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Dynamic bacterial and fungal microbiomes during sweet sorghum ensiling impact bioethanol production

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ABSTRACT

Significant low-cost biofuel production volumes could be achieved from commercial-scale silage by redirecting lactic acid fermentation to ethanol production. A temporal metagenomic analysis on ensiled sweet sorghum inoculated with an ethanologenic yeast has been conducted to understand the underlying microbial processes during bioethanol production. Individual silage buckets approximating silage piles were prepared with freshly harvested material and supplemented with ethanologenic yeast, sulfuric acid or both. The ensiling progress was assessed using high performance liquid chromatography, microbial taxonomic identification and abundance. The combined treatment with *Saccharomyces* and acid led to a steady reduction of bacterial abundance and microbial diversity with *Lactobacillus* becoming the dominant genus during the late timepoints. Furthermore, the addition of acid to inhibit bacterial growth hindered *Saccharomyces* ability to compete with native yeasts like *Candida*. Knowledge of the response of the in-situ microbial community to the various treatments during ensiling will help improve current methodologies for bioethanol production.

1. Introduction

Further expanding biofuel production features in many of the forward paths in the decarbonization of the transportation sector required to keep global temperature increases below 2 °C (IPCC, 2014), as defined by the Paris Agreement. Current first-generation biofuels are dominated by sugar and starch derived bioethanol, and biodiesel from vegetable oils, that are typically sourced from food crops grown on high quality farmland (Ajanovic, 2011). This potential competition with food production (Hill et al., 2006) has led to limitations on the contribution of these first-generation biofuels within overall policy and regulatory environments otherwise explicitly encouraging of expanded biofuel production (U.S.A, 2007; EU Parliament, 2015).

Sweet sorghum (*Sorghum bicolor*) is a promising dedicated energy crop for bioethanol production, due to its ability to grow on many soil types, its drought and heat tolerance, and its high biomass productivity (Barcelos et al., 2016). In contrast to sugarcane and sugar beet, the sugars in sweet sorghum do not crystalize, limiting its food use to the small syrup market. There has been some interest in pressing sweet sorghum canes and then fermenting the juice to bioethanol, similar to sugarcane (Laopaiboon et al., 2009). In the temperate regions where sweet sorghum is better adapted than sugarcane, there is a relatively short crop developmental window to harvest when sugar yields are high. Building a biorefinery that is idle most of the year is an inefficient use of capital, limiting the potential of sweet sorghum juice ethanol plants.

Forage sorghum (*Sorghum bicolor*), a close relative of sweet sorghum, has been developed for high yields of digestible cattle feed. To enable year-round feeding during the similarly short harvest window, forage sorghum is immediately ensiled to prevent spoilage and minimize dry matter loss during storage. Ensiling is achieved by tightly packing the harvested wet biomass in piles or pits that are subsequently covered by plastic sheets to minimize oxygen intrusion. The crop is typically harvested below moisture contents of ~65%, to minimize leachate production during ensiling (Gebrehanna et al., 2014). The available plant carbohydrates in the shredded biomass lead to rapid growth of endogenous aerotolerant lactic acid bacteria (LAB) (Gänzle, 2015) which ferment these sugars to lactic acid causing a pH drop to around 4 (Duniere et al., 2017). There is a rapid depletion of the oxygen trapped at packing; directly as a substrate of microbial metabolism, and indirectly through dilution and off-gassing due to the production of CO_2 and other fermentation gases. In contrast to industrial fermentations in

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steel tanks with microbial hygiene procedures (e.g. corn starch ethanol), biomass is packed with native inoculants from the plants, soil, equipment and residue from prior silage. Depending on their tolerance to oxygen and range of fermentation products, LAB are grouped into obligately homofermentative, obligately heterofermentative and facultatively heterofermentative categories (Gänzle, 2015). Homofermentative LAB utilize the Emden-Meyerhof pathway to produce lactate from glucose, and include the families *Lactobacillaceae* and *Streptococcaceae*, whereas the heterofermentative LAB, like *Leuconostocaceae*, use the phosphoketolase pathway to produce a mixture of lactic acid, acetic acid and ethanol (Gänzle, 2015). The reductions in oxygen concentration and available plant sugars in the pile, in conjunction with the low pH, slows down the fungal and bacterial activity minimizing biomass spoilage and further mass loss (McDonald, 1981). Once ensiled, the piled biomass can remain stable year-round providing that minimal oxygen intrusion takes place during storage.

In analysis of complex microbial communities, culture-dependent enumeration studies tend to grossly underestimate the microbial abundance, as they can only count microorganisms that can grow independently on certain growth media and at specific temperatures (Douterelo et al., 2014; Duniere et al., 2017). Generally, culture-independent methods of analysis have been proven much more valid than culture-dependent methods, since 99% of microorganisms cannot be cultured under lab conditions (Pace, 1997). Amplicon sequencing of the 16S rDNA is a rapid and cost-effective tool for taxonomic classification compared to whole metagenome sequencing methods, despite the loss of genomic detail (Weinstock, 2012). The 16S rRNA gene and the fungal ITS region are well characterized and provide the most detailed microbial resolution (Taylor et al., 2016; Yang et al., 2016). qPCR is the most appropriate method for bacterial and fungal enumeration given its high specificity, speed and lack of culture bias.

Here we attempt to maximize ethanol and minimize organic acid production in sweet sorghum silage through the use of additions of *Saccharomyces* yeast (Serrano-Ruiz, 2015) and/or inorganic acid (Muck, 2010). Expecting a diverse inoculant microbiome at packing and a rapidly changing silage environment, we explore for the first time the bacterial and fungal community successions of conventional silage, and how they are impacted by these additives. This was achieved by amplicon sequencing of the16S rRNA gene for bacterial and ITS for fungal taxa identification, in conjunction with bacterial and fungal enumeration by qPCR. Moreover, HPLC analysis was also performed for quantification of soluble ensiling substrates and products. To maximize relevance the crop was grown, harvested and mixed at a large scale with commercial silage equipment, and ensiled and stored outdoors on farm. Full scale silage piles are not amenable to repeated sampling, due to the risks of oxygen ingress, and so the analysis was completed at a bucket scale to allow destructive sampling at each time point. This combined approach provided novel insights into the role of microorganisms in terms of bioethanol production and undesirable ensiling products such as lactic and acetic acid.

2. Materials and methods

2.1. Ensiling and sampling

The N6G60 hybrid of sweet sorghum (NexSteppe) was chopped with a silage harvester (John Deere 7800) into a horizontal auger silage feed ration mixing wagon (Roto-Mix 420-12) near McAllen, TX in May 2017. Four silage treatments were prepared in the field including i) fresh biomass harvested with no additives termed “None” thereafter, (ii) addition of H_2SO_4 (20% v.v^{-1}) at a rate of 6.3 L.tonne^{-1} fresh biomass (targeting an initial pH of ~4.0) and mixed for 20 min, termed “Acid” treatment thereafter, (iii) spraying of stabilized liquid *Saccharomyces cerevisiae* yeast (Eagle® C6 Fuel, Lallemand Biofuels & Distilled Spirits) on to the biomass in the chute of the harvester at a rate of 0.44 L.tonne^{-1} fresh biomass, termed “Yeast” treatment thereafter, and finally (iv) addition of H_2SO_4 (20% v.v^{-1}) at a rate of 6.3 L.tonne^{-1} fresh biomass to the “Yeast” treated material and mixed for 20 min, termed “Yeast + Acid” thereafter. Prior testing with this mixer had demonstrated that mixing for longer than 10 min had minimal additional homogenization benefits when blue food dye (FD&C Blue 1 34%, Robert Koch Industries Inc.) was blended with post-ensiled sorghum (data not shown), and is consistent with commercial practice in mixing feed rations. Following harvesting and treatment, sorghum was manually packed into 18.9 L buckets (Uline) with a tamper, sealed with a stopper and a three-piece airlock (Northern Brewer), and stored outdoors under cover (mean air temperature = 30 °C). Samples were collected every six hours for 48 h (T0 – T48), with duplicates every 12 h (T12, T24, T36, T48), plus an additional duplicate sample was taken after two weeks of ensiling (T336). High reproducibility between replicate buckets was observed in a pilot study (data not shown), so taking replicates from alternate timepoints in the current study was considered an acceptable compromise given the constraints on the number of treatments, the frequency of destructive sampling and the limited harvest window. For sampling, approximately 30 g material from the centre of the bucket was collected into a sterile 50 mL conical tube (Falcon) and stored at −20 °C. As the anaerobic conditions would be compromised upon opening a bucket preventing its use for additional sampling, a separate bucket was used for each sample at each timepoint.

2.2. Chemical analysis

Chemical composition of juice expressed from the samples was analysed by HPLC. Briefly, 10 g of fresh biomass was removed from −20 °C storage, allowed to thaw completely at room temperature and transferred to a 60 mL syringe (BD Biosciences). The syringe was squeezed using a manual press (Dake Arbor), and the fluid collected in a 15 mL conical centrifuge tube (Falcon). The pH of each fluid sample was measured using a pH probe (InLab Expert Pro, Mettler Toledo), calibrated using pH buffer solution (ThermoFisher). From the approximately 5 mL of fluid produced, 1 mL was transferred to a Costar Spin-X centrifuge tube equipped with a 0.22 µm nylon membrane (Corning) and centrifuged at 13,000*g* for 2 min. HPLC analysis was performed on a Dionex UltiMate 3000 (Thermo Scientific). Post-ensiled samples were analysed using a HPX-87H-column (Bio-Rad). 10 µL of the supernatant was injected into the HPLC, flow rate 0.6 mL per minute, mobile phase 0.005% H_2SO_4, at 60 °C, with a positive refractive index at 50 °C and positive polarity. Soluble sugars (glucose, fructose, sucrose, arabinose, cellobiose and xylose; summed and referred to as “Total sugars”), organic acids (lactic acid and acetic acid), glycerol and ethanol were analysed against standards. For pre-ensiled (T0) samples, the supernatant was first diluted five-fold with water prior to HPLC analysis as before but using a Bio-Rad HPX-87P column with a 20 µL injection volume, column temperature at 85 °C, flow rate 0.6 mL per minute of mobile phase Milli-Q Water, and RI conditions as above.

2.3. DNA isolation

DNA for microbial community analysis was sampled from a large mass of sorghum using a wash method. Samples were thawed at room temperature for 30 min, then approximately 10 g from each sample was transferred into a sterile 50 mL tube (Falcon) containing 30 mL sterile Milli-Q water. The 50 mL tubes were then vortexed for 45 s, inverting every 15 s, and the supernatant was decanted into a clean, sterile 50 mL tube (Falcon), avoiding the transfer of any large plant material. The samples were centrifuged at 15,000*g* for 20 min at room temperature and the supernatant was carefully discarded. Approximately 0.3 g of each pellet was transferred into separate 2 mL PowerBeat tubes included in the DNeasy PowerLyzer PowerSoil Kit (Qiagen) using a sterile disposable spatula (Fisher). DNA extractions were performed using the DNeasy PowerLyzer PowerSoil Kit DNA isolation kit (Qiagen) as per the