Nitrogen amendment of green waste impacts microbial community, enzyme secretion and potential for lignocellulose decomposition

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Abstract

Microorganisms involved in biomass deconstruction are an important resource for organic waste recycling and enzymes for lignocellulose bioconversion. The goals of this study were to examine the impact of nitrogen amendment on microbial community restructuring, secretion of xylanases and endoglucanases, and potential for biomass deconstruction. Communities were cultivated aerobically at 55 °C on green waste (GW) amended with varying levels of NH_4Cl . Bacterial and fungal communities were determined using 16S rRNA and ITS region gene sequencing and PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was applied to predict relative abundance of genes involved in lignocellulose hydrolysis. Nitrogen amendment significantly increased secretion of xylanases and endoglucanases, and microbial activity; enzyme activities and cumulative respiration were greatest when nitrogen level in GW was between 4.13-4.56 wt% (g/g), but decreased with higher nitrogen levels. The microbial community shifted to one with increasing potential to decompose complex polymers as nitrogen increased with peak potential occurring between 3.79-4.45 wt% (g/g) nitrogen amendment. The results will aid in informing the management of nitrogen level to foster microbial communities capable of secreting enzymes that hydrolyze recalcitrant polymers in lignocellulose and yield rapid decomposition of green waste.

Keywords:

Municipal green waste, compost, PICRUSt, biomass deconstruction, carbon to nitrogen ratio

Introduction

Each year, 167 million metric tons of municipal solid wastes are sent to landfills in the U.S and about 44.9% of these wastes are organic [1]. While infrastructure has been developed in states such as California to recycle these materials using composting and anaerobic digestion, utilizing organic wastes for biofuels could also divert biomass from landfills, although the costs associated with waste collection and separation must be considered [2]. Among the currently collected and transported organic wastes, green wastes (GW), which include mixtures of leaves, grass, prunings and trimmings, and branches and stumps, are potential sources for compost and liquid fuel production in or near urban areas and they could be utilized locally to avoid transportation costs [3].

Efficient conversion of lignocellulosic organic wastes such as GW to compost and liquid fuel requires enzymes that function at extreme conditions, such as high temperature, high-solid loading, and low moisture [4-6]. Nitrogen is one of the essential nutrients for the growth of microorganisms and a critical element in enzymes, however nitrogen concentration is typically low in many terrestrial ecosystems and lignocellulosic waste streams [7]. Carbon to nitrogen ratio (C/N) is one of the important factors affecting composting processes; C/N ratios of 25-30 are usually considered ideal and blending of feedstocks is often required to achieve optimum ratios [8-10]. Nitrogen amendment has also been shown to affect activities of enzymes in soil ecosystems; cellulase activity of degrading dogwood, maple, and oak litter was stimulated by nitrogen addition while lignin degrading activity was significantly reduced in high lignin containing oak litter [11]. Although studies have shown the availability of nitrogen can alter structure, function, and enzyme activity of soil microbial communities [7, 11, 12], few studies have elucidated the impact of nitrogen amendment on thermophilic microbial communities found in high solids biomass conversion systems and secretion of enzymes required for the hydrolysis of recalcitrant polysaccharides.

The goals of this study were to elucidate the biological mechanisms of enhanced GW decomposition with nitrogen amendment through examination of xylanase and endoglucanase secretion, microbial community restructuring and potential for GW deconstruction. Experiments were completed in a high-solids and aerobic environment at 55 °C with varying levels of nitrogen amendment. Compositions of both bacterial and fungal communities were examined and PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was applied to predict bacterial genes involved in lignocellulose degradation. The results will aid in informing the management of nitrogen to foster microbial communities capable of secreting enzymes that hydrolyze recalcitrant polymers in lignocellulose and yield rapid decomposition of green waste.

Materials and Methods

Materials

Finished green waste compost was used as inoculum and obtained from a commercial facility that composts agricultural residues including tree and vine prunings (Northern Recycling, Zamora, CA). Compost was solar-dried and stored at 4 °C until applied as inocula.

Green waste was prepared based on composition data reported in the California 2008 Statewide Waste Characterization Study (CSWCS) [13]. In the CSWCS, California was divided into five regions including the Bay Area, Coastal, Mountain, Southern, and Central Valley. To simulate the municipal green waste in urban areas, only the composition data from the Bay Area and Southern Area were applied. The composition data from both regions were normalized to 100% and then averaged, resulting in the GW which had 53.4% (wet basis) leaves and grass, 38.2% (wet basis) prunings and trimmings, and 8.4% (wet basis) branches and stumps (Table S.1). The category of leaves and grass was further separated by assuming that the mass fractions of leaves and grass were 46% and 54% respectively, and therefore the mass fractions of leaves and grass in GW became 24.6 and 28.8% (wet basis) respectively (Table S.1).

High solids incubations

High-solids incubations were conducted as described previously using bioreactors with a 0.2 L working volume loaded with 6 g dry weight of GW (5.4 g) and inocula mixture (0.6 g) [14]. To prepare mixtures NH_4Cl was added to M9 minimal media to the target nitrogen concentrations of 1, 2, 3, 4, and 5 (g NH_4Cl /kg M9) as N-M9 solutions. The pH of each N-M9 solution was adjusted with HCl to match the pH of the standard N-M9 solution (pH 5.5), which was 1 g NH_4Cl /kg M9. Prior to the experiment, GW samples were wetted with the assigned N-M9 solutions to a moisture content of 400 wt% dry basis (g N-M9 solution (g dry solid)⁻¹) and equilibrated at 4°C overnight.

An enriched community (T1) was established prior to the experiment to use as inoculum. Previous studies showed that the largest change in the microbial community composition occurred in the first enrichment [15] so only one enrichment was completed for this study. GW was wetted with 1g NH_4Cl /kg M9 and inoculated with 10 wt% (g dry compost (g dry solid)⁻¹) compost for one week. After the incubation, each N-M9 treated GW sample was inoculated with 10 wt% (g dry enriched sample (g total dry weight)⁻¹) of the enriched community (T1) and transferred to a new bioreactor (T2). The T2 incubation ran for 11 days and the experiment was conducted with three replicates.

DNA extraction and 16S rRNA gene and ITS region sequencing

At the end of the each enrichment, 3 g wet weight enriched feedstock was collected from each reactor, frozen in liquid nitrogen, homogenized with an oscillating ball mill (MM400, Retsch Inc., Newtown, PA), and extracted using the MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The quantity of the purified products was measured with a Qubit® fluorometer (Life Technologies, Grand Island, New York) using the manufacturer's

protocol. Three replicates were analyzed for each treatment. Sequencing was performed on purified DNA as previously described [16].

Enzyme extraction from solid samples

3 g wet weight of incubated GW was collected from each reactor at the end of the experiment. Samples were flash frozen with liquid nitrogen and stored at -20 °C for a week before enzyme extraction. Enzymes were extracted from incubated GW with a buffer containing 1 wt% sodium chloride, 0.1 wt% Tween 80 and 50 wt% ethylene glycol [6]. Endoglucanase (as carboxymethyl cellulose) and xylanase activities were measured as described previously [6]. All assays were completed in triplicate. Activities were reported as IU gdw⁻¹ where one IU = μmol product min⁻¹.

Prediction of gene content

16S rRNA gene sequencing reads were processed using Quantitative Insights Into Microbial Ecology software (QIIME) [17]. Reads were filtered and trimmed following the default parameters ($r = 3$; $p = 0.75$ total read length; $q = 3$; $n = 0$; $c = 0.005\%$) [18]. Closed-reference OTU picking was conducted within QIIME using the `pick_closed_reference_otus.py` command with default parameters. The Greengenes Database (Version 13.8) (pre-clustered at 97% similarity) was used as the reference sequence set [19]. The OTU table generated by QIIME was used as input for gene content prediction in PICRUSt software (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) [20]. To account for variation in the number of 16S rRNA genes between different taxa, OTU counts were normalized within PICRUSt based on predicted 16S rRNA gene copy numbers to better estimate the relative abundance of target genes [21]. Normalized OTU counts were used to predict the presence and abundance of genes belonging to protein families involved in cellulose, hemi-cellulose, lignin, and cello-oligosaccharide degradation.

Data analysis

Respiration data from high solids incubations were used to calculate CO₂ evolution rates (CER) and cumulative respiration (cCER) from CO₂ concentration and mass flow rate measurements of reactor effluents, as described previously [6]. Three replicate measurements were done for all nitrogen concentrations except for 3 g NH₄Cl/kg M9 which had only 2 replicates, due to a water clog inside the tubing connecting the reactor to the CO₂ sensor.

Sequences obtained through high-throughput 16S rRNA gene and ITS region sequencing of isolated DNA were quality trimmed, filtered, assembled and assigned to OTUs using methods described previously [16]. 16S rRNA gene and ITS region and read counts were used to conduct ecological and ordination analyses as described previously [22].

The relationships between cCER, xylanase and endoglucanase activities, gene counts predicted by PICRUSt and nitrogen level were evaluated using a second order polynomial regression model. An F-test was done to evaluate whether a second order polynomial relationship existed between the dependent and independent variable. Also, the significance of estimated regression coefficients were tested and evaluated for multicollinearity. Significant differences between treatments were identified using analysis of variance (ANOVA) and least significant difference with a significance level $\alpha = 0.05$. Data were analyzed using R (version 3.0.2, R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Effect of nitrogen amendment on microbial activity

Carbon dioxide evolution rate (CER) profiles for incubations with different nitrogen concentrations are shown in Figure S.1. The respiration rate increased rapidly at the beginning of incubation, reached a peak activity at approximately 1.4 days and then dropped rapidly. The two highest CER peaks were observed for treatments containing 2 and 3 g NH₄Cl/kg M9 with an average rate of 56 mg CO₂ (g dry matter)⁻¹ day⁻¹. The two lowest peaks for CER were observed

for treatments containing 1 and 5 g $\text{NH}_4\text{Cl/kg}$ M9 with an average rate of $46 \text{ mg CO}_2 (\text{g dry matter})^{-1} \text{ day}^{-1}$. A higher peak respiration indicates higher metabolic activity, suggesting that the communities in treatments with 2 and 3 g $\text{NH}_4\text{Cl/kg}$ M9 were more adapted for GW decomposition than communities in treatments with 1 and 5 g $\text{NH}_4\text{Cl/kg}$ M9. The lower peak respiration for 1 g $\text{NH}_4\text{Cl/kg}$ M9 compared to treatments with 2 and 3 g $\text{NH}_4\text{Cl/kg}$ M9 may have been due to nitrogen limitations and the resulting impact on microbial growth and synthesis of proteins required for substrate metabolism. The lower peak respiration for treatments containing 5 g $\text{NH}_4\text{Cl/kg}$ M9 may have been due to inhibition of microbial and enzymatic activity by nitrogen.

The ability of the microbial community to decompose GW under different levels of nitrogen amendment was also assessed by measuring cumulative respiration (cCER). The cCER on days 3, 7, and 11 increased as nitrogen content increased from 1 to 3 g $\text{NH}_4\text{Cl/kg}$ M9 and decreased as nitrogen increased from 3 to 5 g $\text{NH}_4\text{Cl/kg}$ M9 (Table 1). The relationship between cCER and nitrogen concentration was evaluated using a second order polynomial regression model (Table 2). Only the relationship between 11-day cCER and nitrogen level was significant; the maximum cCER was predicted at 2.76 g $\text{NH}_4\text{Cl/kg}$ M9.

Enzyme activity

Xylanase activity increased from 14.65 to 26.44 IU $(\text{g dry matter})^{-1}$ as the concentration of nitrogen increased from 1 to 3 g $\text{NH}_4\text{Cl/kg}$ M9 and decreased to 8.04 IU $(\text{g dry matter})^{-1}$ as the concentration of nitrogen increased to 4 g $\text{NH}_4\text{Cl/kg}$ M9 (Table 1). Further decrease in xylanase activity was observed as the concentration of nitrogen increased to 5 g $\text{NH}_4\text{Cl/kg}$ M9. Similar trends were observed for endoglucanase (CMC) activity. The endoglucanase activity increased as the concentration of nitrogen increased from 1 to 3 g $\text{NH}_4\text{Cl/kg}$ M9 and activity in the sample treated with 5 g $\text{NH}_4\text{Cl/kg}$ M9 was much lower than the other treatments.

The relationships between the activities of both xylanase and endoglucanase and nitrogen level were well characterized by second order polynomial functions (Table 2). The activities of xylanase and endoglucanase reached their maxima when the concentrations of nitrogen were 2.40 and 2.38 g NH₄Cl/kg M9, respectively.

Similar results to those observed in this study were found in a previous study which evaluated the xylanase and endoglucanase activities during composting of sugarcane trash with cattle dung; xylanase activity increased as the C/N ratio decreased from 51 to 30, reached the peak at C/N 30, but decreased as C/N continued decreasing, while the endoglucanase activity had a similar trend but the peak was reached at a C/N ratio of 36 [23]. Prior studies also observed that endoglucanase activities increased as the nitrogen concentration increased in maple and oak leaf composts although the nitrogen concentrations were in a much lower range than the present study [11, 24].

The cCER on day 11 and xylanase and endoglucanase activities reached their maxima between 2.4 and 2.8 g NH₄Cl/kg M9. A previous study also showed that increases in xylanase and endoglucanase activities corresponded with increases in cCER indicating a link between the microbial activity and enzyme activity [6]. However, in this study xylanase activity was significantly higher in samples amended with 2 g NH₄Cl/kg M9 compared to those with 4 g NH₄Cl/kg M9 while cCER was similar between samples (Table 1). It is possible the secreted enzymes detected in our assays were not sufficiently active on GW amended with 2 g NH₄Cl/kg M9 to be able to yield hydrolysis products that could be accessed by microorganisms and result in higher microbial activity. It is also possible that microbial activity was inhibited in samples with 2 g NH₄Cl/kg M9.

Effect of nitrogen amendment on green waste degrading bacterial community

The bacterial communities for treatments with 1, 2, 3, and 4 g NH₄Cl/kg M9 tended to cluster together on the NMDS plot (Figure S.2). The bacterial communities for treatments with 5 g NH₄Cl/kg M9 were similar within the three replicates, but diverged from the other nitrogen treatments.

The bacterial communities shifted in composition as the concentration of NH₄Cl changed. The dominant phyla were *Firmicutes* and *Proteobacteria* (Table 3). *Firmicutes* was the most abundant phylum in all treatments, and its relative abundance increased (85 to 97%) as the nitrogen concentration increased from 1 to 5 g NH₄Cl/kg M9. The relative abundance of *Proteobacteria* decreased from 11% to 2% as the nitrogen concentration increased from 1 to 5 g NH₄Cl/kg M9. The relative abundance of *Actinobacteria* significantly decreased from 4% to 2% as the nitrogen concentration increased from 1 to 2 g NH₄Cl/kg M9, and was not significantly different in treatments from 2 to 5 g NH₄Cl/kg M9. Three dominant families, *Bacillaceae*, *Paenibacillaceae*, and *Sporolactobacillaceae* were identified under *Firmicutes* (Table S.2). The relative abundance of *Bacillaceae* remained between 27-29% in treatments with 1 to 4 g NH₄Cl/kg M9 but increased to 50% as the concentration of NH₄Cl increased to 5 g NH₄Cl/kg M9. This was primarily due to changes in the relative abundances of the genera *Bacillus* (OTU 0), *Bacillus* (OTU 3), and *Geobacillus* (OTU 13) which were 3.5%, 14%, and 1.4% respectively in treatments with 1 g NH₄Cl/kg M9 and significantly increased to 7%, 33%, and 3.8%, respectively, in the treatment with 5 g NH₄Cl/kg M9 (Table S.3). Contrary to trends observed for *Geobacillus* (OTU 13), the relative abundance of *Geobacillus* (OTU 5) decreased from 9% to 7% as the concentration of nitrogen increased from 1 to 3 g NH₄Cl/kg M9 and remained between 5% and 7% in treatments from 3 to 5 g NH₄Cl/kg M9 (Table S.3). *Chelatococcus* was the most abundant genus under the family *Beijerinckiaceae* (under the class *Alphaproteobacteria*). The relative abundance of *Chelatococcus* in treatments with 1 g NH₄Cl/kg M9 was significantly higher than the sample with 5 g NH₄Cl/kg M9 (Table S.3).

The relative abundances of families *Sporolactobacillaceae* and *Paenibacillaceae* were not significantly affected by the nitrogen levels tested in this study (Table S.2). The relative abundance of family *Streptosporangiaceae* (under the phylum *Actinobacteria*) significantly decreased from 3% to 1.3% as the nitrogen concentration increased from 1 to 2 g NH₄Cl/kg M9 and was not significantly different in treatments between 2 to 5 g NH₄Cl/kg M9. At the genus level, the relative abundances of *Microbispora* (under family *Streptosporangiaceae*), *Sporolactobacillus* (OTU 2), and *Sporolactobacillus* (OTU 24) were around 2, 20, and 1%, respectively, for the nitrogen levels tested (Table S.3). The abundance of *Sporolactobacillus* (OTU 1) significantly decreased from 30% to 19% as the nitrogen concentration increased from 1 to 5 g NH₄Cl/kg M9 (Table S.3). The abundance of *Thermobacillus* significantly decreased from 3.4% to 1.2% as the nitrogen concentration increased from 1 to 5 g NH₄Cl/kg M9 (Table S.3).

Several studies also reported that *Firmicutes*, *Proteobacteria*, and *Actinobacteria* to be the dominant three phyla in green waste and municipal solid waste composts [25-28]. However, the relative abundances of microbial communities found in the present study were different from previous studies done in our lab which applied the same green waste compost as inocula in switchgrass and rice straw decomposition under thermophilic conditions with treatments containing 1 g NH₄Cl/kg M9 [14, 15]. The relative abundances of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were about 20%, 15%, and 44% respectively in a rice straw degrading community while the relative abundances of *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were about 8%, 38%, and 15%, respectively, in switchgrass degrading communities enriched from the same inoculum [14, 15]. Unlike the GW in this study, both rice straw and switchgrass were washed with ethanol and water prior to enrichments, and their C/N ratios (after adding M9 minimal media) were about 49 and 170 in washed rice straw and washed switchgrass, respectively. It is possible that the low C/N ratio in GW may favor the growth of *Firmicutes* but

not *Proteobacteria* and *Actinobacteria*. For example, the relative abundance of *Firmicutes* has been shown to be 90.6% in poultry litter compost with a C/N ratio of 9.2 [26].

Effect of nitrogen amendment on green waste degrading fungal community

The fungal communities for treatments with 1 and 4 g NH₄Cl/kg M9, and for treatments with 2, 3, and 5 g NH₄Cl/kg M9) formed two separate clusters on the NMDS plot (Figure S.2).

Ascomycota was the most abundant phylum (>99% in relative abundance) in the fungal communities followed by *Basidiomycota* (<1%). Three classes, *Dothideomycetes*, *Eurotiomycetes*, and *Sordariomycetes*, were identified under *Ascomycota*. *Eurotiomycetes* was the most abundant class followed by *Sordariomycetes* and *Dothideomycetes* (Table 3). The relative abundance of *Dothideomycetes* was not significantly affected by the nitrogen levels tested in this study. The relative abundance of *Eurothiomycetes* significantly increased from 56% to 91% as the concentration of NH₄Cl increased from 1 to 2 g NH₄Cl/kg M9, decreased from 91% to 62% as the concentration of NH₄Cl increased from 2 to 4 g NH₄Cl/kg M9, and then increased when nitrogen concentration increased from 4 to 5 g NH₄Cl/kg M9. Contrary to trends observed for *Eurothiomycetes*, the relative abundance of *Sordariomycetes* decreased significantly from 43% to 6% as the concentration of nitrogen increased from 1 to 2 g NH₄Cl/kg M9, increased from 6% to 37% when the nitrogen concentration increased from 2 to 4 g NH₄Cl/kg M9, and dropped significantly to 5% as the nitrogen concentration increased from 4 to 5 g NH₄Cl/kg M9.

Prior studies have shown that both *Ascomycota* and *Basidiomycota* to be two dominant phyla found in composts and soil communities in general [29, 30]. For instance, studies showed that the abundances of *Ascomycota* and *Basidiomycota* were 69% and 30% respectively in hardwood composts, and 60% and 35% respectively in hay composts [30]. Similar results were found in a prior study which reported that the abundances of *Ascomycota* and *Basidiomycota* were 95.1% and 1%, respectively, in the compost from forestry and botanical prunings, and 83% and 12%,

respectively, in the compost from a mixture of botanical prunings and municipal solid waste [31]. These studies also showed that the abundance of *Ascomycota* increased after enrichment under thermophilic conditions [30, 31]. Other studies reported on the occurrence of different eukaryotic members in the different stages of rice straw, lignocellulosic material (sun-dried tomato plant waste and pine woodchips), and chestnut green waste composting, however the phylum *Ascomycota* was ubiquitous throughout the composting process [27, 28, 32]. The results suggest that thermophilic conditions are more favorable for the growth of *Ascomycota* than other fungal phyla.

PICRUSt prediction of enzyme production potential

The NSTI is the sum of phylogenetic distances for each organism in the OTU table to its nearest relative with a sequenced reference genome, measured in terms of substitutions per site in the 16S rRNA gene and weighted by the frequency of that organism in the OTU table [20, 33]. A low NSTI value indicates a more accurate and reliable metagenome prediction. The NSTI values of samples from all treatments were in the range of 0.07 ± 0.003 (data not shown), suggesting that the predictions were very good.

The sum of abundance of predicted genes associated with cellulose, hemicellulose, lignin, and cello-oligosaccharide degradation changed with nitrogen level (Figure S.3). Results were tabulated to observe the influence of nitrogen level on individual genes involved in lignocellulose degradation (Table 4). Of the 53 genes examined, 10 genes related to cellulose degradation were found to vary significantly between treatments. For 6-phospho-beta-glucosidase [EC:3.2.1.86] (K01223), alpha-glucosidase [EC:3.2.1.20], glucan endo-1,3-beta-D-glucosidase [EC:3.2.1.39], and oligo-1,6-glucosidase [EC:3.2.1.10], the abundance of genes increased as the concentration of nitrogen increased and reached peak levels in samples with 5 g $\text{NH}_4\text{Cl/kg}$ M9. For beta-glucosidase [EC:3.2.1.21] (K05350) and cellulose 1,4-beta-cellobiosidase [EC:3.2.1.91], the abundance of genes decreased as the concentration of nitrogen

increased and reached the lowest levels in samples with 5 g NH₄Cl/kg M9. Other predicted genes reached their peak levels when the concentration of NH₄Cl was between 2 and 4 g NH₄Cl/kg M9 although the results were not statistically significant.

12 out of 22 predicted genes which relate to hemicellulose degradation increased in abundance as the concentration of NH₄Cl increased from 1 to 2 and 3 g NH₄Cl/kg M9 but decreased as the concentration of NH₄Cl increased to 4 and 5 g NH₄Cl/kg M9 (Table 4). For alpha-galactosidase [EC:3.2.1.22] (K07406), alpha-L-fucosidase [EC:3.2.1.51], alpha-mannosidase [EC:3.2.1.24], alpha-N-arabinofuranosidase [EC:3.2.1.55], beta-galactosidase [EC:3.2.1.23] (K01190), beta-glucuronidase [EC:3.2.1.31], beta-mannosidase [EC:3.2.1.25], endo-1,4-beta-xylanase [EC:3.2.1.8], levanase [EC:3.2.1.65], and xylan 1,4-beta-xylosidase [EC:3.2.1.37], the abundance of genes reached peak levels in samples with 2 and 3 g NH₄Cl/kg M9 but significantly decreased when the concentration of NH₄Cl increased to 5 g NH₄Cl/kg M9, suggesting that the higher concentration of NH₄Cl may be harmful to organisms containing these genes. For alpha-amylase [EC:3.2.1.1] (K01176), arabinogalactan endo-1,4-beta-galactosidase [EC:3.2.1.89], carboxylesterase [EC:3.1.1.1] (K03928), carboxylesterase 1 [EC:3.1.1.1] (K01044), endo-1,3(4)-beta-glucanase [EC:3.2.1.6], lysophospholipase [EC:3.1.1.5], and mannan endo-1,4-beta-mannosidase [EC:3.2.1.78], the abundance of predicted genes increased significantly when the concentration of NH₄Cl in treated samples increased to 5 g NH₄Cl/kg M9. In contrast, the abundance of predicted genes encoding carboxylesterase 2 [EC:3.1.1.1] (K03927) significantly decreased as the concentration of NH₄Cl in treatments increased from 1 to 5 g NH₄Cl/kg M9.

Six predicted genes related to lignin degradation reached their highest levels in treatments with 1 g NH₄Cl/kg M9 and significantly decreased when the concentration of NH₄Cl in treatments increased to 5 g NH₄Cl/kg M9. These include catalase [EC:1.11.1.6] (K00429), chloride peroxidase [EC:1.11.1.10], cytochrome c peroxidase [EC:1.11.1.5], glycolate oxidase

[EC:1.1.3.15], vanillate monooxygenase [EC:1.14.13.82] (K03862), and vanillate monooxygenase [EC:1.14.13.82] (K03863) (Table 4). The abundance of catalase [EC:1.11.1.6] (K03781) and thiol peroxidase, atypical 2-Cys peroxiredoxin [EC:1.11.1.15] increased significantly as the concentration of NH_4Cl in treatments increased from 1 to 5 g $\text{NH}_4\text{Cl}/\text{kg}$ M9. For catalase/peroxidase [EC:1.11.1.6 1.11.1.7] and glutathione peroxidase [EC:1.11.1.9], the abundance of predicted genes reached peak levels in samples with 2g $\text{NH}_4\text{Cl}/\text{kg}$ M9.

Beside alpha-L-rhamnosidase [EC:3.2.1.40] and maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141], other predicted genes related to cello-oligosaccharide degradation significantly increased as the concentration of NH_4Cl increased from 1 to 5 (g $\text{NH}_4\text{Cl}/\text{kg}$ M9) (Table 4). In contrast, the abundance of predicted genes encoding maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141] significantly decreased as the concentration of NH_4Cl in treatments increased from 1 to 5 g $\text{NH}_4\text{Cl}/\text{kg}$ M9. For alpha-L-rhamnosidase [EC:3.2.1.40] the peak abundance of predicted genes was observed when the concentration of NH_4Cl in treatments was 2 g $\text{NH}_4\text{Cl}/\text{kg}$ M9.

Levels of each gene predicted by PICRUSt were also fitted to the second order polynomial model. Only 10 out of 53 predicted genes could be represented by a second order polynomial model, and these 8 genes were associated with the degradation of hemicellulose (Table 5). The results showed that the predicted maximum number of these 10 genes occurred when the concentration of nitrogen was between 2.08 and 2.66 g $\text{NH}_4\text{Cl}/\text{kg}$ M9.

A number of bacterial genes involved in hemicellulose deconstruction showed peak enrichment at the same level where peak xylanase activity and cumulative respiration were observed. In contrast, there was not a significant enrichment of bacterial cellulases at the nitrogen level where peak endoglucanase activity was measured. This could be due to fungal cellulases contributing more to cellulose degradation or it could highlight the fact that enrichment of certain

deconstruction genes within the community is only a partial predictor of peak activity for those enzymes. While a certain gene may be prominent in a given community, the culture conditions must also promote the viability of the corresponding microbe and the expression of that gene for the activity to ultimately arise.

For hemicellulose hydrolysis, alpha-L-fucosidase [EC:3.2.1.51] was abundant in GW amended with 2 to 4 g NH₄Cl/kg M9 and its activity significantly dropped by 40% as the concentration of nitrogen increased to 5 g NH₄Cl/kg M9. Alpha-L-fucosidase is an important enzyme that catalyzes the hydrolysis of alpha-l-fucosyl residues in agricultural waste [34-36]. Alpha-amylase [EC: 3.2.1.1] (K01176), an enzyme that hydrolyses alpha-linked polysaccharides such as starch, was found to be abundant in GW amended with nitrogen at all levels [37]. Beta-galactosidase [EC: 3.2.1.23], an enzyme that hydrolyzes beta-galactosidic bonds between galactose, was most active in GW treated with 2 g NH₄Cl/kg M9 [38].

Beta-mannosidase is important in the hydrolysis of terminal, non-reducing beta-D-mannosyl residues in beta-D-mannosides [39]. The abundance of beta-mannosidase [EC: 3.2.1.25] genes remained high in treatments between 1 and 2 g NH₄Cl/kg M9, but dropped to 50% of its peak abundance when the concentration of nitrogen increased to 5 g NH₄Cl/kg M9. Genes encoding endo-1,4-beta-xylanases [EC:3.2.1.8], which perform the endohydrolysis of 1, 4-beta D-xylosidic linkages in xylan [36], were most abundant when nitrogen level was 2 g NH₄Cl/kg M9 but the abundance of predicted gene counts dropped by 46% as the concentration of nitrogen increased to 5 g NH₄Cl/kg M9. Xylan 1,4-beta-xylosidase [EC:3.2.1.37] is an exotype glycosidase that hydrolyzes short xylo-oligomers to single xylose units [40]. It was found that the abundance of genes encoding xylan 1,4-beta-xylosidase peaked when the concentration of nitrogen was between 2 and 4 g NH₄Cl/kg M9 but abundance dropped by 37% as the concentration of nitrogen increased to 5 g NH₄Cl/kg M9. Alpha-L-rhamnosidase [EC: 3.2.1.40], an enzyme which cleaves terminal alpha-l-rhamnose from a large number of natural glycosides

and has potential applications in citrus fruit juice and wine industries [41], reached peak numbers when the concentration of nitrogen was 2 g NH₄Cl/kg M9.

Among the categories of PICRUSt predicted genes involved in lignocellulose degradation, the sum of abundance of predicted genes involved in lignin degradation was the highest. Of these genes, peroxidases [EC:1.11.1.-], classified as “auxiliary activities” (AA2 family) in the CAZy (Carbohydrate-Active enZYmes Database), are involved in oxidation of phenolic and non-phenolic aromatic compounds and have ability to modify lignin polymers [42, 43]. Glycolate oxidase [EC: 1.1.3.15] is classified in CAZy as family AA7 and its predicted gene abundance was highest when the concentration of nitrogen was 1 g NH₄Cl/kg M9. In family AA7 [EC: 1.1.3.-], studies have shown that gluco-oligosaccharide oxidases are able to oxidize a variety of carbohydrates, including D-glucose, maltose, lactose, cellobiose, malto- and cello-oligosaccharides, with the associated reduction of O₂ to H₂O₂ [44, 45]. AA7 enzymes are likely involved in the biotransformation or detoxification of lignocellulosic compounds [43, 46].

Overall, a majority of the PICRUSt predicted genes involved in lignocellulose degradation reached peak counts when the concentration of nitrogen was between 2 and 3 g NH₄Cl/kg M9. These results corresponded with measured xylanase and endoglucanase (CMC) activities, suggesting that PICRUSt could be a valuable tool for identifying genes and associated enzymes involved in lignocellulose degradation.

Conclusions

Carbon to nitrogen ratio (C/N) is an important factor affecting bioconversion [8, 10]; C/N ratios of 25-30 are considered ideal for thermophilic biological deconstruction, and intensive microbial and enzymatic activities have been observed at such C/N ratios [8, 10]. The C/N ratios of the GW after nitrogen amendment ranged between 25 and 32, however, even within this range, significant shifts in the microbial community, enzyme production and potential for lignocellulose

hydrolysis were observed. The results suggest nitrogen levels in bioconversion processes could be better tuned to achieve more efficient deconstruction of recalcitrant polysaccharides and discovery of enzymes for bioconversion.

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Table 1. Cumulative carbon dioxide evolution rate (cCER) for each treatment, and xylanase and endoglucanase (CMC) activities extracted from samples after 11 days of enrichment.

g NH ₄ Cl/kg M9	C/N	Nitrogen in amended GW, % dry matter	Mean cCER, mg CO ₂ (g dry matter) ⁻¹			Xylanase IU (g dry matter) ⁻¹	Endoglucanase IU (g dry matter) ⁻¹
			3-day	7-day	11-day		
1 (T2)	32	2.57	73 (11)	143 (19)	186 (26)	14.65 (3.78) A,B	1.16 (0.31) A
2 (T2)	30	3.70	99 (9)	156 (13)	210 (27)	19.89 (6.36) A,C	1.40 (0.32) A
3 (T2)	28	4.83	100 (19)	159 (25)	225 (31)	26.44 (3.06) C	1.39 (0.25) A
4 (T2)	26	5.96	84 (8)	152 (32)	209 (27)	8.04 (2.22) B,D	1.09 (0.22) A
5 (T2)	25	7.09	85 (12)	134 (15)	153 (17)	3.12 (0.04) D	0.02 (0.005) B

Standard deviations in parentheses. Three replicates for all treatments except 3 g NH₄Cl/kg M9 with 2 replicates. Means followed by the same letter within columns are not statistically different at $\alpha = 0.05$ based on Tukey-Kramer HSD test.

Table 2. Prediction of cCER, enzyme activity, and optimum nitrogen level using a second order polynomial equation.

	Polynomial equation	p-values	Optimum nitrogen level, g NH ₄ Cl/kg M9
cCER on day 3	$Y_1 = -4.64X^2 + 28.92X + 52.45$	0.11	3.11
cCER on day 7	$Y_1 = -5.14X^2 + 28.70X + 119.37$	0.26	2.79
cCER on day 11	$Y_1 = -13.61X^2 + 75.10X + 121.03$	<0.01	2.76
Xylanase	$Y_2 = -2.92X^2 + 14.02X + 4.03$	0.002	2.40
Endoglucanase	$Y_2 = -0.21X^2 + 0.99X + 0.33$	<0.0001	2.38

*X is the concentration of nitrogen in g/kg M9, Y_1 is the cumulative carbon dioxide evolution, and Y_2 is the enzyme activity IU (g dry matter)⁻¹.

Table 3. Mean relative abundance (%) of phyla in bacterial communities and classes in fungal communities.

g NH ₄ Cl/kg M9	C/N	Bacteria			
		Actinobacteria	Firmicutes	Proteobacteria	Other bacteria
1	32	4.12 (1.00) A	84.50 (2.98) A	11.03 (2.10) A	0.35 (0.15) A
2	30	1.84 (0.58) B	92.46 (1.16) A,B	5.21 (1.97) A,B	0.49 (0.27) A
3	28	1.51 (0.73) B	92.17 (1.83) A,B	5.77 (1.33) A,B	0.55 (0.22) A
4	26	1.82 (0.97) B	91.33 (6.61) A,B	6.44 (5.70) A,B	0.41 (0.13) A
5	25	0.49 (0.08) B	97.09 (0.52) B	1.61 (0.34) B	0.81 (0.18) A

g NH ₄ Cl/kg M9	C/N	Fungi			
		Dothideomycetes	Eurotiomycetes	Sordariomycetes	Other fungi
1	32	0.75 (0.22) A	56.20 (1.90) A	42.93 (1.71) A	0.12 (0.04) A
2	30	2.97 (3.54) A	90.70 (4.76) B	5.84 (3.51) B	0.49 (0.57) A
3	28	2.30 (0.93) A	88.45(3.38) B	8.91 (4.47) B	0.33 (0.17) A
4	26	1.32 (0.95) A	61.93 (8.02) A	36.55 (8.56) A	0.20 (0.15) A
5	25	5.73 (1.39) A	88.68 (3.15) B	4.60 (1.45) B	0.99 (0.39) A

*Means followed by the same letter within columns are not statistically different at $\alpha = 0.05$ based on Tukey-Kramer HSD test. Three replicates for all treatments except 3 g NH₄Cl/kg M9 with 2 replicates.

Table 4. Selection of 53 genes involved in lignocellulose degradation and mean percent abundance* of predicted genes by PICRUSt.

	KEGG gene description [EC number] (KEGG number)	T2_1	T2_2	T2_3	T2_4	T2_5	p-value	CAZy, GH, or AA
Cellulose	6-phospho-beta-glucosidase [EC:3.2.1.86] (K01222)	162	172	155	159	132	0.07	GH (1,4)
	6-phospho-beta-glucosidase [EC:3.2.1.86] (K01223)	34 A	41 A	46A	45 A	87 B	ND	GH (1,4)
	alpha-glucosidase [EC:3.2.1.20]	675 A	695 A	706 A,B	716 A,B	777 B	ND	GH (4,13,31,63,97,122)
	beta-glucosidase [EC:3.2.1.21] (K01188)	95	87	95	95	121	0.06	GH (1,3,5,9,30,116)
	beta-glucosidase [EC:3.2.1.21] (K05349)	550 A,B	609 A	552 A,B	570 A,B	457 B	0.02	GH (1,3,5,9,30,116)
	beta-glucosidase [EC:3.2.1.21] (K05350)	547	536	524	519	514	0.17	GH (1,3,5,9,30,116)
	cellulose 1,4-beta-cellobiosidase [EC:3.2.1.91]	30 A	14 A,B	14 A,B	16 A,B	4 B	ND	GH (5-9,12,16,44,45,48,74,124)
	endoglucanase [EC:3.2.1.4]	574	597	583	598	558	0.19	GH (16,17,55,64,81,128)
	glucan endo-1,3-beta-D-glucosidase [EC:3.2.1.39]	0.19 A	0.23 A	0.28 A,B	0.31 A,B	0.39 B	ND	GH (13,31)
	oligo-1,6-glucosidase [EC:3.2.1.10]	430 A	539 A,B	539 A,B	550 A,B	645 B	ND	GH (13,31)
Hemi-Cellulose	alpha-amylase [EC:3.2.1.1] (K01176)	260 A	308 A	320 A	310 A	432 B	ND	GH (13,14,57,119)
	alpha-amylase [EC:3.2.1.1] (K07405)	0.06	0.05	0.03	0.08	0.11	0.08	GH (13,14,57,119)
								GH (4,27,32,36,57,97,110)
	alpha-galactosidase [EC:3.2.1.22] (K07406)	176 A,B	207 A	186 A,B	200 A	112 B	<0.01	GH (4,27,32,36,57,97,110)
								GH (4,27,32,36,57,97,110)
	alpha-galactosidase [EC:3.2.1.22] (K07407)	190	204	185	185	175	0.41	GH (29,95)
	alpha-L-fucosidase [EC:3.2.1.51]	168 A	198 A	182 A	198 A	119 B	<0.01	GH (31,38,92)
	alpha-mannosidase [EC:3.2.1.24]	115 A	118 A	110 A,B	118 A	80 B	0.01	GH (3,10,43,51,54,62)
	alpha-N-arabinofuranosidase [EC:3.2.1.55]	256 A	278 A	246 A,B	257 A	168 B	<0.01	GH (53)
	arabinogalactan endo-1,4-beta-galactosidase [EC:3.2.1.89]	3.7 A	3.3 A	4.1A,B	4.5 A,B	6.9 B	ND	GH (1,2,3,35,42,50)
	beta-galactosidase [EC:3.2.1.23] (K01190)	94 A,B	110 A	101 A,B	108 A	71 B	<0.01	GH (1,2,3,35,42,50)
	beta-galactosidase [EC:3.2.1.23] (K12308)	372	418	385	402	330	0.08	GH (1,2,79)
	beta-glucuronidase [EC:3.2.1.31]	94 A,B	114 A	98 A,B	101 A	58 B	ND	GH (1,2,5)
	beta-mannosidase [EC:3.2.1.25]	143 A	149 A	130 A	133 A	74 B	<0.01	GH (5)
	carboxylesterase [EC:3.1.1.1] (K03928)	267 A	314 A	325 A	319 A	431 B	ND	

	carboxylesterase 1 [EC:3.1.1.1] (K01044)	72 A	88 A	87 A	80 A	143 B	ND	GH (5)
	carboxylesterase 2 [EC:3.1.1.1] (K03927)	264 A	201 B	181 B	194 B	83 C	ND	GH (5)
	endo-1,3(4)-beta-glucanase [EC:3.2.1.6]	0.8 A	0.8 A	1.3 A	1.2 A	2.5 B	ND	GH (5,8,9,10,11,12,16,30,43,44)
	endo-1,4-beta-xylanase [EC:3.2.1.8]	376 A	414 A	345 A,B	364 A	223 B	ND	GH (1,2,3,35,42,50)
	evolved beta-galactosidase subunit alpha [EC:3.2.1.23]	0.8	1.3	0.9	0.8	1.3	0.81	GH (32)
	levanase [EC:3.2.1.65]	75 A	90 A	81 A	90 A	52 B	0.03	GH (5)
	lysophospholipase [EC:3.1.1.5]	313 A	328 A,B	345 B	336 A,B	406 C	ND	AA10, GH (5,9,26,44,113)
	mannan endo-1,4-beta-mannosidase [EC:3.2.1.78]	1.2 A	1.3 A,B	1.3 A,B	1.4 A,B	1.7 B	ND	GH (1,3,30,39,43,52,54,16,120)
	xylan 1,4-beta-xylosidase [EC:3.2.1.37]	92 A,B	108 A	99 A,B	106 A	68 B	<0.01	
Lignin	(s)-2-hydroxy-acid oxidase [EC:1.1.3.15]	0.09	0.05	0.04	0.07	0.07	0.42	AA7
	catalase [EC:1.11.1.6] (K00429)	88 A	50 A,B	53 A,B	52 A,B	7 B	ND	AA2
	catalase [EC:1.11.1.6] (K03781)	691 A	763 A,B	758 A,B	768 A,B	837 B	ND	AA2
	catalase/oxidase [EC:1.11.1.6 1.11.1.7]	654	674	664	666	647	0.20	AA2
	chloride peroxidase [EC:1.11.1.10]	299 A	227 A	217 A	223 A	86 B	ND	AA2
	cytochrome c peroxidase [EC:1.11.1.5]	186 A	106 A,B	111 A,B	108 A,B	24 B	ND	AA2
	glutathione peroxidase [EC:1.11.1.9]	605 A	611 A	602 A,B	601 A	555 B	ND	AA2
	glycolate oxidase [EC:1.1.3.15]	559 A	529 A,B	531 A,B	533 A,B	509 B	ND	AA7
	laccase [EC:1.10.3.2]	0.05	0.08	0.11	0.08	0.08	0.22	
	peroxiredoxin (alkyl hydroperoxide reductase subunit C) [EC:1.11.1.15]	256	230	244	232	283	0.13	AA2
	thiol peroxidase, atypical 2-Cys peroxiredoxin [EC:1.11.1.15]	314 A	384 A,B	384 A,B	385 A,B	459 B	ND	AA2
	vanillate monooxygenase [EC:1.14.13.82] (K03862)	97 A	60 A,B	63 A,B	63 A,B	28 B	ND	NC
	vanillate monooxygenase [EC:1.14.13.82] (K03863)	93 A	56 A,B	60 A,B	59 A,B,	21 B	ND	NC
Cello-oligosaccharides	alpha,alpha-trehalase [EC:3.2.1.28]	5 A	6 A	7 A	7 A	14 B	ND	GH (13,15,37,65)
	alpha-L-rhamnosidase [EC:3.2.1.40]	124 A	129 A	113 A	117 A	67 B	<0.01	GH (13,37,106)
	beta-fructofuranosidase [EC:3.2.1.26]	244 A	299 A	312 A	305 A	417 B	ND	GH (32,68,100)

glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12]	723 A	785 A	799 A	795 A	937 B	ND	NC
maltooligosyltrehalose trehalohydrolase [EC:3.2.1.141]	118 A	65 A,B	65 A,B	65 A,B	11 B	ND	GH (13)
maltose-6'-phosphate glucosidase [EC:3.2.1.122]	30 A	43 A	50 A	41 A	103 B	ND	GH (4)
PTS system, 2-O-A-mannosyl-D-glycerate-specific IIC component	0.24 A	0.28 A	0.36 A	0.35 A	0.85 B	ND	NC
trehalose-6-phosphate hydrolase [EC:3.2.1.93]	96 A	113 A	131 A	122 A	209 B	ND	GH (13)

The selection of genes was based on the study reported by Jimenez et al. [36]

*Values show 10,000x the percent abundance of the gene relative to the total number of genes in the community.

Three replicates for all treatments except 3 g NH₄Cl/kg M9 with 2 replicates.

AA: Auxiliary activities; GH: Glycosyl hydrolase; NC: Not classified.

p-value: significance of the prediction of gene count using a second order polynomial equation.

ND: presence of multicollinearity

Table 5. Prediction of gene percent abundance using a second order polynomial equation.

Gene description	Polynomial equation*	p-value	Optimum nitrogen concentration, g/L
alpha-galactosidase [EC:3.2.1.22]	$Y = -1.56 \times 10^{-3}X^2 + 7.99 \times 10^{-3}X + 1.10 \times 10^{-2}$	<0.01	2.56
alpha-L-fucosidase [EC:3.2.1.51]	$Y = -1.43 \times 10^{-3}X^2 + 7.56 \times 10^{-3}X + 1.05 \times 10^{-2}$	<0.01	2.65
alpha-L-rhamnosidase [EC:3.2.1.40]	$Y = -6.95 \times 10^{-4}X^2 + 2.89 \times 10^{-3}X + 1.00 \times 10^{-2}$	<0.01	2.08
alpha-mannosidase [EC:3.2.1.24]	$Y = -5.19 \times 10^{-4}X^2 + 2.43 \times 10^{-3}X + 9.32 \times 10^{-3}$	0.01	2.34
alpha-N-arabinofuranosidase [EC:3.2.1.55]	$Y = -1.39 \times 10^{-3}X^2 + 6.40 \times 10^{-3}X + 2.04 \times 10^{-2}$	<0.01	2.29
beta-galactosidase [EC:3.2.1.23]	$Y = -6.92 \times 10^{-4}X^2 + 3.68 \times 10^{-3}X + 6.31 \times 10^{-3}$	<0.01	2.66
beta-glucosidase [EC:3.2.1.21]	$Y = -2.12 \times 10^{-3}X^2 + 1.05 \times 10^{-2}X + 4.70 \times 10^{-2}$	0.02	2.47
beta-mannosidase [EC:3.2.1.25]	$Y = -8.38 \times 10^{-4}X^2 + 3.50 \times 10^{-3}X + 1.14 \times 10^{-2}$	<0.01	2.09
levanase [EC:3.2.1.65]	$Y = -6.92 \times 10^{-4}X^2 + 3.69 \times 10^{-3}X + 4.40 \times 10^{-3}$	0.03	2.66
xylan 1,4-beta-xylosidase [EC:3.2.1.37]	$Y = -7.03 \times 10^{-4}X^2 + 3.72 \times 10^{-3}X + 6.11 \times 10^{-3}$	<0.01	2.64

*X is the concentration of nitrogen in g/kg M9 and Y is the percent abundance of the gene relative to the total number of genes in the community.

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