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Evaluation of Copper-Contaminated Marginal Land for the Cultivation of Vetiver Grass (*Chrysopogon zizanioides*) as a Lignocellulosic Feedstock and its Impact on Downstream Bioethanol Production

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Featured Application: Vetiver grass is a suitable candidate for phytoremediation of copper-contaminated soil. The harvested biomass has the potential to be used as feedstock for bioethanol production.

Abstract: Metal-contaminated soil could be sustainably used for biofuel feedstock production if the harvested biomass is amenable to bioethanol production. A 60-day greenhouse experiment was performed to evaluate (1) the potential of vetiver grass to phytostabilize soil contaminated with copper (Cu), and (2) the impact of Cu exposure on its lignocellulosic composition and downstream bioethanol production. Dilute acid pretreatment, enzymatic hydrolysis, and fermentation parameters were optimized sequentially for vetiver grass using response surface methodology (RSM). Results indicate that the lignocellulosic composition of vetiver grown on Cu-rich soil was favorably altered with a significant decrease in lignin and increase in hemicellulose and cellulose content. Hydrolysates produced from Cu exposed biomass achieved a significantly greater ethanol yield and volumetric productivity compared to those of the control biomass. Upon pretreatment, the hemicellulosic hydrolysate showed an increase in total sugars per liter by 204.7% of the predicted yield. After fermentation, 110% of the predicted ethanol yield was obtained for the vetiver grown on Cu-contaminated soil. By contrast, for vetiver grown on uncontaminated soil a 62.3% of theoretical ethanol yield was achieved, indicating that vetiver has the potential to serve the dual purpose of phytoremediation and biofuel feedstock generation on contaminated sites.

Keywords: phytoremediation; third-generation biofuels; lignocellulosic biomass; response surface methodology; optimization

1. Introduction

Mineral mining practices have contributed to potential toxic metal (PTM) pollution of soils worldwide [1]. Stamp milling was a common method used for extracting minerals from host rock. It is a process in which large mineral containing rock is crushed, or stamped, into smaller pieces for mineral extraction [2]. The fragmented rock, or mine waste product is called *stamp sand* because of its sand-like properties. Stamp sand has elevated levels of many PTMs, low organic matter, low water content, and does not support most vegetative growth [3]. In the Upper Peninsula of Michigan, during

the Cu mining boom period of late 19th and early 20th centuries, millions of tons of stamp sand were disposed of into Lake Superior and surrounding areas [4]. The sites that contain the mine waste are now considered marginal land, containing elevated levels of PTMs including Cu, Pb, Zn, As, Fe, Ni, Cd, Ag, Hg, Cr, Al, and Mo [5]. Due to the lack of vegetative cover, Cu contaminated stamp sands are eroding back into the lakes, thus affecting the aquatic ecosystem.

A potential remedy to make use of marginal, metal-contaminated land includes the application of phytoremediation; the use of plants to accumulate or stabilize contaminants. Following harvest, the contaminated biomass is conventionally disposed of at landfill sites or incinerated [6]. Depending on the nature of the plant species used for phytoremediation, the biomass harvested after phytoremediation could potentially be used as a second- or third-generation biofuel feedstock for sustainable land management.

Vetiver (*Chrysopogon zizianioides* L. Nash) grass is a lignocellulosic species that has been reported to be tolerant of various xenobiotics [7]. It has been classified as a hyperaccumulator of metals such as Pb and Zn [7,8]. It also accumulates many other PTMs [7]. Vetiver is non-invasive, has high biomass and an extensive root system [9,10]. Its biomass composition is low in lignin and rich in xylose, a highly fermentable pentose sugar [11–13]. Specifically, its lignocellulosic composition consists of 34.4% (m/m) cellulose, 39.4% (m/m) hemicellulose, and 7.9% (m/m) lignin [14,15]. The cellulose and hemicellulose composition make bioethanol production possible by yeast fermentation. The lignin content of vetiver is less than that of miscanthus and switchgrass, common second-generation bioethanol feedstocks [16,17]. We hypothesized that these characteristics would make vetiver a practical feedstock for bioethanol production.

In the current study, vetiver grass was grown in a greenhouse in columns containing stamp sand collected from the Torch Lake area in Upper Peninsula, Michigan. Metal uptake and leaching from the columns was monitored for a period of 60 days. At the end of the experiment, biomass was harvested and used for bioethanol extraction. The objectives of the study were to (1) evaluate the potential of vetiver grass to grow in stamp sand containing elevated levels of Cu, (2) evaluate the effectiveness of vetiver roots to control stamp sand erosion, (3) investigate the effects of PTM exposure on the lignocellulosic composition of the grass for downstream fermentation, (4) optimize the fermentative processes for vetiver biomass, and (5) study the effects of Cu exposed biomass on bioethanol yield.

2. Materials and Methods

2.1. Soil Collection and Characterization

Stamp sand was collected from the shoreline of Torch Lake in Lake Linden, Michigan (USA). The shoreline was divided into plots 25 m in length, two meters from the water table. Six sites were randomly selected for metal analysis. After the initial metal analysis, soil was collected in bulk from the site with the highest level of Cu (47°11’17.39” N, 88°24’26.05” W). The site for collecting the control soil was selected from a nearby site using the Michigan State University Extensions soil survey map from Atlantic Mine, MI (coordinates N 47.18529, W 88.69616). The basis for control soil selection was minimal Cu content (<50 mg/kg), but similar physico-chemical properties as those of the stamp sand, including particle size distribution, pH, and organic matter content. The physiochemical characterization of the stamp sand and control soil were performed in triplicates and included particle size distribution (ASTM method D6913), water content using an oven drying method [18], soil organic matter (SOM) content by loss on ignition [19] and water holding capacity described by Harding & Ross [20]. pH and electrical conductivity were measured using an Orion pH meter and conductivity probe. Toxicity characterization leaching procedure (TCLP) was performed according to EPA method 1311 [21]. Tamm’s reagent extraction for plant available iron and aluminum [22], sequential extraction for metal speciation [23], anion analysis by ion chromatography, and metal content by acid digestion (EPA Method 3050B) [24] were performed. Metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

2.2. Greenhouse Study

A 60-day phytoremediation study was performed in a greenhouse setting. Eighteen columns were assembled for the study (n = 9). The columns were constructed from PVC pipes 45 cm in height and 15 cm in diameter with a nylon suspension and a Tygon® tube at the bottom of each column for collecting leachate [25].

Vetiver plantlets were obtained from Agriflora Tropicals (Puerto Rico). The plantlets were potted in potting soil and acclimated for 30-days with regular watering in a greenhouse, prior to the experiment.

Nine columns were set up for each treatment group. Six of the nine columns were biotic (planted with vetiver). The remaining three columns were set up as abiotic (unplanted) to test the impact of vetiver on the leaching of metals from stamp sands. The stamp sand and control soil were dried and sieved (<2 mm). Each column was filled using 5.0 L of soil and vetiver grass plantlets were planted. Before planting, the vetiver plantlets were trimmed to achieve uniform shoot lengths, and biomass. Day zero measurements included vetiver biomass, root and shoot lengths, whole plant chlorophyll content by extracting with 80% acetone [24], lignocellulosic analysis of vetiver shoot tissue and metal analysis of the soils. Cu content was measured in root, shoot, and soil by acid digestion using EPA method 3050B [25] and ICP-MS analysis of the digests. Lignocellulosic composition was evaluated using National Renewable Energy Laboratory (NREL) method "Determination of Structural Carbohydrates and Lignin in Biomass" [26] and a crystalline cellulose assay [27].

During the 60-day study, the plants were maintained at 80% water holding capacity and rotated weekly [28]. Deionized water was used to water the plants as needed. Leachates were collected and analyzed for metals. Total suspended solids (TSS) and total dissolved solids (TDS) were estimated in the leachate to determine particulate leaching [29].

On day 60, shoot, root, soil, and leachate samples were collected; plant metal uptake, lignocellulosic composition of vetiver tissue and soil metal analysis was performed as described for day zero. For the leachate samples TSS, TDS, and metal content were determined. Bioconcentration factors (BCF) and translocation factors (TF) were calculated for Cu to measure its translocation [30,31].

2.3. Optimization of Fermentation Parameters

The RSM (Response Surface Methodology) optimization studies for dilute acid pretreatment, enzymatic saccharification, and fermentation were carried out using vetiver grass grown on control soil. Minitab® 17, a statistical design of experiments (DOE) software was used for experimental design by response surface methodology for the sequential lignocellulosic biomass to ethanol processes [32]. Factors for optimization of dilute acid pretreatment included acid concentration, biomass loading, and time and temperature of treatment. Table S1a displays treatment factors and levels of treatment used in the DOE with a central composite design (CCD). Values for levels of treatment were based on the NREL standard protocol for determination of carbohydrates and lignin from lignocellulosic biomass [26]. Replicates of two were built in. The total number of experimental combinations was equal to n = 60.

Each of the experimental combinations was carried out in 125 mL Erlenmeyer flasks using an automatic Yamato autoclave for varying time and temperature factors. The total volume of sulfuric acid was 50 mL for each trial. Following dilute acid pretreatment, the hemicellulosic hydrolysate was analyzed for monomeric carbohydrate and inhibitor content using high performance liquid chromatography (HPLC) following methods described by Groves, 2009 [33].

Total monosaccharide, furfural, 5-hydroxymethyl furfural, and acetic acid concentrations (g/L) were the response variables entered into Minitab® 17 following chromatogram analysis. The software's Response Optimizer was used to manipulate the factor levels simultaneously for maximum total monosaccharides and targets of 0.1 g/L, 0.5 g/L and 2.0 g/L for furfural, 5-hydroymethylfurfural, and acetic acid, respectively.

Prior to RSM optimized DOE for enzymatic saccharification, the biomass was washed three times, each with a volume of deionized water 3× that of the biomass, with 10 min of agitation on a rotary

shaker set to 150 rpm, and vacuum filtered [34]. Optimization of the process was based on buffer pH, time and temperature of treatment, and cellulase and β-glucosidase loading [34]. Table S1b shows the evaluated factors and their levels of treatment used in a central composite design (CCD). Minitab®17 RSM DOE created 66 different factor combinations for response analysis for treatment, according to Selig et al., (2008) [34]. Based on the control lignocellulosic content, 0.15 g (dry weight) of vetiver biomass was added to 20 mL scintillation vials at 105 °C [34]. Five milliliters of 0.1 M sodium citrate buffer were added to the biomass. 100 µL of 2% sodium azide were added to the vials for antimicrobial effects. The contents of the vial were incubated and brought to the appropriate temperatures. Cellulase and β-glucosidase were obtained from MP Biomedicals, LLC., Ohio, USA. Enzyme volumes of cellulase and β-glucosidase were loaded, and the vial contents were brought to a final volume of 10 mL with deionized water. The vials were capped and incubated for the indicated length of treatment time on a rotary shaker set to 160 rpm.

Following enzymatic treatment, the vials were heated at 80 °C for 10 min. One milliliter samples were drawn and filtered, using a 3 mL Luer-lock syringe a 0.45 µm nylon syringe filter, into HPLC vials for analysis. Glucose concentrations in g/L were entered into Minitab® 17 as the response variable. The Response Optimizer was used to determine which level of treatment for each factor would liberate the most glucose from cellulose. The determined time and temperature of treatment, pH, and enzyme loading concentrations for optimal glucose yield were performed on dilute-acid pretreated vetiver to obtain cellulosic hydrolysate for use in fermentation optimization and the achieved glucose yield was compared to the predicted value.

Optimization of yeast fermentation was based on maximum ethanol yield as a response, and experimental factors for fermentation time, temperature, inoculation percentage, nutrient additions, pH, and yeast species were investigated. A CCD design incorporated the levels of treatment for each treatment factor into a regression equation for fermentation optimization (Equation S2c), generated a total of 162 factor combinations. Combined hydrolysates created from RSM optimized DAP and enzymatic hydrolysis were used. Fermentations were carried out in 50 mL falcon tubes with a total volume of 15 mL. Nutrient supplements were added and mixed into the hydrolysate, including yeast extract for vitamins and minerals and peptone as a nitrogen source, according to the experimental design. Appropriate yeast species, grown 24 h prior to fermentation at 30 °C in general-purpose broth (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose), were added at indicated inoculum percentages. The fermentation tubes were incubated at the appropriate temperature on rotary shakers set to 160 rpm for the indicated length of time. Samples (1 mL) were drawn from the fermentation tube and syringe filtered (0.45 µm nylon filter) and analyzed by HPLC. The interactive Response Optimizer was used to predict factor levels that would achieve the maximum ethanol yield from the vetiver hydrolysates (Equation S2c).

2.4. Effects of Copper Exposure on Lignocellulosic Biomass

Experimental and control vetiver biomass, harvested at day-60 of the greenhouse study, was pretreated using dilute sulfuric acid under the conditions predetermined using RSM. The hemicellulosic hydrolysates were collected for fermentation and the biomass was used for further extraction of glucose by RSM optimized enzymatic hydrolysis. The combined hydrolysates and waste biomass were analyzed for Cu content using EPA method 3050B [25]. The hydrolysates were also examined for sugar and inhibitor yield using HPLC. The fermentations were performed under the RSM optimized conditions and the total sugar concentrations and ethanol yields were measured using HPLC every 12 h. Volumetric productivity (Q) and theoretical yields were calculated at the time of peak ethanol; 0.51 g of ethanol can be produced per gram of fermentable sugar [35].

2.5. Statistical Analysis

Statistical analysis was performed using *t*-test with Welch's correction and alpha = 0.05 for measuring significance between control and experimental data using GraphPad Prism version 6.04

for MAC, GraphPad Software, La Jolla, San Diego, CA, USA, www.graphpad.com. The significance of factors and the interactions between factors observed in RSM optimizations were evaluated using 2-way ANOVA (alpha = 0.05) (Table S4a–c).

3. Results and Discussion

3.1. Growth, Lignocellulosic Composition and Metal Uptake by Vetiver Grass Grown in Stamp Sand

The control soil was chosen such that it was similar in texture to that of the stamp sand, but had significantly lower PTM-content, since the sand-like texture of stamp sand highly influences water retention and soil organic matter [36]. Both soils are classified as a sandy loam according to the USDA-NRCS web soil survey. Stamp sand and control soil were characterized prior to the 60-day uptake study (Table 1). Particle size analysis showed that both the stamp sand and control soil had 70% sand content, and their water holding capacities were statistically similar using Welch's *t*-test with $\alpha = 0.05$ (Table 1).

Table 1. The physical and chemical properties of stamp sand and control soil. Shown as averages from triplicates ± standard deviation. Particular to the selection of the control soil was the sand fraction/texture of the soil and the differences in copper concentrations. The % sand fraction of particle size distribution was statistically similar, and the levels of copper were significantly different between soil types using Welch's *t*-test with $\alpha = 0.05$.

			Stamp Sand	Control Soil
Particle Size Distribution		% Clay	6.23 ± 2.36	15.20 ± 0.06
		% Silt	3.77 ± 0.80	10.20 ± 0.08
		% Sand Total	69.74 ± 1.28	70.81 ± 0.09
		% Sand 1000 µm	4.48 ± 1.15	0 ± 0
		% Sand 500 µm	14.45 ± 0.95	1.29 ± 0.05
		% Sand 400 µm	12.06 ± 0.16	1.70 ± 0.02
		% Sand 250 µm	38.76 ± 1.45	85.75 ± 0.03
		% Course silt	20.25 ± 1.88	5.01 ± 0.02
pH			6.82 ± 0.02	5.44 ± 0.02
Electrical conductivity (µS)			12.7 ± 0.21	20.9 ± 1.23
Water holding capacity (% WHC)			32.12 ± 1.44	29.7 ± 0.01
Water content (%)			22.96 ± 2.63	46.01 ± 0.01
Organic matter content (% LOI)			0.92 ± 0.20	3.45 ± 0.12
Tamm's Reagent (mg/kg)		Al	22.45 ± 0.04	11.65 ± 0.01
		Fe	89.00 ± 0.02	93.97 ± 0.94
Toxicity Characteristic Leaching Procedure (mg/kg)		Cu	394.32 ± 118.18	96.55 ± 73.43
		Fe	47.20 ± 13.94	64.20 ± 12.69
		Al	252.7 ± 28.22	39.27 ± 13.65
Total Metal Analysis (mg/kg)		Cu	1551 ± 28.28	27.05 ± 9.68
		Fe	44830 ± 806.00	12212.5 ± 333.08
		Al	7586.62 ± 761.88	3777.52 ± 558.34
Sequential Extraction (mg/kg)	Acid-soluble	Cu	397.37 ± 3.18	BDL
	Reducible	Cu	367.00 ± 1.18	BDL
	Oxidizable	Cu	126.60 ± 2.94	BDL
	Residual	Cu	1150.67 ± 10.26	0.65 ± 0.09

While stamp sand had more coarse silt (20.3%) when compared to the control soil (5%), the control soil had more clay (15.2%) compared to stamp sand (6.2%). The control soil was more acidic (5.4) compared to the stamp sand (6.8). Stamp sand had high Cu (1551 mg/Kg), Fe (44,830 mg/Kg) and Al (7886 mg/Kg) levels and Toxicity Characteristic Leaching Procedure (TCLP) showed that both Cu and Al had high potential to leach (Table 1). Sequential extraction was done to quantify the operationally-defined forms of Cu, which showed that acid-soluble and reducible fractions of Cu constituted about 20% of the total Cu each, whereas 50% of the Cu was in the residual fraction (silicate

bound). The stamp sand and the control soil had significantly different levels of PTMs, including copper, using Welch's *t*-test with $\alpha = 0.05$. The physico-chemical properties of stamp sand indicate that it is not favorable for plant growth due to elevated metals, low water holding capacity, and low organic matter content [37,38]. Stamp sand analyzed by sequential extraction revealed that the largest fraction (other than residual) for Cu was the acid-soluble fraction. This raises environmental and health concerns due to the high potential of the PTMs to leach into surface and groundwater. This concern is supported by the TCLP data, which revealed high concentrations of Cu far exceeding those recommended by the USEPA [39]. The reducible forms of Cu were also predominant, which allows for mobilization of metals through the formation of soluble metal salts from metal oxides [6]. The rich mineral content of stamp sand could also lead to the inhibition of soil microbiota, resulting in low SOM from decomposition [40]. Nitrate, phosphate and sulfate content of the stamp sand was also analyzed. Stamp sand has low phosphate (7 mg/kg), and although the nitrate concentration in stamp sand was adequate (222 mg/kg), nitrate uptake is likely to be severely reduced due to the inhibition of nitrate reductase from exposure to Cu toxicity [41].

The phenotypic differences were compared between the control and stamp sand grown vetiver after 60-days to determine the impact of metals on plant growth (Figure 1). The initial (day zero) biomass of each vetiver plant used for the stamp sand treatments was 19.8 g and each vetiver plant used for the control soil treatments was 15 g. The average root length for the vetiver plants used for the stamp sand treatments was 34.4 cm, and that of the control vetiver plants was 37.4 cm. The shoots were all trimmed to 30 cm length. The average chlorophyll content of the vetiver plants at day zero was 18.8 mg/kg for the stamp sand treatments and 22.0 mg/kg for the control treatments. The differences between the experimental and control groups for changes in mass of whole plant and root and shoot lengths after 60 days were significantly different with $p = <0.0001$, $p = 0.0408$, and $p = 0.003$, respectively (Figure 1). There was no significant difference between the chlorophyll content between the experimental and control groups ($p = 0.0940$) (Figure 1), however, the impact of Cu on chlorophyll was visible, with the stamp sand-grown plants showing much paler yellowish green leaves compared to the dark green leaves in the control plants (Figure S4).

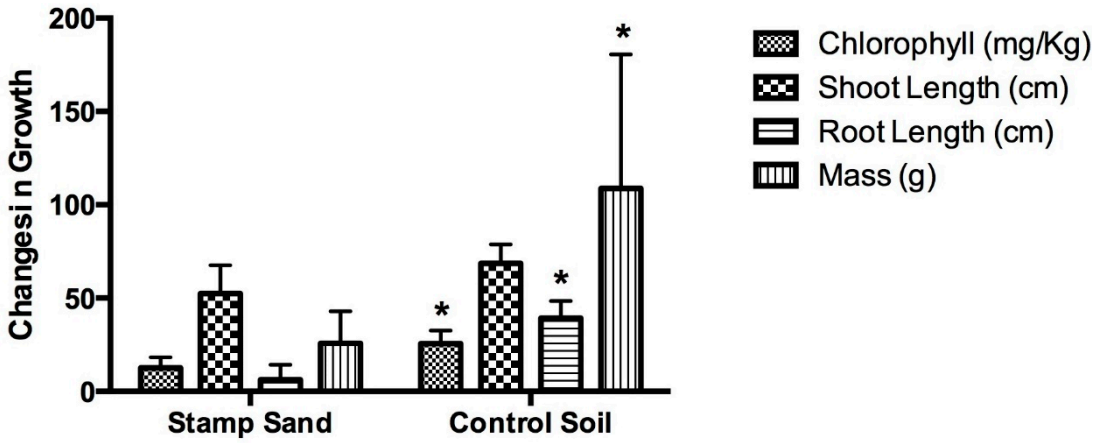

Figure 1. Effect of stamp sand on the growth of vetiver grass compared to control soil. Error bars represent standard deviation with n = 6. Statistical significance was determined using Welch's *t*-test and with $\alpha = 0.05$. (*) denotes *p*-value < 0.05.

Chlorophyll biosynthesis in plants is not directly impaired by Cu toxicity, but is affected by elevated levels of Mg, Mn, and/or Fe [41]. Rates of various photosynthetic processes, however, can be impaired directly due to Cu toxicity. At high Cu concentrations, biosynthesis of photosynthetic proteins such as Cu-containing plastocyanin PetE, are reduced [42]. Within plant cell organelles such as the chloroplast and nucleus, Cu (II) is often reduced to Cu (I), a reactive species that leads to oxidative stress [42–47]. Chloroplasts are a major storage location for Cu ions in plant cells [44,47]. Copper (I)

ions readily bind the thylakoid membrane, and impact photosystems I and II, inhibiting the function of chloroplasts in general. In the nucleus, Cu (I) binds to DNA in the presence of hydrogen peroxide and further reacts to form Cu (II) and hydroxylated-DNA, which damages the chromatin [43]. The reduced rate of photosynthesis leads to stunted growth in both aerial and root structures [42,46] and can explain the observed significant decreases in whole plant mass, shoot length, and root length. The present study did not specifically investigate the effects of copper speciation. The lack of plant available phosphorus could also contribute to the decreased root length of vetiver grown in stamp sand [48].

There were differences in Cu content in the stamp sand and control soil over the 60-day period, due to Cu uptake by vetiver as well as Cu leaching out of the column. However, the changes were not significant for the stamp sand or control soil. Vetiver roots contained increased concentrations of Cu by day 60 (Figure 2a). The increase in Cu content of the roots was greater than 14-fold, which was significant for the experimental biomass (p = 0.0002). The average Cu accumulation in the root tissue (n = 6) for the experimental group was 538 mg/kg and for the control group it was 60 mg/kg over the 60 days. The shoots of vetiver grass did not accumulate significant concentrations of Cu over the course of the study, in either the control or experimental group (Figure 2b).

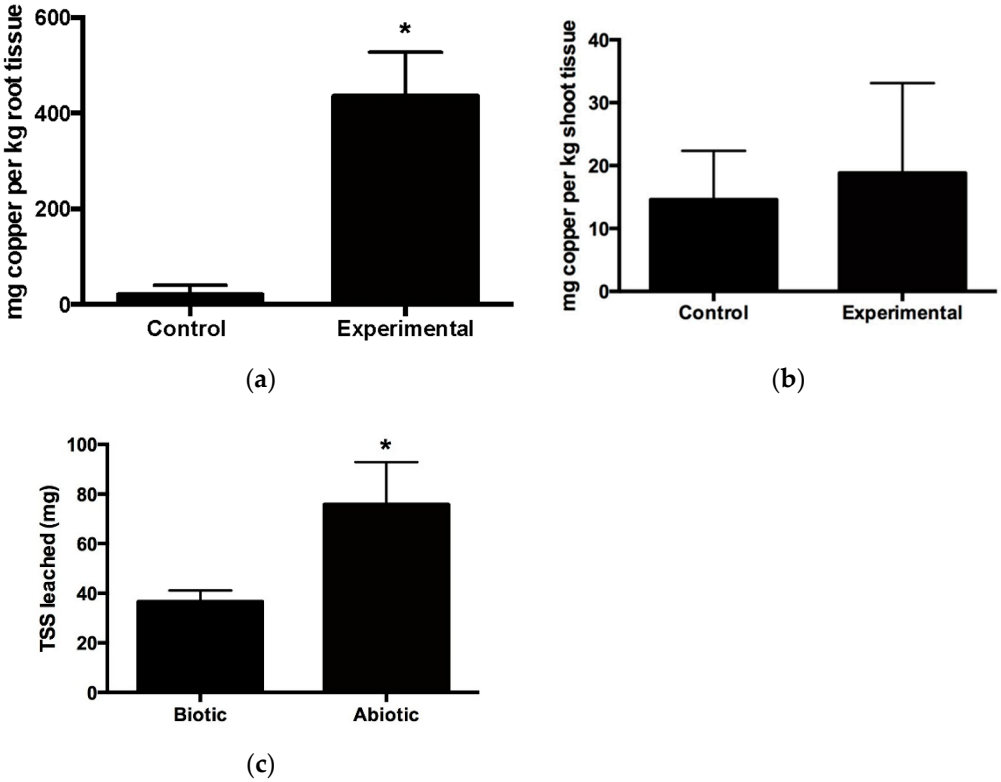

Figure 2. Copper accumulation in vetiver grown (biotic columns) in control soil versus stamp sand in (**a**) root and (**b**) shoot. "Control" = vetiver grown in control soil; "Experimental" = vetiver grown in stamp sand. (**c**) Total suspended solids (TSS) in leachate (mg) between biotic and abiotic columns. Error bars show standard deviation (n = 6). (*) indicates p-value = <0.05, using t-test with Welch's correction and α = 0.05.

Our results are consistent with previous reports showing that vetiver grass is capable of tolerating Cu toxicity, but accumulation of Cu in shoots and roots is limited [10]. The efficiency of metal removal by vetiver grass was quantified using bioconcentration factor (BCF) and translocation factor (TF). The BCFshoot, BCFroot, and TF were 0.0106 (± 0.0098), 0.3781 (± 0.1814), and 0.0175 (±0.0064), respectively. Bioconcentration factors and translocation factor for Cu indicate that Cu accumulates mainly in the root tissue. Vetiver has been reported to be able to accumulate large amounts of metals in their root, but prevent translocation to the shoot to protect against toxicity in aerial structures [49,50].

The leachates collected from day 30 to day 60 was used for measuring differences in particulate leaching between the biotic and abiotic columns. There was a significant difference between the TSS leached from biotic and abiotic columns (p = 0.0187) (Figure 2c). This result indicated that the root structures significantly reduced leaching of TSS, which would potentially reduce Cu leaching from the columns in the presence of vetiver.

The aerial shoots of vetiver from both treatment groups of the uptake study (n = 6) were harvested to evaluate lignocellulosic composition. Lignin content significantly decreased (p = 0.0006) (Figure 3), fermentable carbohydrates comprising the hemicellulose fraction significantly increased (p = 0.0125) (Figure 3), and crystalline cellulose content significantly increased (p = 0.0002) (Figure 3) in experimental shoots.

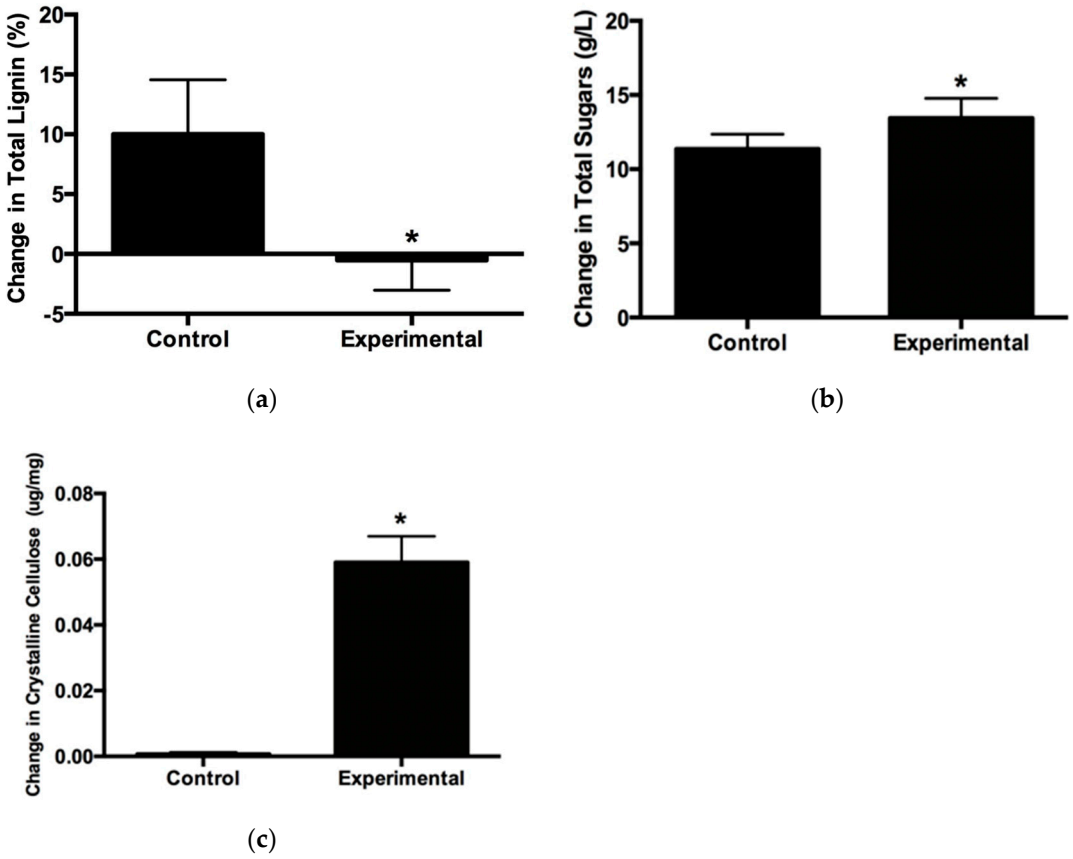

Figure 3. Changes in the lignocellulosic composition of vetiver biomass grown in control (control) soil and stamp sand (experimental) soil over 60 days: (**a**) percent changes in total lignin, (**b**) changes in total hemicellulose (sugar) levels in g/L, and (**c**) changes in crystalline cellulose in μg/mg. Error bars show standard deviation (n = 6). (*) indicates p-value = <0.05, using t-test with Welch's correction and α = 0.05.

These changes in the lignocellulosic composition of Cu-treated vetiver favor fermentability and compensate for the reduction in biomass caused by metal exposure. Significant reductions in acid insoluble and acid soluble lignin were found. Monosaccharides that comprise the hemicellulose and crystalline cellulose content both increased significantly. Lignin production is carried out by phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol-NADPH-dehydrogenase (CAD), which are regulated by extracellular Cu-dependent laccases and peroxidases [51,52]. Kannaiyan et al. (2012) found that there is a peak Cu concentration at which laccase activity switches from being promoted to repressed and/or terminated [53]. This threshold concentration reflects the Cu tolerance of the organism; at lower concentrations Cu acts as a cofactor and at higher concentrations Cu toxicity is observed. Copper tolerance of laccase enzymes among basidiomycetes can vary 100-fold in fungal cell walls [54].

The high Cu content within the stamp sand is the likely reason for the significant reduction in lignin levels observed in vetiver shoots. During the pretreatment of lignocellulosic biomass for bioethanol production, the biomass undergoes delignification [55]. Less lignin and greater hemicellulose and cellulose content within the biomass contributes to a hydrolysate richer in fermentable monosaccharides for conversion into ethanol. This leads to increased fermentability and bioethanol yield by microbial fermentation [10,11,13].

3.2. Optimization of Fermentation Processes

3.2.1. RSM Optimization of Dilute Acid Pretreatment

The central composite design (CCD) model with four factors for optimization of dilute acid pretreatment was used to create a regression equation (Equation S2a) as a prediction for maximum monosaccharide yield after input of values for response variables. The significance of factors and the interactions between factors on the composition of the hemicellulosic hydrolysate sugars are shown in Figure S3a. Surface plots of interactions between factors on monosaccharide yield and production of inhibitors are shown in Figure S3b.

The optimal parameters for efficient dilute acid pretreatment include a treatment time of 45 min, temperature of 125 °C, use of 1% (*v*/*v*) sulfuric acid, and 6.44% (*m*/*v*) biomass loading. Using these parameters for dilute acid pretreatment of vetiver grass, it is predicted that 20.07 g of total sugars per liter can be achieved for the hemicellulosic hydrolysate. Upon pretreatment of vetiver biomass (n = 3), the hemicellulosic hydrolysate was found to have 41.085 ± 0.046 g of total sugars per liter, a 204.7% percent yield of the predicted. Inhibitors were produced above the targeted values at 0.305 ± 0.031 g/L furfural, 0.197 ± 0.035 g/L 5-hydroxymethylfurfural, and 4.859 ± 0.070 g/L acetic acid.

Optimization of dilute acid pretreatment of vetiver grass using RSM proved that acid concentration was the only evaluated treatment factor that had a negative impact on monosaccharide recovery from hemicellulose. Acid concentration also had the greatest effect on furfural production of the four tested treatment factors. The optimum sulfuric acid concentration of 1.0% (*v*/*v*) was most dilute out of the acid concentrations evaluated. Higher acid concentrations have been shown to decrease xylan hydrolysis, which results in lower xylose recovery during pretreatment [56]. However, inhibitor (furfural and acetic acid) yields have been found to increase with increasing acid concentration for dilute acid pretreatment [55–57]. Decreases in concentrations of 5-hydroxymethylfurfural were observed as acid concentration increased. Acidic conditions cause 5-hydroxymethylfurfural to degrade to levulinic acid and formic acid [58].

Temperature was also found to greatly impact furfural production. Furfural is a degradation product of xylose which constitutes the majority of hemicellulose as the polymer xylan [56]. The toxicity of furfural was observed in Saccharomyces cerevisiae, specifically through the inhibition of dehydrogenases, which negatively impacts glycolysis [59]. Higher treatment temperature decreased the presence of 5-hydroxymethylfurfural, most likely due to further degradation to levulinic acid and/or formic acid [58]. Acetic acid formation was not significantly impacted by changes in temperature. Increases in treatment temperature have also been shown to decrease reducing sugar yields [56]. The optimal temperature for maximum reducing sugar recovery and production of below target concentrations of inhibitors for the delignification of vetiver biomass was 125 °C.

3.2.2. RSM Optimization of Enzymatic Hydrolysis

The regression equation (Equation S2b) was used to determine factor treatment levels for glucose solubilization by enzymatic hydrolysis. Statistical significance of factors and interactions between factors on glucose yield is shown in Table S4b. No treatment factor or interaction of factors significantly impacted glucose yield. Surface plots for evaluating factor interaction effects on glucose values are shown in Figure S3e.

Optimum conditions for enzymatic hydrolysis of pretreated vetiver biomass were determined to be a treatment pH of 2.0, temperature of 30 °C, and time of 5.5 days with β-glucosidase loading at 120 pNPGU/g cellulose. The prediction of glucose liberation under these parameters is 13.125 g/L. After carrying out the optimized conditions for enzymatic hydrolysis of pretreated vetiver biomass 19.197 ± 0.090 g/L glucose was achieved, a 146.3% yield.

The effects of pretreatment time on desirable sugar and inhibitor concentrations mimicked trends observed by temperature with respect to formation of furfural and degradation of 5-hydroxymethylfurfural. Increasing treatment time leads to increased formation of xylose degradation products [60]. Monomeric carbohydrate concentrations were observed to increase with increasing length of treatment. A greater recovery of sugars has been observed with longer dilute acid pretreatment times [60].

Relationships were positive between biomass loading and each of the four response variables (Figure S3a–d). Out of the responses, monosaccharide and acetic acid formation were most significantly affected. The relationship between monosaccharide production and biomass loading was linear with an R2 value equal to 1. Optimal biomass loading of 5–15% was reported by Kim et al. [61]. Although biomass loading above 6.44% (*m*/*v*) achieves greater sugar yield, greater concentrations in inhibitor response variables was observed. Acetic acid is a weak acid that disrupts fermentation by causing an imbalance in the proton motive force as acids cross the cellular membrane [59]. ATP depletion results from the energy requiring process of proton efflux and renders the cells unviable [59]. Hemicellulose contains acetyl groups, which are hydrolyzed simultaneously as xylose is liberated from xylan [62]. Thus, the correlation between increasing sugar and acetic acid formation can be explained. Dilute acid pretreatment of sugarcane with varying solid loading showed similar results [63].

The pretreated vetiver biomass was used for RSM optimization of glucose recovery from cellulose by enzymatic saccharification. There were no significant interactions between treatment factors in this study (Table S4b and Figure S3c). It was observed that as pH increased, glucose recovery decreased. A low pH of 2.5 was deemed optimal. Cellulase and β-glucosidase activities are known to be supported by a wide pH range [64]. Hydrolysis temperature was optimal at 30 °C, with a severe decrease in glucose recovery at temperatures exceeding 30 °C. Similar results were observed in the enzymatic saccharification of rice straw, with an optimum hydrolysis temperature of 35.4 °C [65]. Time of enzymatic hydrolysis was optimum at 5.5 days. Above or below the optimized time, there was a significant decrease in glucose recovery, this was also observed for the enzymatic hydrolysis of sugarcane tops and maize starch [63,66]. There was a positive correlation between β-glucosidase loading and glucose yield. β-glucosidase alone was responsible for maximum glucose recovery, predicted at 13.124 g/L, at 120 pNPGU/g cellulose. The lack of cellulase activity and cellulolytic role of β-glucosidase was also observed for the enzymatic hydrolysis of softwood [67]. A similar threshold for β-glucosidase loading and activity was also observed [67]. Recovery of glucose from the cellulosic fraction of vetiver exceeded the predicted concentrations under the optimized conditions. The prediction of glucose liberation RSM optimized parameters was 13.125 g/L. After carrying out the optimized conditions for enzymatic hydrolysis of pretreated vetiver biomass 19.197 ± 0.090 g/L glucose was achieved, a 146.3% yield.

3.2.3. RSM Optimization of Fermentation

Ethanol concentration (g/L) was the response values entered into Minitab® 17 following fermentation of RSM generated samples. Polynomial regression equations were generated for each categorical factor, or yeast species to predict maximum ethanol production (Table S1c). The significance of interaction effects between treatment factors on ethanol yield are shown in Table S4c and surface plots in Figure S3f.

Investigated fermentation parameters included five continuous factors (pH, time, temperature, inoculum percentage, and nutrient addition percentage) and 1 categorial factor (yeast species). Optimal levels of treatment were determined using Minitab®17 for RSM design of experiment, using maximum

ethanol concentration (g/L) as the targeted response variable. Fermentation conditions for maximum ethanol yield include an initial pH of 8.1, nutrient addition of 8.1% (*w*/*v*) for peptone and 4.05% (*w*/*v*) for yeast extract, 2 2% (*v*/*v*) inoculation with *Scheffersomyces stipitis*, at a temperature of 33.4 °C for 104.8 h (4.3 days). Under these parameters (n = 3), 4.86 ± 0.31 g/L ethanol were produced. The predicted concentration of ethanol was to be 6.8 g/L, providing a 71.47% yield.

As fermentation parameters increased their respective level of treatment, increased ethanol yield was observed. The categorical factor of yeast species had significant interaction effects with pH, time, and inoculum percentage. *Scheffersomyces* (*Pichia*) *stipitis* showed significantly greater ethanol yield than *Kluyveromyces marxianus*, and *Pachysolen tannophillus*. *Scheffersomyces stipitis* is a native xylose fermenter, and the combined vetiver hydrolysate was rich in xylose from the hemicellulose fraction [13,68,69]. A pH of 8.1 was optimal for maximum ethanol yield. As pH increased, ethanol yield increased, as well. This trend was also observed for the fermentation of sunflower hulls [70]. By increasing pH, the amount of undissociated acetic acid molecules decreases, which minimizes their intracellular ATP depleting capacity [58,69]. A fermentation temperature of 33.4 °C was predicted to produce maximum ethanol. This temperature is suitable for the yeast species selected; typically, a fermentation temperature of 30 °C is used [68,71]. Fermentation time of 5.5 days was determined to be optimal, however, peak ethanol as observed at 35 h. Similar results have been noted for peak ethanol achievement [69]. An inoculation percentage of 22% (*v*/*v*) was predicted to yield maximum ethanol. Higher inoculation rates increase ethanol productivity [72,73]. Peptone and yeast extract additions were determined to be optimal at 8.1% (*m*/*v*) and 4.05% (*m*/*v*), respectively. Peptone and yeast extract provide nitrogen and minerals to support yeast health, components that the hydrolysate lacks [72].

3.3. Monitoring Copper within Hydrolysates

The optimized processes were carried out on biomass harvested from a Cu contaminated soil with 1464.1 ± 250.18 mg/kg Cu at the beginning of the experiment. For the control, biomass was harvested from a control soil with 15.77 ± 2.60 mg/kg Cu at the beginning of the experiment (n = 6). Hence, we analyzed Cu content in the waste hydrolysate. The experimental and control hemicellulosic hydrolysates had Cu concentrations of 0.2 mg/kg and below detection limit, respectively. The experimental biomass was found to have 51.6 ± 11.9 mg/kg Cu and the experimental cellulosic hydrolysate contained 0.10 ± 0.02 mg/L Cu. The Cu content between the control cellulosic hydrolysate and experimental cellulosic hydrolysate was significantly different ($p = < 0.0001$).

3.4. Effects of Copper Exposure on Ethanol Yield

The hemicellulosic and cellulosic hydrolysates were combined to yield a total sugar concentration of 17.73 ± 0.07 g/L (64.7% of theoretical) from the experimental hydrolysates and 16.87 ± 0.05 g/L (61.6% of theoretical) for the control hydrolysates. Inhibitor concentrations in the Cu exposed hydrolysate were 0.16 ± 0.10 g/L furfural, 0.16 ± 0.03 g/L 5-hydroxymethylfurfural, and 5.47 ± 0.07 g/L acetic acid. Furfural, 5-hydroxymethylfurfural, and acetic acid concentrations for the control hydrolysate were 0.11 ± 0.07 g/L, 0.14 ± 0.02 g/L, and 4.56 ± 0.07 g/L, respectively. There were no significant differences between the experimental and control furfural and 5-hydroxymethylfurfural concentrations. The differences between the acetic acid ($p = < 0.0001$) and total sugar ($p = <0.0001$) concentrations were significant in favor of the control. Figure 4 shows comparisons the bioconversion of sugar to ethanol for control and experimental groups over time. Peak ethanol for the Cu exposed treatment group was 15.41 ± 1.81 g/L at 23.5 h (110% of theoretical) and 8.71 ± 1.08 g/L at 35 h (62.3% of theoretical) for the control.

The calculated volumetric productivity for the experimental and control groups were 0.613 $g*hr^{-1}$ and 0.249 $g*hr^{-1}$, respectively. There was a significant difference in ethanol yield between the control and experimental hydrolysates ($p = <0.0001$) at times of peak ethanol.

Fermentation of biomass harvested from Cu contaminated soil produced significantly greater ethanol yield than control biomass. This could be attributed to the presence of Cu ions which act as

cofactors during yeast metabolism [74,75]. In addition, the alternation in the lignocellulosic composition of vetiver biomass when cultivated on Cu loaded soils, as discussed in previously is likely to have played a role in increased ethanol yield. The presence of less lignin and greater sugar content was previously reported to lead to an increased yield of ethanol via yeast fermentation [76].

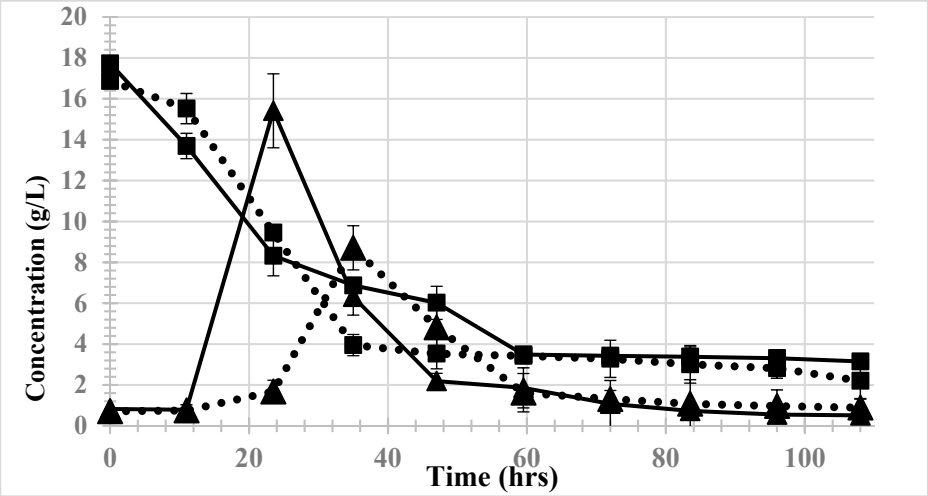

Figure 4. Total sugars (ν) consumed and ethanol (Δ) produced during fermentation of the vetiver hydrolysates. Solid lines represent the experimental group and dashed lines represent the control group. Error bars show standard deviation with n = 6.

A preliminary cost analysis of ethanol for the processing of biomass to hydrolysate, and its conversion to ethanol (0.15 g dry weight; 125 mL) was performed based on the total cost of consumables for these sequential processes. Commercially available Fisher brand reagent pricing, found on their website (www.fishersci.com) [77], was used for cost determination; the option for the smallest quantity of each Fisher brand reagent was selected for use in determining cost due to the small-scale quantities used in each fermentation. The optimal quantity of each reagent was factored into the cost determination per bench scale fermentation, and pricing was found to be $31.66. For the experimental ethanol produced from vetiver biomass grown on stamp sand, the cost per gram of ethanol is $2.03 ± $0.07, and the cost per gram of ethanol from the control biomass is $3.61 ± $0.04. The expenses to produce a gram of ethanol are significantly different (p = 0.0002) between the two treatment groups of vetivers using Welch's t-test with α = 0.05. Thus, ethanol produced from vetiver biomass harvested from stamp sand is significantly cheaper than ethanol produced from vetiver biomass harvested from control soil. Since our experiments were performed on a bench-scale, no attempt was made to compare the cost to the current market price of ethanol.

Increasing interest in the use of biomass grown in marginal, nutrient poor, and contaminated soil has resulted in a few recent reports where various plant species were investigated for phytoremediation of PTM-contaminated soil, followed by bioethanol extraction from the metal-laden biomass. Dhiman et al. [78] studied the potential use of canola for phytoremediation of Zn and Ni, followed by bioethanol production from the harvested biomass. They reported a saccharification yield of 74% and ethanol yield of 67% using Ni-contaminated biomass. Cheng et al. [79] reported the use of sweet potato plants for remediation of lead-contaminated soil, followed by use of the tubers for bioethanol production. Ko et al. [80] used napier grass for phytoremediation of Zn, Cd and Cr. They reported that metals generally lowered fermentation efficiency, but Cr had a positive impact on enzyme hydrolysis. Our study is the first report of the use of vetiver grass for the production of bioethanol, after optimization of bioethanol production conditions using RSM technology. Our results show that growing vetiver in Cu-contaminated soil affected lignin production, which had a positive impact on bioethanol yield. It was previously reported that the lignin content of vetiver is lower than other commonly used biofuel crops [16,17]. This indicated that vetiver biomass would potentially

provide higher ethanol yield from fermentation. Our studies show for the first time that growing vetiver in Cu-contaminated soil had a further positive impact on bioethanol yield. Studies are currently in progress in our lab to dissect the impact of Cu on lignocellulosic biomass composition.

4. Conclusions

Our study is the first report of using biomass harvested from vetiver grass grown in Cu contaminated soil for bioethanol production. Planting vetiver grass on stamp sand had a positive impact on reducing soil erosion. The harvested biomass showed significant and favorable changes in lignocellulosic composition and downstream bioethanol yield. The hydrolysates and residual biomass waste had low levels of Cu, which would eliminate any additional cost of disposal. Further scaled-up studies and life cycle analysis are required to assess the feasibility of using metal contaminated biomass for second-generation biofuel production.

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Author Contributions: E.M.G., D.S. and R.D. were responsible for study conception and design, E.M.G. was responsible for the acquisition of data, E.M.G., D.S. and R.D. were responsible for analysis and interpretation of data, E.M.G. was responsible for the drafting of the manuscript, and R.D. and D.S. were responsible for critical revision.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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