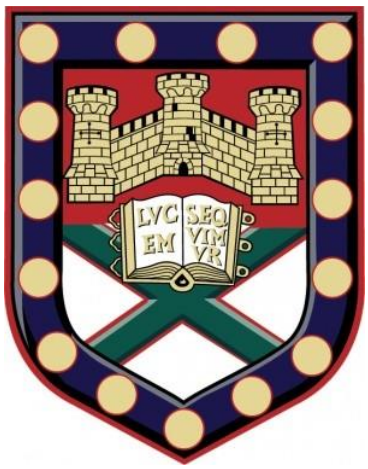

Optimisation of Low-Cost Lignocellulose Reprocessing for Industrial Ethanol Production

Submitted by Lydia Nurse to the University of Exeter as a thesis for the degree of Masters of Science by Research in Biological Sciences in April 2018

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

ABSTRACT

The expanding global demand for transportation fuels and stringent governmental pollution policies have prompted energy supplier investment in the development of renewable energy technologies. Bioethanol is a renewable, market-competitive petroleum additive, produced from the fermentation of plant-derived sugars. First generation ethanol is produced from the fermentation of edible hexoses, whilst second generation ethanol is produced from the saccharification of inedible lignocellulosic fractions of waste plant residues. Due to the recalcitrant nature of lignocellulose, second generation ethanol production requires intensive biomass thermochemical pre-treatment, which incurs significant capital and operating expenditure. Ensiling is an alternative, low-technology route to ethanol, and requires two steps: Primary ensiling, which produces first generation ethanol, and Secondary ensiling, which produces second generation ethanol. Although ensiling associated industrial costs are lower, the process produces significantly reduced ethanol yields in comparison to thermochemical pre-treatment. Therefore, increasing ensiling ethanol production whilst maintaining low technology associated costs is paramount to making it an economically competitive technology.

In this investigation, the optimisation of six ensiling components, biomass cultivar, lignocellulosic degrading enzyme, fermenting yeast species, operating temperature, and process contamination control were explored for increased ethanol production efficiency. The data collected from this study suggests that the use of *Sorghum bicolor* cultivar Topper 76-6, addition of cellulolytic enzyme CTec3 at half the conventional industrial dose, employment of yeast *Saccharomyces cerevisae* strain C6 FUEL, the antibiotic Lactrol, and an operating temperature of 30 ˚C, increased second generation ethanol production efficiency. A novel, 'Consolidated' ensiling approach, which combined production of both first and second generation ethanol in a one-step reaction, was then developed. Utilisation of the determined optimal conditions in Consolidated ensiling resulted in increased ethanol production, decreased process contamination, and the potential to decrease ensiling associated industrial costs in comparison to the two-step Primary – Secondary ensiling process previously employed in industry.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BMR	brown midRib
CAPEX	capital expenditure
CARD	Centre for Agricultural and Rural Development
DoE	design of experiments
EPA	Environmental Protection Agency
g	gramme
g	relative centrifugal force
HAP	hazardous air pollutant
hr	hour
hg	Inch of mercury
HPLC	high performance liquid chromatography
kg	kilogramme
l	litre
ml	millilitre
min	minutes
mol	mole
MON	motor octane number
OPEX	operating expenditure
pH	potential hydrogen
RFS	Renewable Fuels Standard
RIN	renewable identification number
RON	research octane number
RTFO	Renewable Transport Fuel Obligation
SLY	stabilised liquid yeast
STCH	Shell Technology Centre
VOC	volatile organic compound
1G	first generation biofuel
2G	second generation biofuel
3G	third generation biofuel
4G	fourth generation biofuel

INTRODUCTION

Fossil Fuels and Transportation

Fossil fuels are hydrocarbons derived from the continuous heating and pressurization of decaying animal and plant tissues and marine organisms (Schobert, 2013). Besides the essential elements hydrogen and carbon, fossil fuels contain sulphur, nitrogen and oxygen, as well as trace metallics, such as mercury and selenium (Vouk & Piver, 1983). The main fossil fuels utilised today are coal, natural gas, and crude oil (Scott & Stephens, 2015). Crude oil is extracted from underground oil fields by well drilling (North, 1994), and separated into marketable products by fractional distillation (Williams, 1963). Petroleum distillates are further processed by cracking or unification (Fahim, 2010) to convert them into liquid aviation and automotive fuels (Fahim, 2010). One of the most crucial uses of fossil fuels is the provision of retail transport fuels (Wilcox, 2014). At the end of the 20th century, transportation accounted for 33 % of primary fossil fuel derived energy consumption in the United Kingdom (UK), a value predicted to rise to 41 % by 2020, with passenger vehicles accounting for over 43 % of total UK petroleum consumption (Williamson *et al.*, 1997).

Fossil fuels are non-renewable, finite resources, with coal (BP, 2017a), oil (BP, 2017b), and natural gas (BP, 2016) reservoir exhaustion predicted to occur by 2100. In 2010, over 1 billion passenger vehicles were in use globally, and vehicle ownership is predicted to rise, with annual increases of up to 8 % (Sperling, 2009). Continued upward trends in fuel consumption, buoyed by global vehicle ownership increases puts additional strain on already rapidly depleted reserves (BP, 2017c), and exacerbates concerns surrounding fossil fuel resource longevity (Speirs *et al.*, 2015).

Combustion of fossil fuels produces atmospheric pollutants including carbon dioxide (Hiete *et al.*, 2001), sulphur and nitrogen oxides (Gschwandtner *et al.*, 1986), toxic heavy metals (mercury, lead, cadmium, and uranium), ozone (Goldemberg, 2009), methane, volatile organic compounds (VOCs), and particulate matter (Cho, 2008, Miller *et al.*, 2013). Some of these fossil fuel combustion products have been categorised by the US Environmental Protection Agency (EPA) as hazardous air pollutants (HAPs), which are compounds with known human toxicological consequences (Perera, 2018). Particular significant are mercury, ground-level ozone and lead, exposure to which can result in respiratory, cardiovascular, and neuropsychiatric conditions (Liu & Lewis, 2014, O' Lenick *et al.*, 2017).

These pollutants also have detrimental environmental impacts including soil erosion (Lei *et al.*, 2012) dust, noise, and groundwater pollution (Dontala *et al.*, 2015), and acid deposition (Goldemberg, 2009).

The severity of health and environmental impacts associated with fossil fuel combustion, combined with concerns surrounding resource scarcity prompted formulation of stringent governmental energy policies (Shaheen & Lipman, 2007). Programmes such as the Energy Policy Act (United States Congress, 2005), the Clean Air Act (United Kingdom Parliament, 1956), and the Renewable Fuels Standard (RFS) (US EPA, 2006) aim to hold energy suppliers accountable for their fossil fuel derived pollutant outputs by providing financial incentives for development of renewable technologies. These policies reduce taxes associated with renewable energy sources, including nuclear wind, and solar power, and alternative liquid fuels, such as biodiesel and bioethanol (US EPA, 2007). Schemes such as the Renewable Identification Number (RIN) incentive and the Cellulosic Waiver Credit system were additionally implemented to encourage energy entities to meet volume biofuel production standards set by the RFS (US EPA, 2007).

Biofuels

Biofuels are solid, liquid and gaseous fuels (Sims *et al.*, 2008), derived from heterogeneous biotic resources (Janda *et al.*, 2012). Primary biofuels are unprocessed, organic energy resources such as wood (Hood, 2016), animal dung (Iregbu *et al.*, 2014), and municipal waste, (Matsakas *et al.*, 2017), and are burnt for heat (Aro, 2016). Secondary biofuels are combustible biofuels, produced from the processing of primary biofuels (Balan, 2014). Secondary liquid biofuels are further subdivided into first, second, and third and fourth generations according to their source and synthesis (Ben-Iwo *et al.*, 2016).

First generation liquid biofuels are typically defined as alcohols, fatty acid methyl esters, or hydrogenated vegetable oils, derived primarily from food crops (Naik *et al.*, 2010). First generation bioethanol, or simply “ethanol” as the ‘bio’ prefix is largely used for marketing purposes, is produced by the fermentation of hexose sugars, extracted from sugar rich plants such as *Saccharum officinarum* (Sugar cane) and *Beta vulgaris* (Sugar beet) (Zhu *et al.*, 2012). First generation biodiesels are derived from the transesterification of triglycerides from *Brassica napus* (Rapeseed), *Elaneis guineesis* (Oil Palm) and *Arachis hypogaea* (Peanut) (Nguyen *et al.*, 2010, Mekhilef *et al.*, 2011, Jang *et al.*, 2012).

Second generation liquid biofuels are alcohols and oils derived from non-food crop sources (Saini *et al.*, 2015). These sources include 'energy crops' that are specifically grown for use in the biofuel industry, including varieties of the plant genera *Micanthus* (Morandi *et al.*, 2016), *Sorghum* (Monk *et al.*, 1984), *Panicum* (Amaducci *et al.*, 2017), *Jatropha* (Maghuly & Laimer, 2013), and *Salix* (Volk *et al.*, 2016). Waste agroforestry residues (Sharma *et al.*, 2016) and inedible fractions of foodcrops, such corn stover (Sheehan *et al.*, 2004), are also important second generation biofuel resources.

Second generation ethanol production involves the saccharification of lignocelluose to sugar monomers via a variety of pre-treatment processes, such as Fischer-Tropsch synthesis, culminating in the fermentation of soluble sugars to ethanol by select industrial yeast species (Leibbrandt *et al.*, 2013). Second generation biodiesel is produced via the transesterification of triglycerides in waste or inedible plant oils, cooking oils, and animal fats, including neem, karanja, and camelina oil, beef tallow, pork lard, and chicken fat (Bhuiya *et al.*, 2014).

Advanced biofuels include third and fourth generation (3G and 4G) biofuels, and are derived from microbes other than yeast, including green algae (3G) (Slade & Bauen, 2013, Cheng *et al.*, 2014, Fortier *et al.*, 2014, Hossain *et al.*, 2015) and oleaginous heterotrophs, or synthetically engineering microbes (4G) (Howard *et al.*, 2013).

First generation ethanol

Ethanol is a high octane index biofuel used as an additive or substitute for petroleum derived fuels in auto motives (Balat & Balat, 2009). Approximately 99 % of bioethanol produced is first generation, derived from yeast fermentation of simple food-crop sugars, which constitute up to 25 % of dry plant biomass (Barros-Rios *et al.*, 2015). In Brazil, first generation ethanol is utilised in unadulterated and in a blended form, denoted 'gasohol', and consisting of 24 % bioethanol, and 76 % gasoline (Belincanta *et al.*, 2016). Worldwide, first generation ethanol is combined with gasoline to form E85, a compound composed of between 51 % and 85 % denatured ethanol (Christensen & Siddiqui, 2015). In the UK, according to requirements established by the Renewable Transport Fuel Obligation (RTFO) (HM Government, 2007), and the European Standard for petrol EN 228, ethanol is blended with gasoline up to a maximum of 5 % weight to volume (CEN, 2008).

Ethanol has several advantages as a fossil fuel transport fuel substitute. Dependant on market fluctuation, ethanol has the opportunity to be economically competitive with conventional fuels (petroleum), and alternate fuels (biodiesel) (Winchester & Reilly, 2015).

The current production cost per barrel of crude petroleum is $64.51, however, market forecasts predict production costs may rise to $70 per barrel by the end of 2018 (BBC, 2018). In 2018, the Centre for Agricultural and Rural Development valued the production cost for a gallon of ethanol at $1.22, equating to $51.24 per barrel (Iowa State University CARD, 2018). On an oil-equivalent energy basis (ethanol has 23 % lower energy value than gasoline) a barrel of ethanol costs $76.86 to produce, 15 % higher than the production cost for a barrel of gasoline (Biofuels Digest, 2017). However, under the RFS, ethanol has a carbon credit of D6 RIN (US EPA, 2007). A D6 RIN credit holds a trading value of $0.41 per gallon, and reduces the net cost of ethanol production to $55 dollars per barrel (Biofuels Digest, 2017). Comparatively, the production cost for a barrel of oil equivalent biodiesel stands at $115, and after applying RFS D4 biomass-based diesel RINs (trading value, $1.03 per gallon), net production costs are reduced to $72 per barrel (US EPA, 2007, Biofuels Digest, 2017). Therefore, in the current economic climate, ethanol (per barrel) is 29 % cheaper than biodiesel, and 21 % cheaper than conventional crude petroleum to produce (Biofuels Digest, 2017, Iowa State University CARD, 2018).

Further advantages of ethanol utilisation as a transport fuel include emission reductions of up to 40 % for VOCs, and up to 55 % for nitrogen oxide emissions, when compared with gasoline (Suarez-Bertoa *et al.*, 2015, Tibaquirá *et al.*, 2018). These reductions in pollutant emissions may reduce detrimental environmental and health impacts associated with petroleum combustion. Furthermore, ethanol production processes, such as biomass ensiling, are relatively simple compared to multistep industrial procedures involved in production of alternate fuel sources such as liquefied coal, or biodiesel (Fukuda *et al.*, 2001, Mitchell, 2008). Ethanol can also be produced from a wide array of globally available resources (corn, sugarcane, alternate biomass) (Ramos *et al.*, 2016), whilst petroleum reserves are confined to specific nations, and access to reserves is often influenced by inter-nation political relations (Rastogi, 2014).

Despite these advantages, first generation ethanol production is a controversial issue. Ethanol producing crop cultivation diverts essential resources away from food propagation (Tenenbaum, 2008), resulting in artificial commodity price inflation, and food market volatility (Drabik *et al.*, 2016). Although bioethanol exhibits some suitable properties for spark ignition internal combustion engines, such as gasoline-comparable octane, motor octane (MON) (Anderson *et al.*, 2010), and research octane numbers (RON) (Anderson *et al.*, 2012), has a low cetane number, and therefore neither burns efficiently nor is easily blended with diesel fuels (Kang *et al.*, 2014a, Mofijur *et al.*, 2015). Furthermore, as firstgeneration ethanol is formed from simple sugars only, the majority of plant biomass (> 75 % mass), including

lignin, hemicellulose, cellulose and ash fractions, remain unutilised, and are categorised as process waste (Lennartsson *et al.*, 2014). In the face of problems associated with first generation ethanol production, research has increasingly focused on utilisation of inedible waste residues as a starting product for further ethanol production.

Second generation ethanol

Second generation ethanol is produced from the hydrolysis and fermentation of complex recalcitrant crop polysaccharides, called lignocelluloses (Im *et al.*, 2016) (Figure 1). Lignocelluloses are bio-fibres that provide structural integrity to plant cell walls (Gallos *et al.*, 2017), and consist of three components; cellulose, hemicellulose, and lignin (Mohanty *et al.*, 2000). Cellulose is composed of polymers of beta-linked D-glucose units (Mohanty *et al.*, 2000) which form either unorganised amorphous aggregates, or structured crystalline micro fibril agglomerates (Hattori & Arai, 2016). Hemicellulose consists of linked glucose epimers, including mannose, galactose, arabinose, and glucuronic acids (Thanapimmetha *et al.*, 2011). The lignin component is composed of polymers of phenyl propane derivatives arranged in a three-dimensional veneer, which serves to protect hemicellulose and cellulose from environmental stressors (He *et al.*, 2017).

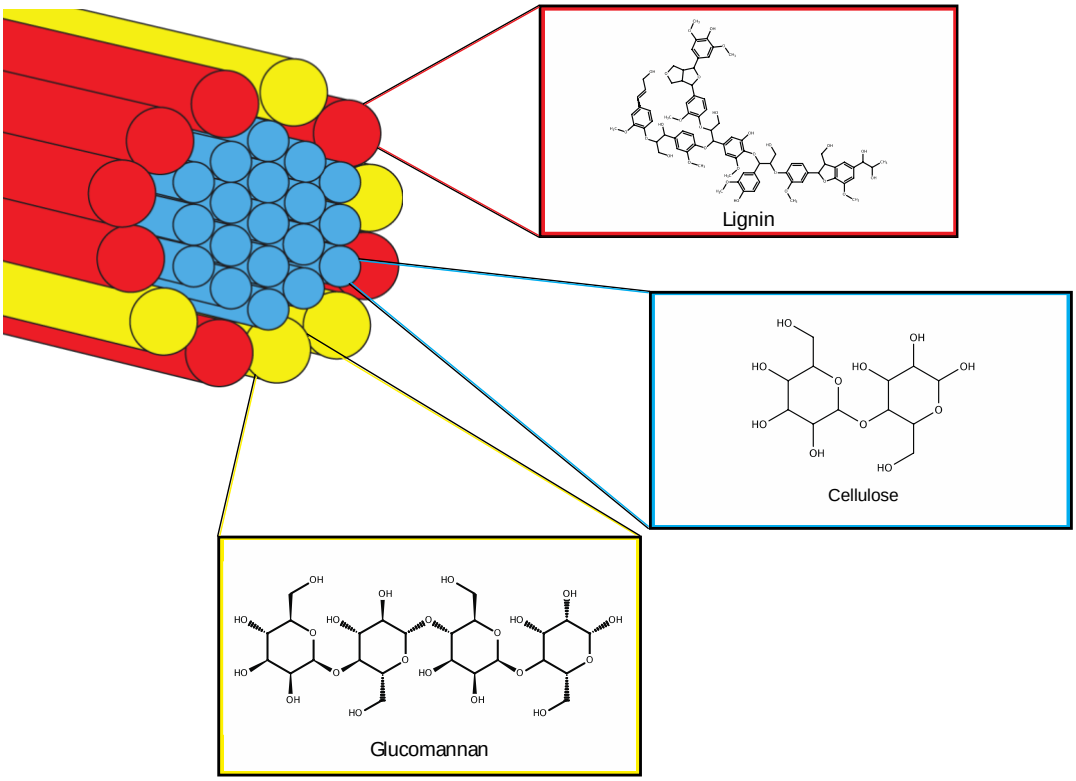

Figure 1. Structural configuration of lignocellulose

Diagrammatic representation of the complex structure of lignocellulose. Cellulose fibrils (blue) are surrounded by a protective sheath of randomly distributed hemicellulose (yellow) and lignin (red) fibrils. The chemical structures of lignin, cellulose, and hemicellulose are shown.

Lignocellulose (Figure 1) is recalcitrant to degradation (Mohanty *et al.*, 2000) and requires intensive bio- or thermochemical pre-treatment to be hydrolysed into constituent sugar monomers (Jönsson & Martín, 2016). The objective of pre-treatment is to solubilise lignin, hemicellulose, and crystalline cellulose (Van den Bosch *et al.*, 2015) and expose amorphous cellulose to enzymatic activity, producing C6 sugars for subsequent yeast fermentation to bioethanol (Heredia-Olea *et al.*, 2015). Lignocellulose hydrolysis strategies are subdivided into three groups: biological hydrolysis using lignin-hydrolysing Fungi, Bacteria and Archaea; chemical hydrolysis using concentrated acid, alkali, ionic liquids, or eutectic solvents (Kumar & Sharma, 2017); thermochemical hydrolysis, including mechanical extrusion via milling, pyrolysis, microwaving, and steam or carbon-dioxide based explosion (Shen *et al.*, 2015). Intensive pre-treatment yields high sugar and ethanol yields: up to 80 % lignocellulose to ethanol conversion has been previously documented (Neil, 2016). However, high temperatures, pressures, and chemical loadings associated with intensive lignocellulose pretreatment (Bensah & Mensah, 2013) require additional energy inputs, and incur substantial

capital (CAPEX) and operating (OPEX) costs (Amin *et al.*, 2017). Thermochemical pretreatment also produces compounds including phenols, aliphatic acids, furan aldehydes, inorganic ions, and bio-alcohols (Rasmussen *et al.*, 2017), which inhibit both enzyme biocatalyst and fermentative organism activity, resulting in reduced process efficiency and ethanol production (Heer & Sauer, 2008).

This high-input process is one route to ethanol. Biomass ensiling offers an alternate, reduced-step, less process-intensive route to ethanol, with lower associated capital (CAPEX) and operating (OPEX) costs (Bhatia *et al.*, 2012), and reduced production of process-impeding by-products (Neil, 2016) (Figure 2).

Figure 2. Comparative workflow of the two processes involved in the production of second generation ethanol

Blue box: Processes required for the conventional production of ethanol from lignocellulosic biomass. Firstly, biomass is reaped using a combine harvester, and biomass is combined with yeast, antibiotic, and acid. Biomass is ensiled in a pile, to produce first generation bioethanol. Waste biomass residues from this process are thermos-chemically pre-treated, to produce a lysate. This lysate undergoes enzymatic hydrolysis to convert complex long chain sugars to shorter sugar monomers. This hydrolysate then undergoes fermentation in which short chain sugars are metabolised to ethanol by the added yeast population. Ethanol is distilled from the lysate and distributed for use, most commonly as a fuel additive. Green workflow steps: Processes required for production of first generation bioethanol from thermochemical pretreatment of lignocellulosic biomass. Blue workflow steps: Processes required for production of second generation bioethanol from thermochemical pretreatment of biomass. Red box: Processes required for the reduced-energy reduced-cost production of ethanol from lignocellulosic biomass. Again, biomass is harvested, combined with yeast, acid, and antibiotic, and ensiled to produced first generation bioethanol. Waste biomass residues from this process are then dried, and ensiled again (denoted ‘Secondary ensiling’) with additional yeast and antimicrobial additives, as well as cellulosic enzymes. During this stage, long chain sugars are hydrolysed to shorter monomers by enzymes and simultaneously metabolised to ethanol by the active yeast population. Ethanol is then distilled from post-ensiled biomass residues and distributed for use. Orange workflow steps: Processes required for production of first generation bioethanol from ensiling. Red workflow steps: Processes required for production of second generation bioethanol from ensiling. Images obtained from John Deere and Shell.

Ensiling for ethanol production

Biomass ensiling has been utilised in agriculture for many years as a nutrient preserving storage method for forage crops (Weinberg & Ashbell, 2003). At Shell Technology Centre Houston (STCH), the ensiling process under investigation is a two-step procedure for ethanol production (Figure 3). During the first stage (Primary ensiling), yeast are added to harvested, chopped biomass to ferment simple sugars to first generation ethanol (Oleskowicz-Popiel *et al.*, 2011). Biomass, however, contains an endemic bacterial load, notably, bacterial species from the *Lactobacillaceae* family (Liu *et al.*, 2013). During industrial ensiling for ethanol, *Lactobacillaceae* species ferment C6 sugars to lactic acid, thereby reducing ethanol production (Kang *et al.*, 2014b). At STCH, these endemic microbial biomass communities are controlled by antibiotic and concentrated acid addition, a useful, but costly method for boosting ethanol production. After addition of antibiotic and acid, biomass is sealed into an anaerobic fermentation chamber for a period determined by industrial constraints. At STCH, sealed anaerobic biomass buckets are utilised in large-scale field experiments. Conical tubes are used in the laboratory to replicate the anaerobic fermentation field environment at a manageable scale (Figure 3). Post fermentation, biomass is dried to extract ethanol, which is prepared for commercial use via distillation, adsorption, pervaporation, and ozonation (Yang *et al.*, 2012, Onuki *et al.*, 2016). The dry waste biomass residues are then re-processed in the second ensiling stage (Secondary ensiling) to produce second generation ethanol.

Figure 3. Workflow of Primary and Secondary ensiling laboratory process for production of ethanol

Processes required for the production of First and Second generation ethanol from Primary and Secondary ensiling at laboratory scale are shown. Biomass is harvested ensiled with yeast, acid and antibiotic in 50 ml centrifuge tubes with bungs and airlocks to maintain an anaerobic environment. This Primary ensiling process produces First generation bioethanol. After 14 days, biomass is unpacked from the 50 ml centrifuge tubes, and dried in a laboratory vacuum oven to remove first generation ethanol. Dried biomass is reconstituted with water, and acid, antibiotic, yeast and cellulolytic enzymes are added. Biomass is mixed with additives and packed into new 50 ml centrifuge tubes, which are ensiled for 14 days. Biomass is decanted, sampled for ethanol content and moisture content and discarded. Images obtained from John Deere, Shell, and Thermofisher. Scientific.

During Secondary ensiling, dried, post-Primary ensiled material is reconstituted to its original moisture content (> 75 %) by water addition. Commercial cellulolytic enzymes are then added to hydrolyse exposed amorphous cellulose into fermentable sugars, and yeast are added to ferment C6 sugars to second generation ethanol. Antibiotic is also added to control microbial contamination. Microbial contamination is a more significant issue in Secondary ensiling than for Primary ensiling as contaminant acid yields in Secondary ensiling can be up to 100 % higher than ethanol yields, compared to Primary ensiling, where acid yields are conventionally less than 10 % of the total ethanol yield. The increased microbial contamination observed in Secondary ensiling is potentially due to environmental exposure of biomass during unpacking, drying, and repacking. Disruption of the controlled anaerobic environment established by industry practices during two-stage ensiling may allow domination of biomass endemic microbial communities and activation of aerobic yeast respiration, resulting in decreases in ethanol yields and increases in acid yields. To avoid environmental exposure and associated enhanced acid production, a process termed ‘Consolidated ensiling’ has been developed at STCH.

During Consolidated ensiling (Figure 4), yeast, acid, antibiotic and cellulolytic enzyme are added to fresh harvested chopped biomass. Biomass is packed into a silage chamber, where yeast ferments both simple and hydrolysed lignocellulose derived sugars to first and second-generation ethanol simultaneously. Post-fermented biomass is dried, and ethanol is distilled and separated into commercial products, including transport fuel substitutes or additives. As biomass unpacking, drying, and repacking involve energy expenditure through labour and fuel burning, the Consolidated ensiling process offers a route to ethanol with potentially further reduced CAPEX and OPEX associated costs in comparison to the already reduced CAPEX and OPEX two-step Primary-Secondary ensiling process.

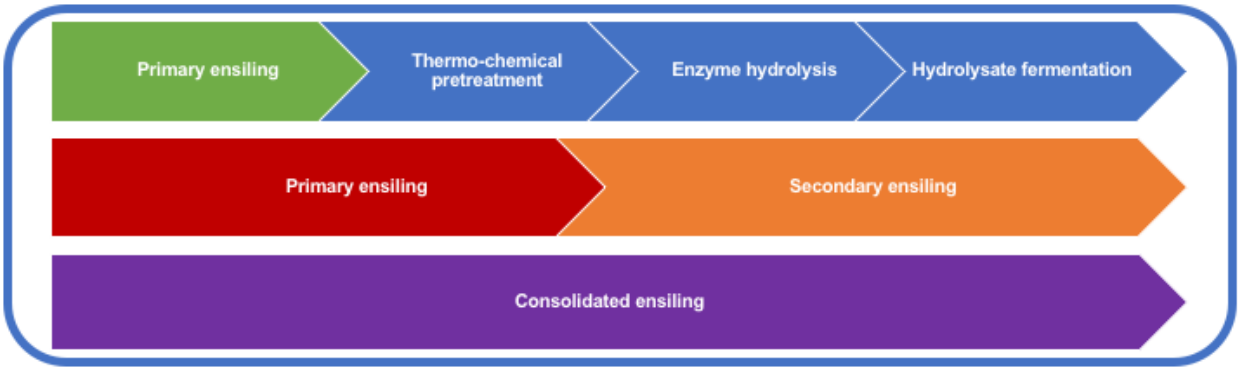

Figure 4. Comparative summary of three possible routes to first and second generation bioethanol

The first work flow shows the conventional high cost, multi-step intensive pre-treatment route to first (green) and second (blue) generation bioethanol. The second workflow shows the lower-cost two-step Primary-Secondary route to first (red) and second (orange) generation bioethanol. The third workflow shows the hypothetically further reduced cost one-step Consolidated ensiling route to both first and second generation bioethanol (purple).

As Consolidated ensiling utilises existing agricultural technology (Horita *et al.*, 2015), and requires reduced physical and thermochemical inputs, it potentially offers a lower-energy, lower-cost route (Bhatia *et al.*, 2012) to second generation ethanol, compared to intensive pre-treatment. However, without the thermochemical extrusion strategies employed in pre-treatment, the majority of cellulose remains inaccessible, trapped in crystalline form or beneath protective lignin and hemicellulose (Karimi & Taherzadeh, 2016) and the naturally exposed, amorphous portion of cellulose is available for enzyme-driven hydrolytic breakdown (Eibinger *et al.*, 2014). This limited proportion of cellulose available for saccharification without pre-treatment is referred to as the 'cellulose barrier'. A consequence of the cellulose barrier is reduced sugar yields: ensiling produces 40 % less fermentable sugars (Jonsson *et al.*, 2013), and 50 % less second generation ethanol, compared to aggressive thermochemical pre-treatment (Oleskowicz-Popiel *et al.*, 2011). Although ensiling offers financial benefits compared with aggressive pre-treatment, increasing ethanol outputs is essential to make Consolidated ensiling competitive with conventional pretreatment.

The production of second generation bioethanol from ensiling relies on two key components; enzymatic access to cellulose, and optimal yeast activity. Increasing ethanol yields from ensiling therefore requires simultaneous circumvention of the cellulose barrier and provision of favourable conditions for the ethanol producing yeast. At STCH, two years of ensiling trials were performed to test the conditions that favour either or both variables. This work initially divided the ensiling trials into Primary and Secondary phases, and investigated alteration of biomass growing location, conditions and cultivar, silage preparation strategies including microwaving, milling, and freezing, addition of silage hydrolysing enzymes, chemicals, and fungi, addition of different yeast species, and duration of ensiling. Analysis of these trials showed that the main factors affecting second generation ethanol yields were biomass variety, yeast species, and enzyme additive. Replicate treatments, however, showed worrying levels of variability that, if repeated at scale would result in unpredictable ethanol yields and an inability to deliver sufficient product to the appropriate market.

HYPOTHESIS AND PROJECT AIMS

We hypothesised that the source of the observed replicate-to-replicate variation in ethanol yields from Secondary-ensiled biomass was due either to inefficient saccharification of the ensiled material or, potentially, to microbial contamination that competed with yeast for the lignocellulose-derived sugars. The separation of the ensiling process into two stages was also proposed as a possible vehicle for increasing contamination from detrimental microbes, so an assessment of ethanol yields from consolidated (*i.e.* one-step) ensiling was performed.

To address this hypotheses, the aims of this project were therefore to:

1. Improve yeast glucose to ethanol conversion efficiency through reduction of microbial competition through identification of proficient antimicrobial additives in Primary ensiling
2. Improve lignocellulose saccharification through identification of a proficient enzyme package and highly digestible *Sorghum bicolor* cultivar in Secondary ensiling
3. Improve yeast ethanol production proficiency through identification of a robust *Saccharomyces cerevisae* strain and optimal yeast activity temperature
4. Develop a Consolidated ensiling approach to producing First and Second generation ethanol in an efficient, potentially contamination and cost-reduced one-step process by combining Primary and Secondary ensiling

MATERIALS AND METHODS

Unless otherwise stated all chemicals were purchased from Thermo-Fisher Scientific (Horsham, UK) and Merck (Feltham, UK). All solutions were prepared using 18 MΩcm3 milli-Q water (Merck, Feltham, UK).

Biomass selection, site selection, field ensiling process, standard loadings for additives, and ensiling period were predetermined by the ongoing projects at Shell Technology Centre - Houston (STCH) prior to the commencement of this research project. Additive volumes are expressed in forms of x, where 1.0 x is one times the conventional STCH loading. Product (ethanol, lactic acid, acetic acid) is expressed as moles per litre per gramme of post-ensiled sorghum biomass (mol l^{-1} g^{-1}). This unit refers to the molar concentration (mol l^{-1})of product produced from ensiling. This unit has been normalised for each gramme of sorghum biomass, as the mass of sorghum ensiled was not consistent between field and laboratory experiments. Without normalisation per gramme of biomass, disparity between biomass mass used in the field (> 15 kg) and in the laboratory (<50 g) prevented direct comparison of the success of different enzyme, yeast, temperature, and antimicrobial treatments. Molar concentration of product, and biomass mass given in results refers only to post-fermentation material.

Ensiling biomass selection

The biomass cultivars used for the ensiling experiments were sweet *Sorghum bicolor* M81E, Topper 76-6, NG6G0. In addition forage *Sorghum bicolor* Brown Mid-Rib (BMR) was used.

Site selection

Shell commissioned a number of farms in agriculturally diverse locations to generate the base biomass upon which field experiments where conducted. Farms were located in Florida and Texas. Critical agricultural parameters were farm location, soil type, biomass cultivar, irrigation type, pest control, fertiliser, growth period and post-harvest chop length (Appendix 1).

Primary ensiling

Primary ensiling trials were set up in the field. Fresh harvested chopped biomass was mixed with acid (H_2SO_4, 12.5 % (w/w) (6.73 ml kg^{-1}), Stabilized Liquid Yeast (1.37 ml kg^{-1}), and lactrol (0.0032 ml kg^{-1}), (Phibro Animal Health Coorporation, USA). The standard Primary ensiling field treatment at STCH was acid (1.0 x), yeast (1.0 x), and lactrol (1.0 x). Standard treatments

were performed on all biomass varieties described in Appendix 1. Each treatment had between 3 and 5 replicates. These biological replicates were distinct aliquots formed from the same biomass sample (the same variety, from the same geographical location, exposed to the same growing environment), constructed to validate the impact of the independent variable (enzyme, yeast, temperature, and antimicrobial treatments) on product (ethanol, lactic acid, acetic acid) production from ensiling. Specific additive loadings and experimental conditions for Secondary ensiling experiments are given in Tables 1.

Contamination Control Treatment	Acid Dose (x)	Lactrol Dose (x)	Hop Oil Dose (x)	Wintergreen Oil Dose (x)	Repeat Number
A	0	0	0	0	5
B	1.0	0	0	0	5
C	0	1.0	0	0	5
D	0	0	1.0	0	5
E	0	0	2.0	0	5
F	0	1.0	1.0	0	5
G	0	1.0	2.0	0	5
H	0	0	0	1.0	5
I	0	0	0	2.0	5
J	0	1.0	0	1.0	5
K	0	1.0	1.0	1.0	5
L	1.0	1.0	1.0	1.0	5

Table 1. Primary ensiling contamination control variation experimental treatments.

Contamination control treatment name relevant to that given in the results, dose of each control method added and repeat number are given. Contamination control additive loadings detailed were added in addition to 1.0x yeast. All other experimental variables were kept the same.

Both before and after fermentation (ensiling), biomass aliquots of approximately 10 g were sampled from each replicate. Biomass aliquots were dried at 78.0 ºC, 24 hr, 25.0 hg to determine biomass moisture content (NAPCO E Series vacuum oven) (Equation 1), and pressed for a liquid effluent sample, from which pH and ethanol, glucose, lactic and acetic acid concentrations (g l^{-1}) were determined by High Performance Liquid Chromatography (HPLC) analysis and final molar concentrations (mol l^{-1} g^{-1}) were determined (Equation 2). In equation

2, ethanol concentration is normalised for glucose added to the system by enzyme addition. This normalisation ensures that observed differences in ethanol concentrations between treatments are not artefacts of increased sugar addition (due to increased enzyme loading) but are due to independent variable variation.

(i) Total Solids (g) = Dry Mass (g) - Control Loss (g) ÷ Wet Mass (g) × 100

(ii) Moisture Content (%) = 100 - Total Solids (g)

Equation 1. Calculating pre- and post-ensiled sample moisture content.

(i) Post ensiled glucose (g l^{-1}) - Pre-ensiled glucose (g l^{-1}) = glucose added (g l^{-1})

(ii) Glucose added (g l^{-1}) × Maximum glucose to ethanol conversion rate (g l^{-1}) = Maximum ethanol produced from glucose added (g l^{-1})

(iii) Post ensiled ethanol (g l^{-1}) - Maximum ethanol produced from glucose added = Net ethanol (g l^{-1})

(iv) Net ethanol (g l^{-1}) ÷ Ethanol molar mass = Moles of ethanol (mol l^{-1})

(v) Moles of ethanol (mol l^{-1}) × Biomass mass (g) = Total ethanol (mol l^{-1} g^{-1})

Equation 2. Calculating ensiling ethanol yield

Biomass was packed into bucket silage chambers (26.5 l) (Letica Corporation, USA) and silage chamber mass was recorded (approximately 10-20 kg $bucket^{-1}$). Each silage chamber was sealed with a size 7 drilled rubber stopper and three-piece airlock (Austin Homebrew Supply, USA) to maintain an anaerobic environment. Sealed silage chambers were fermented *in situ* for 14 or more days. On opening, bucket silage chambers were weighed, emptied, and the biomass was mixed to ensure homogeneity. 10 g biomass aliquots were sampled from each replicate, and as before aliquots were dried at 78.0 ºC, for 24 hr, at 25.0 hg to determine biomass moisture content, and pressed for an effluent samples from which pH, and HPLC derived molar concentrations (mol l^{-1} g^{-1}) were determined (Equation 2).

Secondary ensiling

Secondary ensiling trials were set up in the laboratory. Decanted post Primary-ensiled biomass was dried at 78 ˚C, 25 hg, until a moisture content of 60% was reached. It was previously determined that at a moisture content of 60 %, > 90 % of ethanol derived from

Primary ensiling was removed. Moisture content during drying was tracked via intermittent weighing of biomass, and subsequent calculation of biomass moisture content (Equation 3).

(i) Moisture content (%) = (Wet biomass mass (g) × (Initial biomass moisture content ÷ 100) - Biomass mass loss (g)) ÷ Dried biomass mass (g)

Equation 3. Calculation of biomass moisture content during drying

Dried biomass with a moisture content of 60 % was mixed with a combination of Stabilized Liquid Yeast (SLY) (1.37 ml kg^{-1}), C6 FUEL yeast (107.5 ml kg^{-1}). (Lallemand Biofuels and Distilled Spirits, USA) and AH130 yeast (107.5 ml kg^{-1}) (Codexis, USA), acid (H_2SO_4 12.5 % (w/w), 6.73 ml kg^{-1}), lactrol (0.0032 ml kg^{-1}), CTec3 (17.86 ml kg^{-1}), Viscozyme (17.86 ml kg^{-1}) (Novozymes, Denmark), Lipase (13.84 kg^{-1}), Pectinase (2.9 g kg^{-1}), and Amylase (2.5 g kg^{-1}), depending on selected biomass treatment, and incubated at 20, 30, 40 and 50 ˚C. The control Secondary ensiling laboratory treatment at STCH was 1.0x acid, 1.0x yeast and 1.0x lactrol. All Secondary ensiling treatments were performed using these loadings as a base. Specific additive loadings and experimental conditions for Secondary ensiling experiments are given in Tables 2, 3 and 4.

Experiment	Independent Variable	Enzyme Loading (x)	Replicate Number
Enzyme Variation 1	CTec3 loading	0	5
		0.5	5
		1.0	5
		2.5	5
		5.0	5
Enzyme Variation 2	Lipase loading	0	5
		0.5	5
		1.0	5
		2.5	5
		5.0	5
Enzyme Variation 3	Viscozyme loading	0	5
		1.0	5
		2.0	5
		3.0	5
		4.0	5
		5.0	5
Enzyme Variation 4	Amylase loading	0	5
		0.5	5
		1.0	5
		2.5	5
		5.0	5
Enzyme Variation 5	Pectinase loading	0	5
		0.5	5
		1.0	5
		2.5	5
		5.0	5

Table 2. Table of Secondary ensiling enzyme variation experimental treatments.

Experiment name, independent variable, additive loadings and repeat number are given. Enzyme loadings detailed in column three are in addition to the control treatment of 1.0x yeast, 1.0x acid and 1.0x lactrol. All other experimental variables were kept the same.

Experiment	Independent Variable	Yeast Strain	Replicate Number
Yeast Variation	*S. cerevisiae* strain	LSY	5
		C6 FUEL	5
		AH130	5

Table 3. Secondary ensiling yeast variation experimental treatments

Experiment name, independent variable, yeast strain and repeat number are given. Yeast strains C6 FUEL and AH130 detailed in column three were added instead of the standard LSY in the treatment of 1.0x yeast, 1.0x acid and 1.0x lactrol. All other experimental variables were kept the same.

Experiment	Independent Variable	Temperature (˚ C)	Replicate Number
Temperature Variation	Ensiling temperature	20	5
		30	5
		40	5
		50	5

Table 4. Secondary ensiling temperature variation experimental treatments.

Experiment name, independent variable, ensiling temperature and repeat number are given. Ensiling chambers were incubated at temperatures of 30, 40 and 50 ˚C, detailed in column three, instead of the laboratory control temperature of 20 ˚C. All other experimental variables were kept the same.

Treatments were performed on all biomass varieties. Approximately 50 g of biomass was ensiled per replicate per treatment. Both before and after fermentation (Secondary ensiling), biomass aliquots of approximately 10 g were sampled from each replicate. Biomass aliquots were dried at 78.0 ºC, 24 hr, 25.0 hg to determine biomass moisture content (NAPCO E Series vacuum oven) (Equation 1), and pressed for a liquid effluent sample, from which pH and ethanol, glucose, lactic and acetic acid concentrations (g l^{-1}) were determined by High Performance Liquid Chromatography (HPLC) analysis and final molar concentrations (mol l^{-1} g^{-1}) were determined (Equation 2). Biomass was packed into 50 ml centrifuge tubes, each tube was sealed with a size 6.5 stopper and airlock, and tube mass was recorded (≥ 40 g $tube^{-1}$). Tubes were ensiled for 14 days at laboratory room temperature (20 ˚ C). On opening, tube silage chambers were weighed, emptied, and the biomass was mixed to ensure homogeneity. 10 g biomass aliquots were sampled from each replicate and processed as stated before for moisture content (Equation 1), and pH and HPLC analysis (Equation 2).

Consolidated ensiling

Consolidated ensiling trials were set up in the laboratory. Fresh harvested chopped biomass was mixed with a combination of SLY (1.37 ml kg^{-1}), acid (H_2SO_4 12.5 % (w/w), 6.73 ml kg^{-1}), lactrol (0.0032 ml kg^{-1}), CTec3 (17.86 ml kg^{-1}), Viscozyme (17.86 ml kg^{-1}), Lipase (13.84 kg^{-1}), Pectinase (2.9 g kg^{-1}), Amylase (2.5 g kg^{-1}), Hop oil (0.03 g kg^{-1}) (Kalsec, USA), and Wintergreen oil (0.03 g kg^{-1}), depending on selected biomass treatment. The control Consolidated ensiling laboratory treatment at STCH was 1x acid, 1x yeast, and 1x lactrol. All Consolidated ensiling treatments were performed using these loadings as a base. Specific additive loadings and experimental conditions for Consolidated ensiling experiments are given in Tables 5, 6, 7, and 8.

Experiment	Independent Variable	CTec3 Loading (x)	Replicate Number
CTec3 Loading Variation	CTec3 loading	0	3
		0.5	3
		1.0	3
		2.0	3
		3.0	3
		4.0	3
		6.0	3

Table 5. Consolidated ensiling CTec3 loading variation experimental treatments.

Experiment name, independent variable, ensiling temperature and repeat number are given. Enzyme loadings detailed in column three are in addition to the control treatment of 1.0x yeast, 1.0x acid and 1.0x lactrol. All other experimental variables were kept the same

Enzyme Treatment	CTec3 Dose (x)	Amylase Dose (x)	Pectinase Dose (x)	Viscozyme Dose (x)	Repeat Number
A1	1.0	0	0	0	5
B1	0	1.0	0	0	5
C1	0	0	1.0	0	5
D1	0	1.0	1.0	0	5
E1	0	0	0	2.0	5
F1	0	1.0	1.0	2.0	5
A2	1.0	0	0	0	5
B2	1.0	1.0	0	0	5
C2	1.0	0	1.0	0	5
D2	1.0	1.0	1.0	0	5
E2	1.0	0	0	2.0	5
F2	1.0	1.0	1.0	2.0	5

Table 6. Consolidated ensiling enzyme variation experimental treatments

Enzyme treatment name relevant to that given in the results, dose of each enzyme added and repeat number are given. Enzyme loadings detailed are in addition to the control treatment of 1.0x yeast, 1.0x acid and 1.0x lactrol. All other experimental variables were kept the same.

Experiment	Independent Variable	Yeast Strain	Replicate Number
Yeast Variation	*S. cerevisiae* strain	LSY	5
		C6 FUEL	5
		AH130	5

Table 7. Primary ensiling yeast variation experimental treatments

Experiment name, independent variable, yeast strain and repeat number are given. Yeast strains C6 FUEL and AH130 detailed in column three were added instead of the standard LSY in the treatment of 1.0x yeast, 1.0x acid, 1.0x lactrol and 1.0x CTec3. All other experimental variables were kept the same.

Experiment	Independent Variable	Temperature (˚ C)	Replicate Number
Temperature Variation	Ensiling temperature	20	5
		30	5
		40	5
		50	5

Table 8. Consolidated ensiling temperature variation experimental treatments.

Experiment name, independent variable, ensiling temperature and repeat number are given. Ensiling chambers were incubated at temperatures of 30, 40 and 50 ˚C, detailed in column three, instead of the laboratory control temperature of 20 ˚C. Consolidated ensiling additives for these experiments were 1.0x yeast (LSY), 1.0x acid, 1.0x lactrol and 1.0x CTec3. All other experimental variables were kept the same.

After additive addition, before ensiling, 10 g biomass aliquots were sampled from each replicate and processed as stated before for moisture content (Equation 1), pH and HPLC analysis (Equation 2). Biomass was packed into 50 ml centrifuge tubes, each tube was sealed with a size 6.5 stopper and airlock, and tube mass was recorded (≥ 40 g $tube^{-1}$). Tubes were stored at 20 ˚C and ensiled for 14 days. On opening, tube silage chambers were weighed, emptied, and the biomass was mixed to ensure homogeneity. 10 g biomass aliquots were sampled from each replicate and processed as stated before for moisture content (Equation 1), and pH and HPLC analysis (Equation 2).

Sample preparation

A 10 g biomass aliquot from each Primary, Secondary and Consolidated ensiling treatment replicate was packed into 60 ml catheter-tip syringe and compressed in a Model 1 Dake Arbor Press (Dake Coupling, USA) to produce 5 ml of effluent. A 1 ml aliquot of the effluent was centrifuged at 15,000 *x* g for 5 mins at 15 ˚C. A 0.75 ml aliquot was transferred to and centrifuged in a Costar Spin-X filter centrifuge tube at 15,000 *x* g for 5 mins at 15 ˚C. A 0.20 ml aliquot of the filtered supernatant was diluted with 0.8 ml 12.5 % w/w sulfuric acid in a 2 ml HPLC vial (Aligent, USA).

Analysis of HPLC samples

Prepared Primary, Secondary and Consolidated ensiling samples were analysed by HPLC on a Dionex Ultimate 3000 HPLC system (ThermoFisher Scientific, USA). For quantification of ethanol concentration (g l^{-1}), samples were analysed on an Aminex HPX-87H column (H column) (BIO-RAD, USA). For quantification of glucose concentration (g l^{-1}) samples were analysed on an Aminex HPX-87P Column (P column) (BIO-RAD, USA). For quantification of acetic and lactic acid concentrations (g l^{-1}) samples were analysed on a Rezex ROA-Organic Acid H+ column (ROA column)(Phenomenex, USA). The H, P, ROA column are prepacked HPLC columns, in hydrogen form, with 9 µm particle size, 8% cross linkage and pH ranges of 1 - 9, and are used to quantify concentration of carbohydrates. Samples were analysed on each column at 50 °C, with an isocratic elution of 0.005 % H_2SO_4 at a flow rate of 0.6 ml min^1 for 25 mins. 1, 5, 10, 15, and 20 g l^{-1} standards of ethanol, glucose, lactic and acetic acid were used to determine ethanol, glucose, lactic and acetic acid sample concentrations

Statistical Analysis

An ordinary one-way analysis of variance (ANOVA) with a *post-hoc* Tukey test was performed on ethanol data. The *post-hoc* Tukey test is a single-step multiple comparison statistical test, applied here to compare the mean ethanol concentration produced from each treatment to the mean of the control treatment (Witte & Witte, 2009). The Tukey test enabled simultaneous pairwise comparison all replicate means and identified any difference between two means that was greater than the expected standard error (Witte & Witte, 2009). All data met the assumptions of both statistical tests. Associated p-values of below .05 were considered significant with 95 % confidence and are indicated by * within the relevant figure. Statistical analysis was performed using Prism (GraphPad 7).

RESULTS

Intra- and Inter-experimental Variability

In previous ensiling studies at STCH, ethanol yields were highly variable and inconclusive. Variability was seen both within a set of replicates (aliquots from the same biomass sample from the same treatment), and between replica treatments carried out in entirely separate experiments. This variability was attributed to non-uniformity in biomass cultivation and experimental set up, which was unavoidable due to industrial limitations. Biomass cultivar, irrigation method and volumes, soil composition, nutrient loading, cultivation period, harvest date, and storage method were non-standardised by the farmer, whilst ensiling chamber size, storage location, and experiment duration were pre-determined by STCH and non-standardised between harvests. During this project, Secondary ensiling ethanol yields showed increased variability in comparison to Primary and Consolidated ethanol yields. The increased Secondary ensiling-associated variation was attributed to the additional drying and rehydration steps, which may have exposed the biomass to environmental contaminants. Due to the non-uniformity of biomass characteristics and experimental set up, ethanol yields from each treatment are comparable only to their internal controls and intra-experimental yields are not comparable. As such, it is difficult to determine if experimental trends observed in this work are due to manipulation of the independent variable or due to the influence of non-standardised variables. Observation and conclusions drawn from results shown here are therefore provisional, and require qualification by future studies. Whilst the duration of this project did not permit investigation into the sources of variability or possible solutions, reducing yield variation is essential in future studies, to enable valid conclusions to be drawn.

Effect of antimicrobials on ethanol, lactic acid, and acetic acid production from Primary ensiling

To determine the effect of alternative contamination control additives hop oil and wintergreen oil, in comparison to conventional antimicrobials lactrol (antibiotic) and sulphuric acid on fresh biomass, ethanol content was measured after 14 days (Figure 5). In order to assess yield variations derived only from added contaminant controls, and not from effects of enzyme addition, this study was performed in Primary ensiling. To determine if there was a significant difference between ethanol concentrations from different contamination control additives, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on ethanol data.

Compared to the control condition of no contamination control additive (4.59 ± 0.025 g mol^{-1}), ensiling with contamination control treatments B, C, D, E, F, G, J, K and L increased ethanol production to 5.42 ± 0.050 mol g^{-1}, 5.63 ± 0.079 mol g^{-1}, 5.08 ± 0.019 mol g^{-1}, 5.22 ± 0.013 mol g^{-1}, 5.54 ± 0.040 mol g^{-1}, 5.50 ± 0.091 mol g^{-1}, 5.72 ± 0.021 mol g^{-1}, 5.38 ± 0.013 mol g^{-1}, 5.81 ± 0.033 mol g^{-1}, respectively (Figure 5). Ensiling with contamination control treatments H and I decreased ethanol production to 4.24 ± 0.036 mol g^{-1}, and 4.34 ± 0.029 mol g^{-1} (Figure 5). Ensiling with contamination control treatments A, B, C, D, E, F, G, H, I J, K and L produced an average of 1.25 ± 0.032 mol g^{-1}, 0.59 ± 0.002 mol g^{-1}, 0.25 ± 0.007 mol g^{-1}, 0.60 ± 0.099 mol g^{-1}, 0.40 ± 0.021 mol g^{-1}, 0.57 ± 0.012 mol g^{-1}, 0.42 ± 0.029 mol g^{-1}, 1.42 ± 0.032 mol g^{-1}, 1.41 ± 0.024 mol g^{1}, 0.82 ± 0.017 mol g^{-1}, 1.10 ± .0051 mol g^{-1}, and 0.78 ± 0.022 mol g^{-1}, lactic acid (Figure 5). Ensiling with contamination control treatments A, B, C, D, E, F, G, H, I J, K and L produced an average of 0.50 ± 0.003 mol g^{-1}, 0.26 ± 0.021 mol g^{-1}, 0.22 ± 0.025 mol g^{-1}, 0.52 ± 0.058 mol g^{-1}, 0.49 ± 0.017 mol g^{-1}, 0.44 ± 0.003 mol g^{-1}, 0.45 ± 0.004 mol g^{-1}, 0.53 ± 0.002 mol g^{-1}, 0.54 ± 0005 mol g^{-1}, 0.47 ± 0.005 mol g^{-1}, 0.49 ± 0.010 mol g^{-1}, and 0.31 ± 0.015 mol g^{-1} acetic acid (Figure 5). Within the statistical error, treatments L and J resulted in the largest percentage increases in ethanol production (up to 27 and 25 %, respectively; Figure 5). Treatment C resulted in the lowest lactic and acetic acid yields overall (Figure 5).

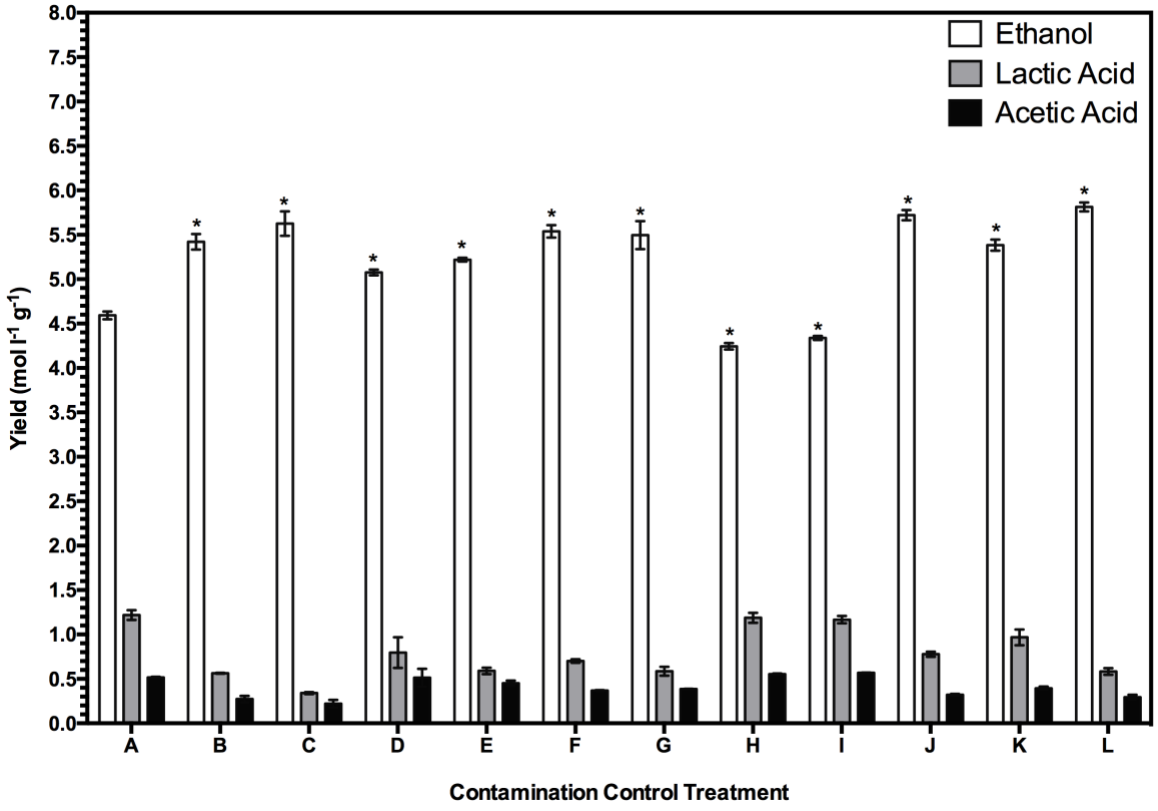

Figure 5. Effect of Contamination Control treatment on ethanol, lactic acid, and acetic acid yields from Primary ensiling

Data were produced from the Primary ensiling of Sorghum bicolor cultivar N6G60 with standard additive loadings of 1.0x acid, 1.0x yeast, 1.0x antibiotic and 1.0x CTec3. Ensiling enzyme loading and additive combination variiables were:
A: No contamination control
B: 1.0x Acid
C: 1.0x Lactrol
D: 1.0x Hop Oil
E: 2.0x Hop Oil
F: 1.0x Lactrol + 1.0x Hop Oil
G: 1.0x Lactrol + 2.0x Hop Oil
H: 1.0x Wintergreen Oil
I: 2.0x Wintergreen Oil
J: 1.0x Lactrol + 1.0x Wintergreen Oil
K: 1.0x Lactrol + 1.0x Hop Oil + 1.0x Wintergreen Oil
L: 1.0x Acid + 1.0x Lactol + 1.0x Hop Oil + 1.0x Wintergreen Oil
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars. Error bars represent the mean of 5 biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of Enzymes on ethanol production from Secondary Ensiling

Ethanol is produced from Secondary ensiling via breakdown of cellulose through the standard treatment of 1x CTec3. To determine the effect of additional enzymes Lipase, Viscoyzme, Amylase and Pectinase on secondary ensiled *Sorghum bicolor* biomass, ethanol content was measured after 14 days (Figure 6). The effect of increasing the loading of enzyme from 0.5 to 5.0 times the standard loading was also investigated, and is referred to as '0.5x'. Lactic acid and acetic acid are major products of endemic microbial biomass contamination, and represent a portion of fermentable sugars for ethanol production lost to contaminant bacterial activity. Therefore, lactic acid and acetic acid concentrations were also. Measured after 14 days (Figure 6). To determine if there was a significant difference between ethanol concentrations from increased enzyme loadings, an ordinary one-way ANOVA with a *posthoc* Tukey multiple comparison test was performed on ethanol data. As neither lactic or acetic acid are goal products of ensiling, statistical analysis was not performed on acid yields.

Compared to the control 0x CTec3, (0.538 ± 0.049 mol g^{-1}), ensiling with 0.5x, 1.0x, 2.5x and 5.0x CTec3 increased ethanol production to 2.50 ± 0.073 mol g^{-1}, 2.84 ± 0.016 mol g^{-1}, 2.63 ± 0.195 mol g^{-1}, 2.49 ± 0.039 mol g^{-1}, respectively (Figure 6A). Ensiling with 0x, 0.5x, 1.0x, 2.5x and 5.0x CTec3 produced an average of $>0.01 \pm 0.010$ mol g^{-1}, 0.40 ± 0.004 mol g^{-1}, 0.90 ± 0.012 mol g^{-1}, 0.90 ± 0.035 and 1.00 ± 0.025 mol g^{-1} lactic acid, respectively (Figure 6A). Ensiling with 0x, 0.5x, 1.0x, 2.5x and 5.0x CTec3 produced an average of $<0.01 \pm 0.0.08$ mol g^{-1}, 0.30 ± 0.003 mol g^{-1}, 0.87 ± 0.009 mol g^{-1}, 1.00 ± 0.027 mol g^{-1}, 0.89 ± 0.009 mol g^{-1}, and 0.90 ± 0.019 mol g^{-1} acetic acid, respectively (Figure 6A).

Compared to the control 0x Lipase + 1.0x CTec3, (3.958 ± 0.16 mol g^{-1}), ensiling with 0.5x, 1.0x, 2.0x, 3.0x, 4.0x and 5.0x Lipase decreased ethanol production to 3.42 ± 0.155 mol g^{-1}, 3.38 ± 0.109 mol g^{-1}, 3.25 ± 0.094 mol g^{-1}, 3.37 ± 0.075 mol g^{-1}, 3.44 ± 0.064 mol g^{-1}, 3.34 ± 0.047 mol g^{-1} respectively (Figure 6B). Ensiling with 0.5x, 1.0x, 2.0x, 3.0x, 4.0x and 5.0x Lipase produced an average of 1.90 ± 0.065 mol g^{1}, 1.70 ± 0.075 mol g^{-1}, 1.60 ± 0.022 mol g^{-1}, 1.60 ± 0.116, 1.90 ± 0.056 mol g^{-1}, 1.75 ± 0.051 mol g^{-1}, 1.65 ± 0.017 mol g^{-1} lactic acid, respectively (Figure 6B). Ensiling with 0.5x, 1.0x, 2.0x, 3.0x, 4.0x and 5.0x Lipase produced an average of 0.80 ± 0.034 mol g^{-1}, 0.70 ± 0.041 mol g^{-1}, 0.70 ± 0.013, 0.75 ± 0.033 mol g^{-1}, 0.75 ± 0.036 mol g^{-1}, 0.69 ± 0.015 mol g^{-1} and 0.71 ± 0.026 mol g^{-1}, acetic acid, respectively (Figure 6B).

Compared to the control 0x Viscozyme + 1.0x CTec3 (2.88 ± 0.166 mol g^{-1}), ensiling with 1.0x, 2.0x, 3.0x, 4.0x and 5.0x Viscozyme increased ethanol production to 3.94 ± 0.266 mol g^{-1}, 4.54 ± 0.091 mol g^{-1}, 4.80 ± 0.179 mol g^{-1}, 4.29 ± 0.202 mol g^{-1}, and 4.14 ± 0.275 mol g^{-1}, respectively (Figure 6C). Ensiling with 1.0x, 2.0x, 3.0x, 4.0x and 5.0x Viscozyme produced an average of 0.8 ± 0.028 mol g^{-1}, 0.75 ± 0.040 mol g^{-1}, 0.75 ± 0.030 mol g^{-1}, 0.81 ± 0.024 mol g^{-1}, 0.78 ± 0.027 mol g^{-1}, and 0.97 ± 0.052 mol g^{-1} lactic acid, respectively (Figure 6C). 1.0x, 2.0x, 3.0x, 4.0x and 5.0x Viscozyme produced an average of 0.4 ± 0.014 mol g^{-1}, 0.4 ± 0.022 mol g^{-1}, 0.39 ± 0.007 mol g^{-1}, 0.72 ± 0.025 mol g^{-1}, 0.69 ± 0.032 mol g^{-1} and 0.95 ± 0.070 mol g^{-1} ,acetic acid, respectively (Figure 6C).

Compared to the control 0x Amylase +1.0x CTec3 (1.64 ± 0.035 mol g^{-1}), ensiling with 0.5x, 1.0x, 2.5x and 5.0x, Amylase decreased ethanol production to 1.06 ± 0.099 mol g^{-1}, 1.50 ± 0.038 mol g^{1}, 1.17 ± 0.083 mol g^{-1}, and 1.32 ± 0.055 mol g^{-1}, respectively (Figure 6D). Ensiling with 0.5x, 1.0x, 2.5x and 5.0x, Amylase produced an average of 1.1 ± 0.047 mol g^{-1}, 1.0 ± 0.035 mol g^{-1}, 0.50 ± 0.016 mol g^{-1}, 1.17 ± 0.046 mol g^{-1}, and 1.30 ± 0.24 mol g^{-1} lactic acid, respectively (Figure 6D). Ensiling with 0.5x, 1.0x, 2.5x and 5.0x, Amylase produced an average of 0.0 mol g^{-1}, 0.0 mol g^{-1}, 0.30 ± 0.022 mol g^{-1}, 0.31 ± 0.044 mol g^{-1}, 0.20 ± 0.003 mol g^{-1} and 0.29 ± 0.020 mol g^{-1} ,acetic acid, respectively (Figure 6D).

Compared to the control 0x Pectinase + 1.0x CTec3 (0.05 ± 0.003 mol g^{-1}), ensiling with 0.5x, 1.0x, 2.5x and 5.0x, Pectinase increased ethanol production to 1.66 ± 0.072 mol g^{-1}, 1.50 ± 0.075 mol g^{-1}, 1.32 ± 0.052 mol g^{-1}, and 1.41 ± 0.022 mol g^{-1}, respectively (Figure 6E). Ensiling with 0.5x, 1.0x, 2.5x and 5.0x Pectinase produced an average of 0.90 ± 0.163 mol g^{-1}, 1.1 ± 0.035 mol g^{-1}, and 0.90 ± 0.121 mol g^{-1}, 0.90 ± 0.020 mol g^{-1}, 0.90 ± 0.006 mol g^{-1} lactic acid, respectively (Figure 6E). Ensiling with 0.5x, 1.0x, 2.5x and 5.0x Pectinase produced an average of 0.1 ± 0.008 mol g^{-1}, 0.2 ± 0.006 mol g^{-1}, 0.2 ± 0.013 mol g^{-1}, 0.30 ± 0.010 mol g^{-1}, 0.35 ± 0.011 mol g^{-1} acetic acid, respectively (Figure 6E).

Significant variations in ethanol yields were observed between experiments A, B, C, D and E (Figure 6). Particularly, from the control treatment (1.0x yeast, acid, lactrol, CTec3), where ethanol yields ranged from 0.05 ± 0.003 (Figure 6E) to 3.958 ± 0.16 (Figure 6C). This variability prevents intra-experimental comparison, and therefore treatments were assessed based upon their success in comparison to the internal experimental control only. Within the statistical error, addition of CTec3 and Pectinase resulted in the largest percentage increases in ethanol

production (up to 428 and 3495 %, respectively; Figure 6A and 6E) compared to their individual experimental controls. Addition of 50 % the conventional dose (defined as 1.0x) of CTec3 and Pectinase are as efficient as the manufacturer's recommended dose, with the concomitant decrease in cost. Within statistical error, addition of Amylase (Figure 6D) and Lipase (Figure 6B) to the Secondary ensiling process resulted in the largest concentration decreases, in comparison to the individual experimental controls. The highest concentrations of lactic and acetic acid were produced by the Lipase Secondary ensiling process (Figure 6B).

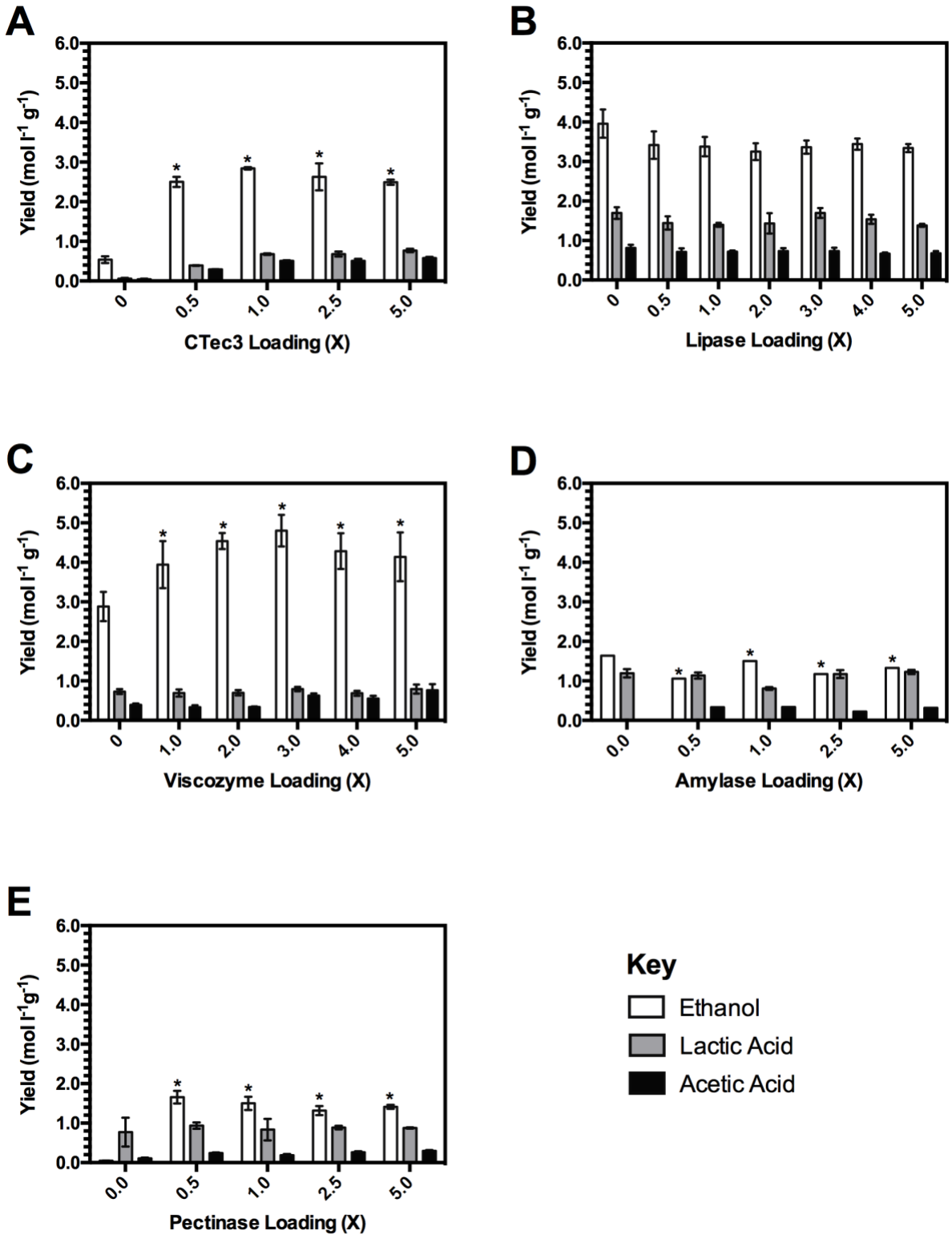
A
Yield (mol l-1 g-1)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
0.5
1.0
2.5
5.0
CTec3 Loading (X)
B
Yield (mol l-1 g-1)
0
0.5
1.0
2.0
3.0
4.0
5.0
Lipase Loading (X)
C
Yield (mol l-1 g-1)
0
1.0
2.0
3.0
4.0
5.0
Viscozyme Loading (X)
D
Yield (mol l-1 g-1)
0.0
0.5
1.0
2.5
5.0
Amylase Loading (X)
E
Yield (mol l-1g-1)
0.0
0.5
1.0
2.5
5.0
Pectinase Loading (X)
Key
Ethanol
Lactic Acid
Acetic Acid

Figure 6. Effect of enzyme type and loading variation on ethanol yield from Secondary ensiled biomass

Data were produced from the Secondary ensiling of Sorghum bicolor cultivar M81E with standard additive loadings of 1.0x acid, 1.0x yeast (LSY) and 1.0x antibiotic. Enzyme type and loading was varied.
A: Ethanol, lactic acid, and acetic acid yield from ensiling with 0x, 0.5x, 1.0x, 2.5x, and 5.0x CTec3, where "x" represents the manufacturer's recommended dose.
B: Ethanol, lactic acid, and acetic acid yield from ensiling with 0x, 0.5x, 1.0x, 2.5x, and 5.0x Lipase.
C: Ethanol, lactic acid, and acetic acid yield from ensiling with 0x, 0.5x, 1.0x, 2.5x, and 5.0x Viscozyme.
D: Ethanol, lactic acid, and acetic acid yield from ensiling with 0x, 0.5x, 1.0x, 2.5x, and 5.0x Amylase.
D: Ethanol, lactic acid, and acetic acid yield from ensiling with 0x, 0.5x, 1.0x, 2.5x, and 5.0x Pectinase.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars. Error bars represent the mean of 5 or more biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of Enzyme addition on alternate Sorghum bicolor cultivar ethanol production from Secondary ensiling

To determine the effect of the enzyme addition on alternate *Sorghum bicolor* cultivars, three additional *Sorghum bicolor* cultivars Topper 76-6, NexSteppe N6G60, Brown Mid Rib (BMR), alongside the conventional cultivar M81E, underwent Secondary ensiling with 1.0x CTec3 (Figure 7) . Ethanol content was measured after 14 days (Figure 7). To assess whether there was a significant difference between ethanol concentrations from different cultivars, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on ethanol data.

Compared to the control M81E (1.81 $\pm$ 0.064 mol g^{-1}), ensiling Topper 76-6 and N6G60 increased ethanol production to 2.94 $\pm$ 0.244 mol g^{-1}, and 2.02 $\pm$ 0.151 mol g^{-1} respectively (Figure 7). Ensiling BMR decreased ethanol production to 0.208 $\pm$ 0.022 mol g^{-1} (Figure 7). Ensiling M81E, Topper 76-6, BMR and N6G60 produced an average of 2.0 $\pm$ 0.007 mol g^{-1}, 0.7 $\pm$ 0.050 mol g^{1}, 1.4 $\pm$ 0.082 mol g^{-1}, 1.4 $\pm$ 0.050 mol g^{-1} lactic acid, respectively (Figure 7). Ensiling M81E, Topper 766, BMR and N6G60 produced an average of 0.5 1.4 $\pm$ 0.082 mol g^{-1}, 0.048 $\pm$ 0.108 mol g^{-1}, 0.6 $\pm$ 0.064 mol g^{1},1.4 $\pm$ 0.069 mol g^{-1} and 1.0 $\pm$ 0.064 mol g^{1} acetic acid (Figure 7). Within the statistical error, ensiling of Topper 76-6 resulted in the largest percentage increases in ethanol production (up to 102 % ; Figure 7) compared to the control of M81E.

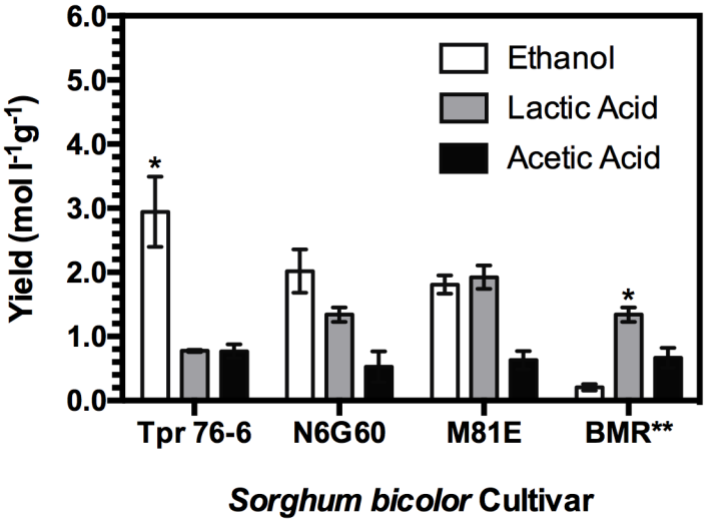

Figure 7. Effect of *Sorghum bicolor* Cultivar variation on ethanol, lactic acid and acetic acids yields from Secondary ensiled biomass

Data were produced from the Secondary ensiling of *Sorghum bicolor* cultivars with standard additive loadings of 1.0x acid, 1.0x yeast (LSY), 1.0x antibiotic and 1.0x CTec3. Biomass *Sorghum bicolor* Cultivar was varied.
M81E: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar M81E.
Tpr 76-6: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar Topper 76-6.
BMR: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar Brown Mid Rib (BMR).
N6G60: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar NextSteppe N6G60.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars.
Error bars represent the mean of 5 biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. **BMR ensiling chambers contaminated with unknown white fungus. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of variation of yeast type on ethanol production from Secondary ensiling

Three commercial strains of *Saccharomyces cerevisae* were investigated for their performance in the production of ethanol when used in the Secondary ensiling of *S. bicolor* biomass. Liquid Stabilised Yeast *S. cerevisae* (LSY) was obtained from Lallemand Biofuels and Distilled Spirits, and contains stabilisers to ensure consistent fermentation activity. C6 FUEL *S. cerevisae* was also obtained from Lallemand Biofuels and Distilled Spirits, and is a commercially selected strain for biomass fermentation. *S. cerevisae* AH130, obtained from Codexis, is an engineered xylose consuming strain. Each *S. cerevisae* strain was added to *S. bicolor* cultivar M81E at identical OD_{600} prior to ensiling. To determine the effect of yeast variation on Secondary ensiled *Sorghum bicolor* biomass, cultivar M81E was ensiled separately with LSY, C6 FUEL and AH130, and ethanol content was measured after 14 days (Figure 8). To ascertain whether the differences between ethanol concentrations from different yeast strains were significant, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on the ethanol data.

Compared to the conventional ensiling yeast LSY (1.79 ± 0.049 mol g^{-1}) ensiling with C6 FUEL increased ethanol production to 2.14 ± 0.063 mol g^{-1}, and ensiling with AH130 decreased ethanol production to 0.802 ± 0.044 mol g^{-1}, (Figure 8). Ensiling with LSY, C6 FUEL, and AH130 produced an average of 0.3 ± 0.018 mol g^{-1}, 0.1 ± 0.004 mol g^{-1}, and 0.7 ± 0.034 mol g^{-1} lactic acid (Figure 8). Ensiling with LSY, C6 FUEL, and AH130 produced an average of 0.1 ± 0.010 mol g^{-1}, 0.15 ± 0.018 mol g^{-1}, and 0.4 ± 0.012 mol g^{-1} acetic acid (Figure 8). Within the statistical error, ensiling with C6 FUEL resulted in the largest percentage increase in ethanol production (up to 20 % ; Figure 8) compared to the control of M81E.

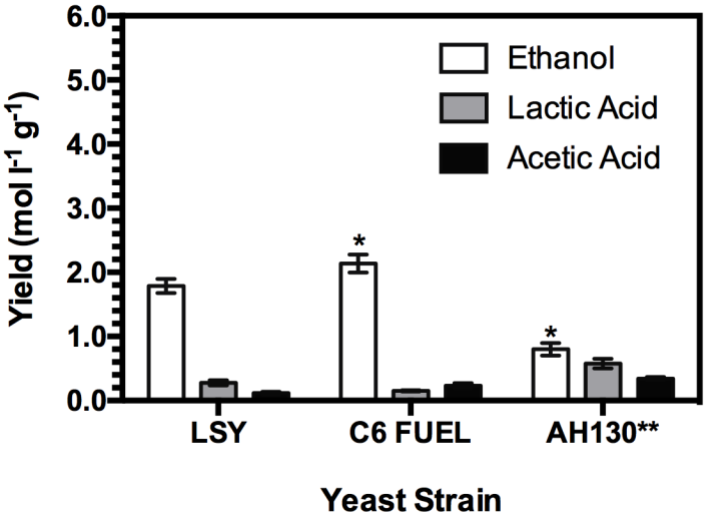

Figure 8. Effect of Saccharomyces cerevisiae strain variation on ethanol, lactic acid and acetic acid yields from Secondary ensiled biomass

Data were produced from the Secondary ensiling of *Sorghum bicolor* cultivar M81E with standard additive loadings of 1.0x acid, 1.0x yeast, 1.0x antibiotic and 1.0x CTec3. Yeast *Saccharomyces cerevisiae* strain was varied**.**
LSY: Ethanol, lactic acid, and acetic acid yield from ensiling with *S. cerevisiae* strain Liquid Stabilised Yeast.
C6 FUEL: Ethanol, lactic acid, and acetic acid yield from ensiling with *S. cerevisiae* strain C6 FUEL.
AH130: Ethanol, lactic acid, and acetic acid yield from ensiling with *S. cerevisiae* strain AH130.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars.
Error bars represent the mean of 5 biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. **OD of AH130 was not comparable to OD of LSY or C6 FUEL. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of different ensiling incubation temperatures on ethanol production from Secondary ensiling

To assess the effect of ensiling temperature on Secondary ensiled *Sorghum bicolor* biomass, cultivar M81E was ensiled and incubated at 20 ˚C, 30 ˚C, 40 ˚C, and 50 ˚C, and ethanol content was measured after 14 days (Figure 9). To determine if there was a significant difference between ethanol concentrations from incubation at different temperatures, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on the ethanol data.

Compared to the laboratory control temperature of 20 ˚C (1.53 ± 0.092 g mol^{-1}), ensiling at 30 ˚C increased ethanol production to 2.21 ± 0.078 mol g^{-1} (Figure 9). Ensiling at 40 ˚C and 50 ˚C decreased ethanol production to 0.889 ± 0.029 mol g^{-1} and 0.352 ± 0.029 mol g^{-1} respectively (Figure 9). Ensiling at 20 ˚C, 30 ˚C, 40 ˚C, and 50 ˚C, produced an average of 0.90 ± 0.021 mol g^{-1}, 0.90 ± 0.052 mol g^{-1}, 0.80 ± 0.026 mol g^{-1}, and 0.80 ± 0.033 mol g^{-1} lactic acid (Figure 9). Ensiling at 20 ˚C, 30 ˚C, 40 ˚C, and 50 ˚C, produced an average of 0.50 ± 0.039 mol g^{-1}, 0.10 ± 0.005 mol g^{-1}, 0.10 ± 0.011 mol g^{-1} and 0.70 ± 0.027 mol g^{-1} acetic acid (Figure 9). Within the statistical error, ensiling at 30 ˚C resulted in the largest percentage increase in ethanol production (up to 45 % ; Figure 9) compared to the control of 20 ˚C.

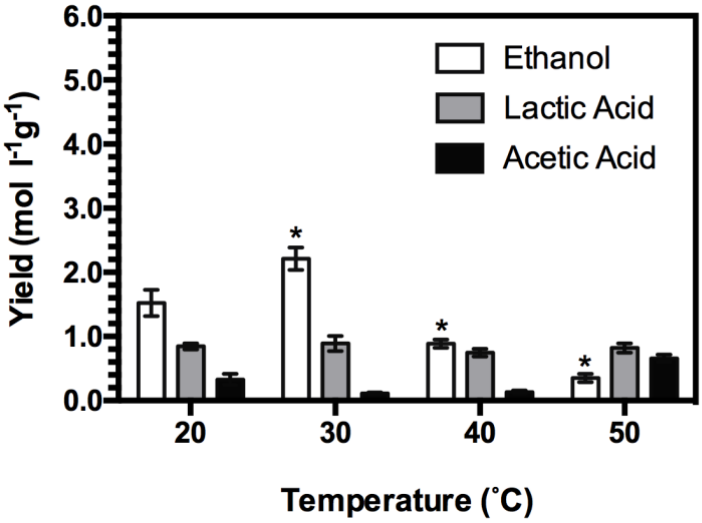

Figure 9. Effect of ensiling chamber temperature variation on ethanol, lactic acid and acetic acid yields from Secondary ensiled biomass

Data were produced from the Secondary ensiling of *Sorghum bicolor* cultivar M81E with standard additive loadings of 1.0x acid, 1.0x yeast, 1.0x antibiotic and 1.0x CTec3. Ensiling temperature was varied.
20: Ethanol, lactic acid, and acetic acid yield from ensiling at 20 ˚C.
30: Ethanol, lactic acid, and acetic acid yield from ensiling at 30 ˚C. 40:
Ethanol, lactic acid, and acetic acid yield from ensiling at 40 ˚C.
50: Ethanol, lactic acid, and acetic acid yield from ensiling at 50 ˚C.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars.
Error bars represent the mean of 5 biological replicates with standard error of the mean shown.
Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of Enzyme addition on alternate Sorghum bicolor cultivar ethanol production from Consolidated ensiling

Based on data from Secondary ensiling studies (Figure 6), optimal Viscozyme, Amylase and Pectinase loadings of 2x, 1x, and 1x, were chosen for application to Consolidated ensiling. 'Optimal' enzyme loadings were defined as the minimum loading with the maximum associated increase in ethanol production compared to the control (0x enzyme loading). Due to industrial constraints and requirements, Lipase was not tested, and the manufacturer's recommended 1x loading for CTec3 was used in Consolidated ensiling. Defined optimal CTec3, Viscozyme, Amylase and Pectinase loadings were added both singularly and in combination to fresh biomass which underwent Consolidated ensiling. Ethanol content was measured after 14 days (Figure 10). To ascertain if there was a significant difference between ethanol concentrations from different enzyme loadings, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on ethanol data.

Compared to the control 0xCTec3, (5.25 ± 0.314 mol g^{-1}), ensiling with 0.5x, 1x, 3x, 4x, 6x CTec3 increased ethanol production to 8.52 ± 0.262 mol g^{-1}, 8.103 ± 0.257 mol g^{-1}, 8.59 ± 0.259 mol g^{-1}, 8.17 ± 0.257 mol g^{-1}, 8.045 ± 0.002 mol g^{-1}, respectively (Figure 10A). Ensiling with 0x, 1x, 3x, 4x, and 6x CTec3 produced an average of 0.39 ± 0.040 mol g^{-1}, 0.64 ± 0.038 mol g^{-1}, 0.747 ± 0.038 mol g^{-1}, 0.628 ± 0.073 mol g^{-1}, 0.317 ± 0.005 mol g^{-1}, 1.226 ± 0.062 mol g^{-1} lactic acid, respectively (Figure 10A). Ensiling with 0x, 1x, 2x, 3x, 4x, 6x, and 15x CTec3 produced an average of 0.175 ± 0.023 mol g^{-1}, 0.368 ± 0.010 mol g^{-1}, 0.429 ± 0.023 mol g^{-1}, 0.307 ± 0.029 mol g^{-1}, 0.305 ± 0.041 mol g^{-1}, 0.436 ± 0.010 mol g^{-1} acetic acid, respectively (Figure 10A).

Compared to the control treatment A, (7.95 ± 0.394 mol g^{-1}), ensiling with treatment B, C, D, E, and F decreased ethanol production to 4.55 ± 0.197 mol g^{-1}, 6.35 ± 0.188 mol g^{-1}, 5.04 ± 0.208 mol g^{-1}, 5.68 ± 0.191 mol g^{-1} and 5.59 ± 0.218 mol g^{-1}, respectively (Figure 10B). Ensiling with treatment A, B, C, D, E and F produced an average of 0.60 ± 0.163 mol g^{-1}, 1.00 ± 0.035 mol g^{1}, 2.00 ± 0.038 mol g^{-1}, 2.00 ± 0.153 mol g^{-1}, 2.00 ± 0.130 mol g^{-1}, 2.00 ± 0.130 mol g^{-1} lactic acid, respectively (Figure 10B). Ensiling with treatment A, B, C, D, E and F produced an average of 0.55 ± 0.026 mol g^{-1}, 0.60 ± 0.028 mol g^{-1}, 0.55 ± 0.049 mol g^{-1}, 1.00 ± 0.130 mol g^{-1}, 1.00 ± 0.090 mol g^{-1}, and 0.90 ± 0.042 mol g^{-1} acetic acid, respectively (Figure 10B).

Compared to the control treatment A, (8.28 ± 0.309 mol g^{-1}), ensiling with treatment B, C, D, and F increased ethanol production to 8.40 ± 0.147 mol g^{-1}, 8.82 ±0.274 mol g^{-1}, 8.93 ± 0.250 mol g^{-1}, 8.93 ± 0.286 mol g^{-1}, and 9.03 ± 0.300 mol g^{-1}, respectively (Figure 10C). Compared to the control treatment A, ensiling with treatment E decreased ethanol production to 8.11 ± 0.286 mol g^{-1} (Figure 10C). Ensiling with treatment A, B, C, D, E, and F produced an average of 0.90 ± 0.075 mol g^{-1}, 0.60 ± 0.021 mol g^{-1}, 0.50 ± 0.019 mol g^{-1}, 0.40 ± 0.026 mol g^{-1}, 0.70 ± 0.051 mol g^{-1}, 0.70 ± 0.055 mol g^{-1} lactic acid, respectively (Figure 10C). Ensiling with treatment A, B, C, D, E, and F produced an average of 0.75 ± 0.069 mol g^{-1}, 0.60 ± 0.019 mol g^{-1}, 0.50 ± 0.028 mol g^{-1}, 0.30 ± 0016 mol g^{-1} and 0.60 ± 0040 mol g^{-1}, 0.60 ± 0032 mol g^{-1}, acetic acid, respectively (Figure 10C).

Within the statistical error, ensiling with 0.5x CTec3 resulted in the largest percentage increase in ethanol production (up to 63 % ; Figure 10A) compared to the control of no enzyme addition. Identical to data shown in Figure 6 with Secondary ensiling, Figure 10A suggests that addition of 50 % the conventional dose of CTec3 is sufficient to produce statistically comparable ethanol yields with the manufacturer's recommended dose.

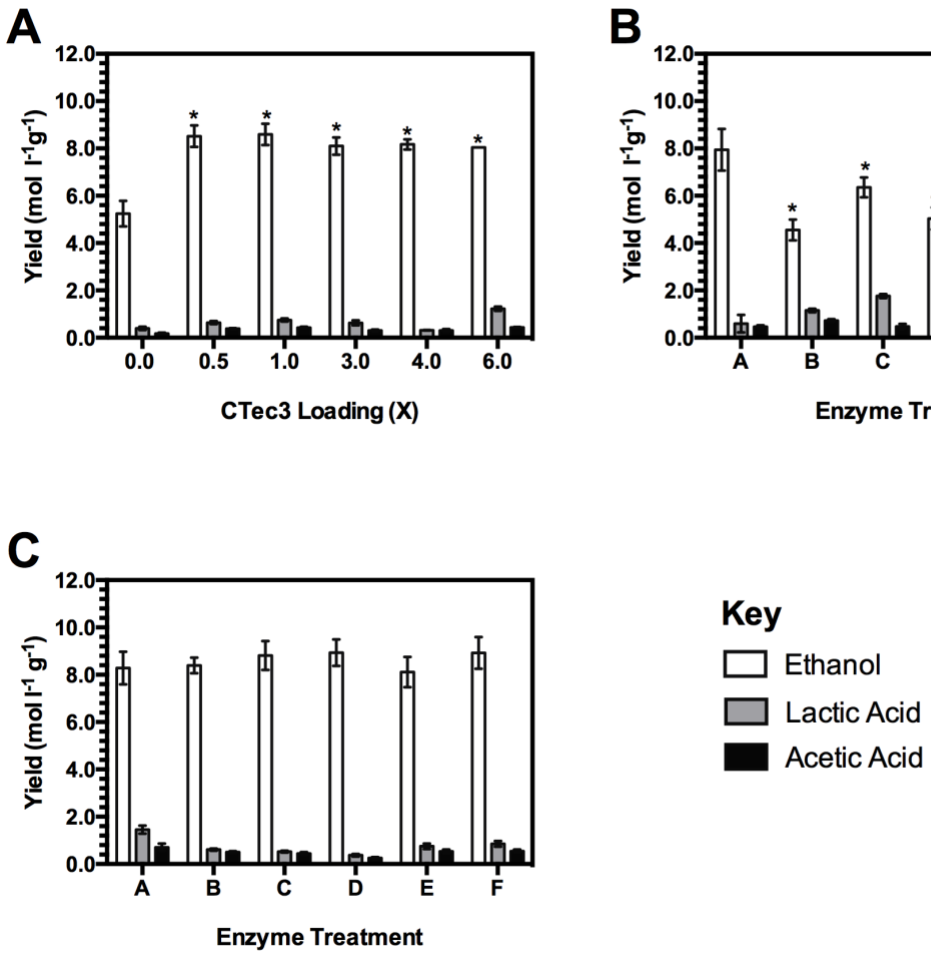
A
Yield (mol l-1g-1)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0.0
0.5
1.0
3.0
4.0
6.0
CTec3 Loading (X)
B
Yield (mol l-1g-1)
A
B
C
D
E
F
Enzyme Treatment
C
Yield (mol l-1 g-1)
Enzyme Treatment
Key
Ethanol
Lactic Acid
Acetic Acid

Figure 10. Effect of enzyme loading and variation on ethanol, lactic acid and acetic acid yields from Consolidated ensiled biomass

Data were produced from the Consolidated ensiling of *Sorghum bicolor* cultivar N6G60 with standard additive loadings of 1.0x acid, 1.0x yeast, 1.0x antibiotic and 1.0x CTec3. Ensiling enzyme loading and additive combination was. varied.

8A: Ethanol, lactic acid, and acetic acid yield from ensiling with 0.0x, 0.5x 1.0x, 3.0x, 4.0x, 6.0x CTec3 loadings.
8B: Ethanol, lactic acid, and acetic acid yield from ensiling with enzyme treatments:
A: 1.0x Ctec3
B: 1.0x Amylase
C: 1.0x Pectinase
D: 1.0x Amylase + 1.0x Pectinase
E: 1.0x Viscozyme
F: 1.0x Amylase + 1.0x Pectinase + 1.0x Viscozyme.
8C: Ethanol, lactic acid, and acetic acid yield from ensiling with enzyme treatments:
A: 1.0x Ctec3
B: 1.0x CTec3 + 1.0x Amylase
C: 1.0x CTec3 + 1.0x Pectinase
D: 1.0x CTec3 + 1.0x Amylase + 1.0x Pectinase
E: 1.0x CTec3 + 1.0x Viscozyme
F: 1.0x CTec3 + 1.0x Amylase + 1.0x Pectinase + 1.0x Viscozyme
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars. Error bars represent the mean of 5 biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of Sorghum bicolor variation on ethanol production from Consolidated ensiling

Based on data from Secondary ensiling studies, it was determined that, in comparison to alternate *S.bicolor* varieties, Topper 76-6 produced the highest ethanol concentration when ensiled with 1x CTec3 (Figure 7). These results were validated through further Consolidated ensiling studies (Figure 11). The comparative response of fresh (not dried, as in Secondary ensiling) *S. bicolor* to CTec3 was assessed via the Consolidated ensiling of four *S.bicolor* cultivars M81E, Topper 76-6, BMR, and N6G60 (Figure 11). Ethanol content was measured after 14 days (Figure 11). To determine if there was a significant difference between ethanol concentrations from enzyme loadings, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on ethanol data.

Compared to the control M81E (5.97 $\pm$ 0.172mol g^{-1}), ensiling Topper 76-6 and N6G60 increased ethanol production to 9.82 $\pm$ 0.072 mol g^{-1}, and 8.68 $\pm$ 0.046 mol g^{-1}, respectively (Figure 11). Ensiling BMR decreased ethanol production to 4.95 $\pm$ 0.207 mol g^{-1} (Figure 11). Ensiling M81E, Topper 76-6, BMR and N6G60 produced an average of 1.50 $\pm$ 0.092 mol g^{-1}, 0.488 $\pm$ 0.092 mol g^{-1}, 1.592 $\pm$ 0.083 mol g^{-1}, 0.134 $\pm$ 0.008 mol g^{-1} lactic acid, respectively (Figure 11). Ensiling M81E, Topper 76-6, BMR and N6G60 produced an average of 0.810 $\pm$ 0.037 mol g^{-1}, 0.230 $\pm$ 0.037 mol g^{-1},0.723 $\pm$ 0.063 mol g^{-1} and 0.049 $\pm$ 0.002 mol g^{-1} acetic acid (Figure 11)***.*** Within the statistical error, ensiling Topper 76-6 resulted in the largest percentage increase in ethanol production (up to 64 % ; Figure 11) compared to the control of M81E.

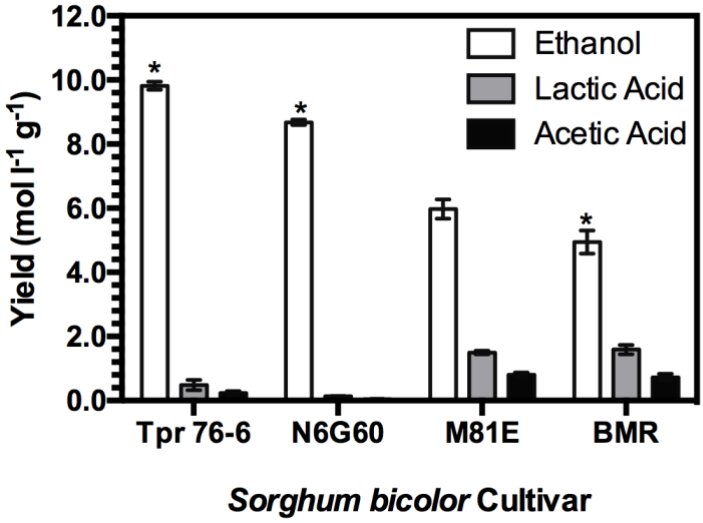

Figure 11. Effect of Sorghum bicolor Cultivar variation on ethanol, lactic acid and acetic acids yields from Consolidated ensiled biomass

Data were produced from the Consolidated ensiling of *Sorghum bicolor* cultivars with standard additive loadings of 1.0x acid, 1.0x yeast (LSY), 1.0x antibiotic and 1.0x CTec3. Biomass *Sorghum bicolor* Cultivar was varied.
M81E: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar M81E.
Tpr 76-6: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar Topper 76-6.
BMR: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar Brown Mid Rib (BMR).
N6G60: Ethanol, lactic acid, and acetic acid yield from ensiling of *Sorghum bicolor* cultivar NextSteppe N6G60.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars. Error bars represent the mean of 5 biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of variation of yeast type on ethanol production from Consolidated ensiling

Based on data from Secondary ensiling studies, it was determined that, in comparison to alternate *S. cerevisae* strains, strain C6 FUEL produced the highest ethanol concentration when Secondary ensiled with 1x CTec3 in comparison with other strains LSY and AH130 (Figure 8). These results were validated through further Consolidated ensiling studies (Figure 12). The comparative response of fresh (not dried, as in Secondary ensiling) *S. bicolor* to CTec3 with three *S.cerevisae* strains LSY, C6 FUEL and AH130 was assessed via the Consolidated ensiling of *S.bicolor* cultivar M81E (Figure 12). Ethanol content was measured after 14 days (Figure 12). To determine if there was a significant difference between ethanol concentrations from enzyme loadings, an ordinary one-way ANOVA with a *post-hoc* Tukey multiple comparison test was performed on ethanol data.

Compared to the conventional ensiling yeast LSY (6.25 $\pm$ 0.058 mol g^{-1}) ensiling with C6 FUEL and AH130 decreased ethanol production to 5.99 $\pm$ 0168 mol g^{-1} and 3.59 $\pm$ 0.068 mol g^{-1}, respectively (Figure 12). Ensiling with LSY, C6 FUEL, and AH103 produced an average of 0.40 $\pm$ 0.010 mol g^{-1}, 0.39 $\pm$ 0.013 mol g^{-1}, and 1.85 $\pm$ 0.198 mol g^{-1} lactic acid (Figure 12). Ensiling with LSY, C6 FUEL, and AH103 produced an average of 0.30 $\pm$ 0.008 mol g^{-1}, 0.31 $\pm$ 0.032 mol g^{-1}, and 1.50 $\pm$ 0.024 mol g^{-1} acetic acid (Figure 12). Within the statistical error, ensiling with LSY produced the highest ethanol yield (Figure 12).

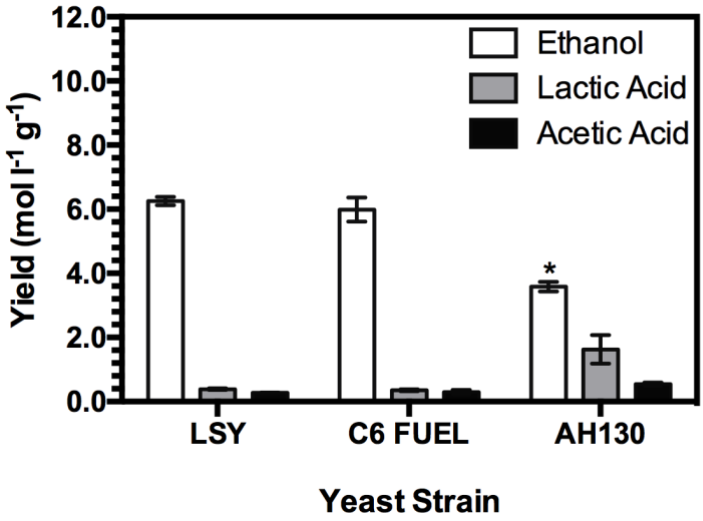

Figure 12. Effect of *Saccharomyces cerevisiae* strain variation on ethanol, lactic acid and acetic acid yields from Consolidated ensiled biomass

Data were produced from the Consolidated ensiling of *Sorghum bicolor* cultivar N6G60 with standard additive loadings of 1.0x acid, 1.0x yeast, 1.0x antibiotic and 1.0x CTec3. Yeast *Saccharomyces cerevisiae* strain was varied.
LSY: Ethanol, lactic acid, and acetic acid yield from ensiling with *S. cerevisiae* strain Liquid Stabilised Yeast.
C6 FUEL: Ethanol, lactic acid, and acetic acid yield from ensiling with *S. cerevisiae* strain C6 FUEL.
AH130: Ethanol, lactic acid, and acetic acid yield from ensiling with *S. cerevisiae* strain AH130.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars. Error bars represent the mean of 5 biological replicates with standard error of the mean shown. Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment. *$p<0.05$. Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

Effect of different ensiling incubation temperatures on ethanol production from Consolidated ensiling

Based on data from Secondary ensiling studies, it was determined that, in comparison to alternate incubation temperatures, ensiling at 30 ˚C produced the highest ethanol concentration when Secondary ensiled with 1x CTec3 (Figure 9). These results were validated through further Consolidated ensiling studies (Figure 13). The comparative response of fresh (not dried, as in Secondary ensiling) *S. bicolor* to CTec3 with incubation at 20 ˚C, 30 ˚C, 40 ˚C, and 50 ˚C was assessed via the Consolidated ensiling of *S.bicolor* cultivar M81E (Figure 13). Ethanol content was measured after 14 days (Figure 13). To determine if there was a significant difference between ethanol concentrations from enzyme loadings, an ordinary oneway ANOVA with a *post-hoc* Tukey multiple comparison test was performed on ethanol data.

Compared to the laboratory control temperature of 20 ˚C (6.64 ± 0.258 g mol^{-1}), ensiling at 30 ˚C and 40 ˚C increased ethanol production to 6.658 ± 0.348 mol g^{-1} and 6.68 ± 0.311 mol g^{-1} (Figure 13). Ensiling at 50 ˚C decreased ethanol production to 0.064 ± 0.003 mol g^{-1} (Figure 13). Ensiling at 20 ˚C, 30 ˚C, 40 ˚C, and 50 ˚C, produced an average of 0.129 ± 0.083 mol g^{-1}, 0.363 ± 0.055 mol g^{-1}, 0.395 ± 0.028 mol g^{-1}, and 2.079 ± 0.553 mol g^{-1} lactic acid, respectivly (Figure 13). Ensiling at 20 ˚C, 30 ˚C, 40 ˚C, and 50 ˚C, produced an average of 0.140 ± 0.019 mol g^{-1}, 0.203 ± 0.016 mol g^{-1}, 0.173 ± 0.007 mol g^{-1}, and 0.838 ± 0.053 mol g^{-1} acetic acid (Figure 13). Within the statistical error, ensiling at 20, 30 and 40 ˚C produced equivalent ethanol yields (Figure 13).

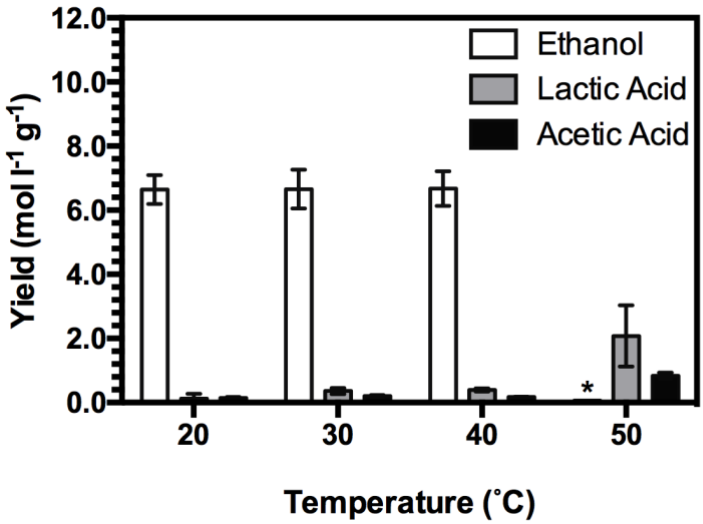

Figure 13. Effect of ensiling chamber temperature variation on ethanol, lactic acid and acetic acid yields from Consolidated ensiled biomass

Data were produced from the Consolidated ensiling of *Sorghum bicolor* cultivar N6G60 with standard additive loadings of 1.0x acid, 1.0x yeast, 1.0x antibiotic and 1.0x CTec3. Ensiling temperature was varied.
20: Ethanol, lactic acid, and acetic acid yield from ensiling at 20 ˚C.
30: Ethanol, lactic acid, and acetic acid yield from ensiling at 30 ˚C. 40:
Ethanol, lactic acid, and acetic acid yield from ensiling at 40 ˚C.
50: Ethanol, lactic acid, and acetic acid yield from ensiling at 50 ˚C.
Ethanol is represented by white bars, lactic acid by grey bars, and acetic acid by black bars.
Error bars represent the mean of 5 biological replicates with standard error of the mean shown.
Replicates indicate distinct aliquots of the same progenitor biomass sample which underwent the same treatment *$p<0.05$.
Due to industrial constraints yield is given in moles per litre of effluent per gram of biomass (mol l^{-1} g^{-1}). An explanation of the derivation of this complex unit is given in the Materials and Methods section.

DISCUSSION

At Shell, *Sorghum bicolor* biomass is enzymatically hydrolysed to produce sugar monomers that undergo fermentation by *Saccharyomyces cerevisa*e to produce ethanol. Ethanol has a commercial value for Shell, primarily as a fuel additive, and maximising the efficiency of the ethanol producing processes employed at Shell is therefore financially imperative. The aim of this project was to explore and identify conditions which enabled maximum ethanol production from the Consolidated ensiling of *Sorghum bicolor* biomass. To achieve this, a range of cellulose degrading enzymes, commercial fermentative *S. cerevisae* strains, *Sorghum bicolor* cultivars and temperature ranges were individually analysed in Secondary ensiling to ascertain the most effective conditions for maximum second generation ethanol production. A range of contamination controls were tested in Primary ensiling to establish a cost effective method for the reduction of undesirable endemic bacterial populations present in *S. bicolor* biomass, which limit first generation ethanol production from this process. The Primary and Secondary ensiling conditions which offered the highest potential in ethanol yields were then applied to Consolidated ensiling, a process which produces both first and second generation ethanol in a single, unified process. As previously mentioned, due to a lack of uniformity in biomass cultivation and experimental set up, ethanol yields were highly variable. Deduced trends, patterns and recommendations for future work must therefore be taken as provisional.

Identification of a cost effective method for the reduction of biomass contamination

Biomass contamination was observed in all Secondary ensiling experiments. Microbial colonisation during the ethanol fermentation of lignocellulosic biomass is a fundamental obstacle in maximising ethanol yields (Beckner *et al.*, 2011, Hernandez-Heredia *et al.*, 2016). The dominant species identified in these microbial consortium include lactic and acetic acid producing bacteria, such as *Lactobacillus sp., Leuconostoc sp.,* and *Acetobacter sp.* (Camu *et al.*, 2007). Lactic acid and acetic acid production is therefore used as a proxy for microbial growth, and was measured during all Secondary ensiling experiments. To reduce bacterial contamination, antimicrobial strategies including the addition of sulphuric acid and the antibiotic, lactrol, are employed during ensiling at STCH. However, these strategies exhibit inefficiencies, including high cost, and detrimental environmental and health and safety risks (Rückle & Senn, 2006). Identification of cost-effective, safe antimicrobials could potentially reduce some of these issues associated with conventional contamination control. Essential plant oils and resins exhibit antimicrobial properties, and are used as conventional, safe, low cost preservatives in alcohol brewing (Preedy, 2016). The antimicrobial capacity of two essential oils, hop oil and wintergreen oil, were investigated in Primary ensiling (Figure 5).

Biomass was ensiled with combinations of sulphuric acid, lactrol, hop oil, and wintergreen oil, and ethanol and acid yields were assessed (Figure 5).

Compared to the control, addition of hop oil reduced total average lactic and acetic acid production (by up to 23 %; Figure 5) whilst addition of wintergreen oil resulted in no significant changes in acid yields. In contrast, addition of either sulphuric acid or lactrol reduced average lactic and acetic acid production (by up 150 % and 257 %, respectively; Figure 5). Only biomass treated with either sulphuric acid or lactrol showed notable, statistically significant increases in ethanol yields (Figure 5). Neither hop oil nor wintergreen oil were therefore successful in reducing microbial contamination and improving ethanol yields in comparison to the conventional antimicrobials (Figure 5).

Although the use of hops as antibiotic is abundant in the brewing industry (Hough *et al.*, 2012), it was found that only the a-acid and b-acid components of hop resins exhibit antimicrobial properties (Patzak *et al.*, 2015). Addition of hop oils, without resins, will therefore not reduce biomass contamination, and should not be used as an alternative to conventional ensiling antimicrobials (Figure 5). Future studies may explore the use of hop acids as antimicrobials, to determine if there is opportunity to reduce costs and environmental and health risks associated with conventional ensiling microbial control methods.

Identification of efficient lignocellulose degrading enzymes

In order to maximise ethanol production from Consolidated ensiling, each of the ensiling components needed to be determined to achieve the maximum yield. The efficiencies of a range of lignocellulose degrading enzymes were explored in Secondary ensiling (Figure 6). Ethanol production on addition of different loadings of Lipase, Viscozyme, Amylase, and Pectinase, in combination with conventional ensiling enzyme CTec3, was assessed (Figure 6). These enzymes catalyse the breakdown of lipids, cellulose, starch and pectin, into fermentable non-reducing sugars, (Sayali *et al.*, 2013, Johansen, 2016).

Addition of Pectinase resulted in the largest significant percentage increase in ethanol production (up to 3,495 %; Figure 6E), in comparison to addition of CTec3 only (Figure 6E). However, it is important to note that the control (addition of CTec3 only) produced an unusually low ethanol yield (0.05 mol l^{-1} g^{-1}). Pectin constitutes up to 50 % of dry plant biomass, and is a major structural component of the plant cell wall (Scheller *et al.*, 2007). It forms an extensive bonding network with lignin and hemicellulose, creating a protective polysaccharide ‘cage’

around cellulose micro fibrils (Dick-Perez *et al.*, 2011). Extraction of pectin has been shown to increase monosaccharide yields from cellulose during processing of hemp (Pakarinen *et al.*, 2012), tobacco, and wheat (Lionetti *et al.*, 2010). Based on this evidence, it appears that during ensiling Pectinase-catalysed pectin degradation may not only produce monosaccharides for fermentation directly, but also enable further CTec3 access to cellulose, thereby enhancing ethanol production. Due to the variable yields from the inter-experimental controls, future work should repeat this study to validate effects of addition of pectinase during ensiling.

Addition of Lipase (Figure 6B), Viscozyme (Figure 6C), and Amylase (Figure 6D) resulted in no significant increases in ethanol production. As Amylases are commercially employed across several industries for the efficient hydrolysis of starch to monosaccharides (de Souza & e Magalhães, 2010), and plant biomass contains up to 80 % starch on a dry weight basis (Pfister & Zeeman, 2016), it was expected that amylase addition would result in significant increases in ethanol production. However, on addition of Amylase, ethanol yields decreased (by up to 35 %; Figure 6D) in comparison to addition of CTec3 only. Starch is a non-structural polysaccharide, and is not bound within the lignocellulose matrix (Liu *et al.*, 2018). As only the lignocellulosic portion of biomass is fermented during Secondary ensiling, it is likely that little or no starch was available for hydrolysis by Amylase, hence addition of amylase had no significant positive impact on ethanol yields (Figure 6D). This hypothesis is supported by composition studies of *Zea mays* (corn), which showed that plant starch content drops from 70 % (w/w) to below 6 % (w/w) during production of first generation ethanol from nonlignocellulosic biomass (Han & Liu, 2010). The remaining low levels (6 % (w/w)) of starch in lignocellulosic biomass are unlikely to have significant impact on yield, even if fully hydrolysed.

Plateaus in ethanol yields occurred past addition of 0.5x or 1.0x for every enzyme, with no significant differences in ethanol production observed (Figure 6). As mentioned before, there is a limited availability of accessible cellulose in *Sorghum bicolor* biomass. The majority of cellulose is present either in crystalline form or trapped within a matrix of recalcitrant lignin polymers. Ethanol plateaus in Figure 6 suggest that at 1.0x, maximum amount of accessible cellulose had been hydrolysed and fermented to ethanol, and increasing enzyme addition past this concentration only served to saturate the limited accessible cellulose further. Observed ethanol yield plateaus are industrially significant: for all enzymes other than viscozyme, only half the manufacturer's dose (0.5x) is required to achieve maximum ethanol production. Halving enzyme doses offers the opportunity to substantially reduce CAPEX costs associated with the conventional ensiling process.

Determination of an optimal Sorghum bicolor cultivar

Selection of a high sugar, highly digestible *Sorghum bicolor* cultivar is imperative in maximising ethanol production from Consolidated ensiling. Ethanol production from four available *S. bicolor* cultivars M81E, Topper 76-6, Brown Mid-Rib (BMR) and N6G60 was assessed in Secondary ensiling (Figure 7).

Ensiling of Topper 76-6 produced the highest ethanol yield in comparison to other cultivars ensiled, at up 102 %, 1,313 %, and 46 % higher than M81E, BMR and N6G60, respectively (Figure 7). Topper 76-6 is a genetically engineered modern hybrid variety of *S. bicolor* (Kuepper *et al.*, 2008). Characteristics of Topper 76-6 include 14 % higher stalk yield (25.6 tons acre^{-1}), comparable disease rating (1.0), 32 % higher sugar production (2439 lbs acre^{-1}), 21 % higher brix (16.0 ˚B) (Day *et al.*, 1995), and 2 % reduction in lignin content compared to M81E, as previously shown at STCH. These characteristics appear to contribute favourably towards Topper 76-6 lignocellulose degradation, hydrolysis and fermentation during Secondary ensiling (Figure 7).

No significant difference in ethanol yields for M81E and N6G60 were observed (Figure 7). BMR produced the lowest ethanol yield (0.208 mol g^{-1}) in comparison to M81E (1.81 mol g^{-1}), Topper 76-6 (2.94 mol g^{-1}), and N6G60 (2.02 mol g^{-1}) (Figure 7). As BMR is genetically engineered to exhibit faulty monolignol synthesis-derived reduced lignin content (Sattler *et al.*, 2010, Li *et al.*, 2015), and has been previously shown to produce increased ethanol compared to wildtype *S. bicolor* during pre-treatment (Dien *et al.*, 2009), it was expected that BMR would produce the highest ethanol yields from ensiling. However, BMR resulted in a significant 89 % yield decrease in comparison to M81E (Figure 7). All five BMR replicates were contaminated with an unknown white fungus. Many species of filamentous fungi hydrolyse lignocellulose via secretory hydrolytic enzymes activity, and consume the resultant sugar products as a nutrient source (Couturier *et al.*, 2012). It appears that here, the unknown fungus consumed the majority of accessible cellulose, thereby limiting substrates for ethanol production from Secondary ensiling. Due to contamination, repetition of the Secondary ensiling of BMR is necessary to assess its true digestibility in comparison to alternate cultivars.

As some fungi are currently being investigated for their use as low-energy biomass pretreatment methods (Wan & Li, 2012), it may be of use to classify the biomass endemic fungi described in this experiment. Utilising endemic fungi may reduce costs associated with creating an adequate nutrient environment for a non-biomass native fungal species during

pre-treatment. To further classify the contaminating fungal species a DNA extraction from the bulk ensiling samples could performed. Subsequent DNA sequencing and taxonomic classification of the sequenced DNA would achieve accurate classification (Raja *et al.*, 2017).

Selection of a robust *Saccharomyces cerevisae* strain

Selection of a robust *S. cerevisae* strain able to cope with the hostile ensiling environment is essential to maximise ethanol production from Consolidated ensiling. Ethanol production from ensiled biomass using three commercially available *S. cerevisae* strains LSY, C6 FUEL and AH130, was assessed (Figure 8).

Ensiling with C6 FUEL produced the highest ethanol yield in comparison to other yeasts ensiled, at 20 % and 167 % higher in comparison to LSY and AH130, respectively (Figure 8). C6 FUEL is a highly concentrated (2.0×10^{10} cells $gram^{-1}$), chemically stabilised, active dry yeast strain, selected specifically for use in solid fermentations (Lallemand Biofuels and Distilled Spirits, USA). C6 FUEL is designed to withstand high temperatures (up to 40 ˚C), wide pH ranges (3.5 – 6.0) and high alcohol concentrations (20 % (w/v) solution). C6 FUEL has a significantly longer shelf life (36 months) at which yeast cells still remain viable, compared to LSY (3 months), a form of fresh liquid yeast designed for use in liquid beverage fermentations (Lallemand Biofuels and Distilled Spirits, USA). It appears that the adaptation of C6 FUEL to solid fermentations, like ensiling, and its extensive viability make it a more robust strain for use in Secondary ensiling in comparison to LSY.

Ensiling with AH130 produced the lowest ethanol yield (0.802 mol g^{-1}) in comparison to LSY (1.79 mol g^{-1}), and C6 FUEL (2.14 mol g^{-1}) (Figure 8). AH130 is a strain of *S.cerevisae* genetically engineered to consume C5 and C6 sugars (Zhang, 2014), so was expected to produce higher ethanol yields due to increased substrate volume. However, ensiling with AH130 resulted in significant percentage decreases in comparison to LSY and C6 FUEL (Figure 8). The culture of AH130 was grown up from a glycerol stock of unknown age, and was not able to reach an OD comparative to that of LSY or C6 FUEL, indicating a lower yeast culture concentration. It appears that the smaller AH130 yeast population was unable to ferment equal volumes of sugar as LSY or C6 FUEL, resulting in lower ethanol yields (Figure 8). In future studies, in order to compare population size and cell viability, methylene blue staining may be used (Kwolek-Mirek & Zadrag-Tecza, 2014).

Optimal yeast activity temperatures

Selection of an ensiling temperature which balances yeast and enzyme activity requirements is necessary to achieve maximum ethanol production from Consolidated ensiling. Four experimental temperatures 20, 30, 40 and 50 ˚C were trialled in Secondary ensiling, and ethanol production was assessed (Figure 9).

Ensiling at 30 ˚C produced the highest ethanol yield in comparison to ensiling at 20, 40, and 50 ˚C, incurring yield increases of 44 %, 149 % and 572 %, respectively (Figure 9). The optimum growth temperature for *S. cerevisae* is 32 ˚C (López-Malo *et al.*, 2013), and deviations from this optimum result in destabilisation of sugar transport systems found in the yeast cell plasma membrane, causing subsequent intracellular toxin build up, loss of cell viability, and reduced fermentation rate (Spencer-Martins, 1994, Bisson, 1999, Ho & Powel, 2014, Bhadana & Chauhan, 2016). The yeast in this experiment, LSY, is recommended for use at 34 ˚C (Lallemand Biofuels and Distilled Spirits, USA). Results from this study, which show the highest ethanol yield (2.21 mol g^{-1}) in comparison to 20 (1.53 g mol^{-1}), 40 (0.889 mol g^{-1}) and 50 ˚C (0.352 mol g^{-1}) indicate that ensiling at 30 ˚C is required for maximum ethanol outputs from Secondary ensiling.

Ensiling at 50 ˚C produced the lowest ethanol yield (0.352 mol g^{-1}) in comparison to other temperatures trialled in this study (Figure 9). The optimum temperature for activity of CTec3, the conventional ensiling enzyme additive, is 50 ˚C (Novozymes, Denmark). At temperatures above or below 50 ˚C, substrate-enzyme hydrolysis kinetics, rate of reaction, and product yields are reduced (Novozymes, Denmark). However, it appears that at 50 ˚C, the yeast population has been rendered unviable, and can no longer ferment sugars, regardless of the potential increase in monosaccharides derived from more efficient enzyme activity.

In order to balance both enzyme and yeast optimum, a thermotolerant yeast with a similar fermentative capacity to *S. cerevisae* needs to be identified. Future studies could investigate species such as *Kluveyveromyces marxianus* and *Arxula adeninivorans,* which grow at approximately 45 ˚C (Urit *et al.*, 2013, Calandra *et al.*, 2016), and may be capable of sugar metabolism at higher temperatures more ideal for efficient CTec3 activity. Thermo-tolerance is also necessary during field ensiling, where silo temperatures can reach 50 ˚C (Jiang & Jofriet, 1988). It is likely that with current yeast species, only a small proportion of the yeast population added is actually functional.

Testing of defined maximal ethanol production conditions in Consolidated ensiling

The efficiencies of lignocellulose degrading enzymes, *Sorghum bicolor* cultivars, commercial *Saccharomyces cerevisae* strains and a range of ensiling temperatures were evaluated in Consolidated ensiling (Figures 10 - 13).

Biomass was ensiled with increasing doses of CTec3, ranging from 0.5x to 15.0x (Figure 10A). No significant difference in ethanol yields was observed between 0.5 and 6.0x CTec3 additions, and it was concluded that 0.5x was the most efficient dose for maximal ethanol production. On addition of 15.0x CTec3, no ethanol was produced, which was attributed to yeast osmotic stress, subsequent metabolism arrest and yeast cell death derived from the high sugar environment (Radmaneshfar *et al.*, 2013). Biomass was ensiled with pectinase, amylase, and viscozyme independently, and in combination with CTec3 (Figure 10B, 10C). There was no experimental combination of enzymes which enhanced ethanol yields in comparison to conventional treatment (1.0x CTec3). The observed plateau of ethanol yields on increased enzyme loading was attributed to enzyme-substrate rate saturation (Michaelis et al., 2011).

Ensiling the *S. bicolor* cultivar Topper 76-6 produced the highest ethanol yields in comparison to the other varieties M81E, N6G60, and Topper 76-6 (Figure 11). The success of Topper 766 in Consolidated ensiling was ascribed to its high sugar content (Day *et al.*, 1995). *S. cerevisae* strains LSY and C6 FUEL performed equally well in Consolidated ensiling, with no significant differences between ethanol yields for either yeast, however, ensiling with AH130 decreased ethanol yields by 74 % in comparison (Figure 12). The poor performance of AH130 in Consolidated ensiling was accredited to its comparatively low ensiling concentration. Ensiling at 20, 30, and 40 ˚ C proved equally successful, with no significant differences between ethanol yields (Figure 13). No ethanol was produced from ensiling at 50 ˚C, which was attributed to the disruption of temperature dependant intracellular processes, and subsequent yeast death (Figure 13) (Spencer-Martins, 1994).

In comparison to Secondary ensiling, Consolidated ensiling showed much lower levels of microbial contamination. The two step Primary – Secondary process involves removal of biomass from the ensiling chamber, biomass drying, and re-ensiling. This step potentially introduces environmental sources of contamination which are avoided during the one step Consolidated ensiling process.

The Consolidated process was designed and implemented to increase ensiling efficiency, by producing first and second generation ethanol in a single, cost-effective process. There was increase in the ethanol yield from Consolidated ensiling when compared to individual Primary and Secondary ensiling, indicating it's potential as a cost effective, unified ensiling process. Furthermore there was an overall reduction in the bacterial contamination observed, allowing more sugar to be fermented to ethanol rather than unprofitable products.

This single factorial analysis has laid the groundwork for a more complex multifactorial experiment using a design of experiments (DoE) approach. The first iteration would include factors such as biomass cultivar, biomass cultivation location, temperature, irrigation, nutrient supplementation, ensiling chamber size, and ensiling fermentation period. Controlling these factors may not represent a "real world" industrial ensiling scenario, but would enable development of a standardised protocol which could produce more uniform yields from ensiling. A second DoE iteration would include the operating ranges defined here for antimicrobial strategy, lignocellulose degrading enzyme, biomass cultivar, fermenting yeast species and ensiling operating temperature, in order to determine "absolute" optimal ensiling conditions in a standardised, repeatable experimental set up.

CONCLUSION

This project aimed to identify optimal Secondary ensiling conditions when using a range of enzyme packages, *Sorghum bicolor* cultivars, commercial *Saccharomyces cerevisae* strains, and operating temperatures. Once optimum conditions had been identified they were applied to a Consolidated ensiling process to assess the ability to produce ethanol from biomass in a more cost-effective and efficient process than the two-stage process currently implemented at STCH. The most effective enzyme was CTec3 at a dose of 0.5x, when utilised with *S bicolor* topper 76-6, *S. cerevisae* C6 FUEL, at an operating temperature of 30 ˚C. Applying these optimal conditions to Consolidated ensiling resulted in a maximum ethanol yield of 9.03 $\pm$ 0.3 mol l^{-1} g^{-1}, in comparison to the maximum ethanol yield from the Primary-Secondary process of 7.44 $\pm$ 0.159 mol l^{-1} g^{-1}. Within the statistical error, utilising the Consolidated process could offer the opportunity to increase ethanol yields from ensiling by up to 28 %. These findings have the potential to reduce the OPEX and CAPEX of secondary biofuel production for Shell, whilst potentially increasing ethanol yields, making the production of ethanol more profitable than the current ensiling process.

In order to achieve the maximum potential of Consolidated ensiling, a lignin degrading enzyme needs to be identified that would enhance the availability of cellulose to the CTec3 enzyme. In addition a more thermo-tolerant yeast species, which is capable of fermentation at ensiling temperatures of 50 ˚C would allow the cellulose degrading enzymes to operate at their optimal rate of conversion. Fundamentally, the ensiling process, from biomass cultivation to fermentation, requires standardisation, and experimental variability must be reduced before accurate conclusions can be drawn from ensiling studies.

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APPENDIX

Agricultural parameters

Location

Three agricultural locations were commissioned by Shell for the production of *S. bicolor.* These locations where: Rio Farms, located in Monte Alto county in Texas state, and University of Florida Institution for Agricultural Studies (UF IFAS), located in Gainesville county in Florida state.

Soil type

At Rio Farms the soil type was sandy loam clay. In UF IFAS the soil type was candler sand.

Crop Variety

At Rio Farms cultivated *S. bicolor* varieties were M81E and N6G60. In UF IFAS cultivated *S. bicolor* varieties were M81E, Topper 76-6, N6G60, and forage sorghum variety Brown MidRib (BMR).

Seed Supplier

S. bicolor variety M81E seeds were supplied by Mississippi State University Seed Programme (MSUSP) (Broadhead et al., 1981), Topper 76-6 seeds were supplied by MSUSP (Broadhead et al., 1981), N6G60 seeds were supplied by NexSteppe (NexSteppe, USA) and BMR seeds were supplied by Richardson Seeds (Richardson Seeds, USA)

Growth period

At both Rio Farms and UF IFAS, *S. bicolor* varieties were grown for 16 weeks.

Fertilizer application

No fertilizers were applied during the growth of any *S. bicolor* variety in any location.

Pesticides

At UF IFAS, *S. bicolor* varieties were treated with Transform® WG (Dow Agrosciences, USA) No pesticide application was recorded at Rio Farms.

Irrigation

At Rio Farms rainwater was the only form of irrigation. At UF IFAS, pivot type of unrecorded volume irrigation was used. Maximum precipitation at Rio Farms was 8.0 mm. Maximum precipitation at UF IFAS was 7.8 mm.

Harvester type and chop length

At both locations, a Claas 960 self-propelled forage harvested with a chop length or 1.27 cm (0.5 inch) (Crisp, USA) and a John Deere Self-Propelled Forage Harvester (John Deere, USA) were used.

Yeast cultivation

The Codexis ethanologen *Saccharomyces cerevisiae* AH130 was grown overnight from a glycerol stock in liquid pre-culture (35 ml) of yeast peptone dextrose (YPD) media, in a vented, baffled flask (125 ml), and incubated in a Multitron Pro Incubator (32 ˚C, 24 hrs., 160 rpm) (INFORS, USA). YPD was prepared according to manufacturer's instructions. On occasion, YPD was also supplemented with xylose (50 ml, 200 g l^{-1}). Growth curves of AH130 were carried out via pre-culture inoculation of fresh media (100 ml) in a vented, baffled flask (250 ml), prior to incubation (32 ˚C, 24 hrs., 160 rpm). OD of the culture was measured using a GENESYS™ 20 Visible Spectrophotometer (600 nm), and HPLC samples were taken at two hour intervals during a twelve-hour period of growth to assess yeast viability and sugar consumption.

The AH130 pre-culture was then used to start a large culture (15 ml AH130, 300 ml YPDX) in a vented, baffled flask (1 l) which was incubated (32 ˚C, 12 hrs., 160 rpm). Post-incubation, the culture was centrifuged at 6000 x g, for 15 mins, at 10 ˚C. Supernatant was discarded and pellet was re-suspended in PBS to reach a target OD (200 AU). Volume of re-suspended culture added to ensiling was calculated based on standard SLY loading.