyl sourced lignin subunits[25]), has been shown to relate to bioconversion efficiency[11] indicating the spread of varieties across Axis 1 may also suggest digestibility.

Guinea grass, on the other hand, not only demonstrated no successful growth at the high elevation of Kula but also displayed high concentrations of V and S lignin (Fig. 1), which have been associated with higher resistance to degradability compared to C lignin[9]. These characteristics, combined with high invasiveness and proclivity to burn[26], may make Guinea grass less suitable for biofuel feedstock production in Hawaii compared to napiergrass.

3.2. Yield and Lignin Concentration

Yield and PM lignin of the four varieties selected as top potential feedstocks differed by plant variety and growth environment. Bana variety had greater yield at Kula compared to Waimanalo ($p=0.0052$ for the site x variety interaction) (Fig. 2a). Kula biomass had less PM lignin compared to Waimanalo for all varieties except PMxD ($p=0.0913$ for the site x variety interaction) (Fig. 2b). Although no significant pairwise differences were detected with Tukey's HSD test, pairwise T-tests between sites

for each variety showed all Kula varieties, except PMxD, having significantly less PM lignin at Kula (Purple: $p < 0.1832$; Green: $p < 0.1984$; Bana: $p < 0.0319$). On average, PM lignin was $15.8 \pm 3.8\%$, ranging from 11.1% to 19.6% in target varieties, or approximately 2.5 times greater than SVC lignin (reported as the addition of syringyl, vanillyl, and cinnamyl CuO extractable lignin monomers). SVC lignin was on average $6.26 \pm 0.08\%$ and ranged from 5.89% to 6.46% of total aboveground biomass (Fig. 2c).

We expect that SVC lignin is a better evaluation metric for biofuel feedstocks, based on the specificity of the CuO oxidation method quantifying only lignin monomers. The difference in total lignin concentration between digestive gravimetric methods such as PM lignin and CuO lignin suggests that approximately 5-14% of aboveground biomass may be misidentified as lignin by similar digestive techniques. Both an underestimation of lignin by SVC lignin due to incomplete breakdown of lignin during the extraction[9], and an overestimation of PM lignin due to inclusion of other unsaturated substances like tannins or suberins[24] contribute to the wide range in potentially misidentified lignin. More detailed determination and differentiation of lignin through CuO oxidation and other similar methods will be a necessary first step in better understanding the mechanisms behind lignin recalcitrance in biofuel conversion.

3.3. Ratios of CuO lignin monomers

The ratios of specific CuO lignin monomers, such as the C/V ratio (Fig. 2d), showed large variations. The measured C/V ratio of Purple and Green showed approximately 15 and 18% larger values at the Kula compared to Waimanalo, respectively, while Bana is about the same between sites, and PMxD is approximately 5% lower at Waimanalo. Ferulic and cinnamic acid, as ester-bound C type lignin structures, are more easily cleaved by hydrolytic enzymes[18] and therefore tissue with greater concentrations of C type lignin are more easily degraded in natural systems than the S and V lignin bonds that require an oxidative enzyme[9]. Therefore, we hypothesize that the greater C/V ratio in Purple and Green napiergrass at Kula will result in more effective hydrolysis during pretreatment and conversion

compared to that of Waimanalo[9, 16-18, 21], while PMxD at Kula may be more resistant to hydrolysis. The wide range in C/V found in this study at both sites (Fig. 3) is indicative of the sensitivity of the CuO oxidation extraction and its ability to capture small changes in lignin composition.

To demonstrate the proposed final use of the C/V ratio, all varieties at both field trials were ranked based on their C/V ratio and thus by their hypothesized digestibility (Fig. 3). Though other metrics for total and extractable sugars will remain important in crop selection and optimization, this study, along with a multitude of other data[7, 8, 10-13], suggests that detailed lignin analysis and existing digestibility relationships have potential in biofuel feedstock selection as well. Compared to derivatization followed by reductive cleavage (DFRC)[27], the CuO oxidation method can resolve multiple breakdown products of each monolignol precursor (H, G, S [25]), resulting in more comparative lignin composition data. Collectively, the high resolution CuO lignin data in this study also suggests that the lignin content and composition of bioenergy feedstocks can be affected by site choice, which could subsequently affect pretreatment and lignin-derived co-products in a biorefinery model.

4. Conclusions

Among the feedstocks identified as having the greatest potential in Hawaii, there were large differences in yield and CuO lignin ratios, such as the C/V ratio (Fig. 1, 2). As indicated by the increase in C/V ratio, Green and Purple varieties at Kula produced lignin that is hypothesized to be more easily degraded and subsequently more efficiently converted to biofuel by lignocellulosic processes[9]. Due to the increased chemical energy of lignin compared to sugars and thermal stability of certain types of lignin[28, 29], lignin amounts and ratios are also expected to influence thermochemical conversion, with pyrolysis oil quality shown to be affected by lignin content[30].

Continued research on CuO lignin correlation to pretreatment and conversion is anticipated to have strong predictive powers in biofuel production. The next stages of research involve directly testing the hypothesized relationship of C/V ratio to conversion efficiency with resulting relationships expected to facilitate selection of the optimal biofuel feedstocks for either lignocellulosic or thermochemical

conversion (Fig 3). Increased biofuel demand may also expand feedstock production into more diverse local landscapes and isolated areas where sensitive indicators of feedstock potential will be important for optimizing environmental suitability of feedstock choices. Continued improvement of tools to identify and quantify lignin in biofuels will be necessary to help researchers better estimate total feedstock composition, identify other co-products to be extracted, and guide decisions on conversion pathways and economies of scale.

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Lignin Composition
Using CuO Oxidation

Lignin Ratios
Suggest Digestibility

Most Easily Degraded
Bioenergy Feedstock

Figure 1

Figure 2

Figure 3

Figure 1 PCA of CuO extractable lignin monomers from 24 C4 grass varieties grown at Waimanalo and Kula field trials. The data shows both species and site shifts between the species/varieties with extracted vanillyl lignin types causing the largest differences.

Figure 2 Data from four top performing varieties of C4 grasses grown as possible biofuel feedstocks: a) Total above ground biomass was collected and measured in Mg/ha; b) Permanganate (PM) lignin percentage was measured as a metric of total lignin of aboveground plant material; c) SVC lignin percent was determined by CuO oxidation extraction as the sum of syringyl (S), vanillyl (V), and cinnamyl (C) lignin monomers. d) C/V ratio was calculated from total C and V lignin monomers quantified by GC/MS.

*error bars represent standard error

**error bars were omitted to avoid confusion between standard error and analytical error

Figure 3 All biofuel feedstock varieties from both Waimanalo and Kula were ranked in order of their C/V ratio and thus their expected digestibility based on literature. Top varieties targeted in this study at Waimanalo are in bold, with Kula varieties also underlined.