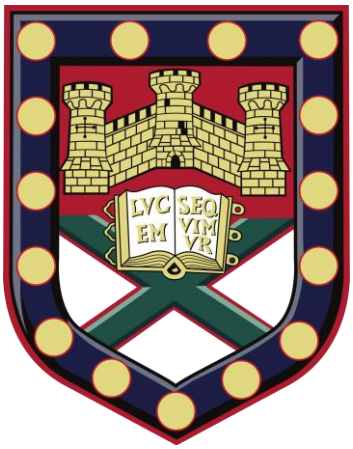

Establishment of an Ensiling System for the Production of Lactic Acid from *Sorghum bicolor* (Sweet Sorghum) Biomass

Submitted by Chloe Brain to the University of Exeter as a thesis for the degree of Masters of Science by Research in Biological Sciences in December 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.

Abstract

Lactic acid is an economically important organic acid that has a wide range of applications in industry, including in the manufacture of sustainable and biodegradable plastic alternative poly-lactic acid (PLA). Transition towards a bio-based economy requires increased production of lactic acid and other industrially important chemicals from biomass. Lignocellulosic biomass is a preferred feedstock for production, as it is inexpensive and abundant, however the recalcitrant nature of lignocellulose makes its utilisation challenging. Therefore, development of a consolidated bioprocessing (CBP) system for efficient conversion of lignocellulosic biomass to value-added products is required.

Lactic acid is currently produced via fermentation of expensive carbohydrate-based feedstocks by lactic acid bacteria (LAB). Application of an ensiling system may overcome problems associated with current fermentation methods and enable conversion of lignocellulosic biomass to lactic acid via CBP. Ensiling is commonly used for the preservation of freshly harvested crops for animal feed, including sweet sorghum, which contains a high concentration of soluble sugar and lignocellulose. In this study, a series of lab-scale ensiling experiments were performed, in which the effects of various conditions and additives on ensiling product yields from sweet sorghum were evaluated. Addition of $CaCO_3$ was identified as an effective method of pH control, resulting in a 2.8 x increase in lactic acid production by endogenous LAB. Inoculation with two different species of LAB (*Lactobacillus plantarum* and *Pediococcus acidilactici*) and addition of a commercial cellulose enzyme package were demonstrated to have minimal influence on lactic acid yield compared to ensiling temperature and time. An ensiling temperature of 45 °C resulted in high lactic acid selectivity and potential degradation of some lignocellulose. However, due to variability between ensiling experiments, further investigation is required before optimal ensiling temperature and time can be confirmed for maximum production of lactic acid from sweet sorghum.

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ANOVA	Analysis of Variance
CBP	Consolidated Bioprocessing
CCR	Carbon Catabolite Repression
cfu	Colony Forming Units
cm	Centimetre
g	Gram
g	Acceleration due to gravity (=9.81ms^{-2})
h	Hour
HPLC	High Performance Liquid Chromotography
kg	Kilogram
l	Litre
LAB	Lactic Acid Bacteria
min	Minute
ml	Millilitre
mol	Mole
pH	Hydrogen potential

1. Introduction

1.1. Bio-based chemical production

The gradual transition towards a bio-based economy is key in reducing environmental pollution and lessening dependence on declining petroleum and coal based feedstocks (Jönsson and Martin, 2016). A variety of fuels and chemicals are currently produced from sugar or starch rich crops, which are a renewable and sustainable source of carbon. However, many of these crops are primarily food resources and so competition exists regarding land and energy use between food crops and those grown for alternative industries. This competition can result in food scarcity and price increases (Haq *et al*., 2016). Hence, there has been increased interest in the development of second-generation bio-products, in which non-food biomass is the basis for production. Potential feedstocks include lignocellulosic biomass, food waste, agricultural waste, and algal biomass (Abdel-Rahman and Sonomoto, 2016). Among these, lignocellulose is considered advantageous as it is the most abundant raw material on the planet (Wyman and Yang, 2008), and therefore offers a large scale and continuous supply for long-term bio-production. Furthermore, since most lignocellulosic biomass is considered as waste or byproduct it is inexpensive compared to other biomass (Perlack *et al*., 2005). Development of an efficient system for the conversion of lignocellulosic carbon to value added products, could transform the basis of fuel and chemical industries and improve efforts towards environmental sustainability.

1.2. Lactic acid demand and application

Lactic acid is one of the most economically important organic acids, with extensive industrial and biotechnological applications (Ghaffar *et al*., 2014). In 2013, global lactic acid demand was estimated to be 714.2 kilo tons and expected to grow annually by 15.5% to reach 1,960.1 kilo tons in 2020 (Abdel-Rahman and Sonomoto, 2016). The food industry accounts for 35% of demand, as lactic acid is essential in the production of fermented foods and is a commonly used acidulant and preservative, flavouring agent, pH regulator and bacterial inhibitor (Komesu *et al*., 2017). Lactic acid also plays a number of roles within the pharmaceutical and cosmetic industries, as well as in the chemical industry, in which lactic acid is converted to ethanol, propylene glycol and acrylic polymers. Polymer production accounts for the greatest proportion of demand. Thirty nine percent of lactic acid is used in the manufacture of poly lactic acid (PLA) (Komesu *et al*., 2017). PLA is a biodegradable alternative to petroleum-based plastics, with applications that include packaging and fibres, foams (Abdel-Rahman, Tashiro and Sonomoto, 2013) and roles in biomedical devices (Lasprilla *et al*., 2012). In 2013 PLA demand was estimated to be 360.8 kilo tons and

expected to grow annually by 18.8% to reach 1,205.3 kilo tons in 2020 (Abdel-Rahman and Sonomoto, 2016). Manufacture of lactic acid-based environmentally friendly solvents is another area for predicted growth as there is the potential to use lactate esters from alcohols with low molecular weights in formulation of pesticides (Sasson et al., 2005; Baur et al., 2008) and other bioactive components, due to its low toxicity. However, high costs associated with lactic acid production are limiting further solvent development as well as the manufacture of PLA (Wee *et al*., 2006). Despite increased PLA demand, it was estimated that only 450 million kg of PLA is produced annually compared to 200 billion kg of total plastics (Okano *et al*., 2010). If PLA is to compete with petroleum based plastics then the price must decrease by approximately half, which makes the targeted manufacture cost of lactic acid less than 0.8 US $/kg (Okano *et al*., 2010).

1.3. Lactic acid production

Lactic acid can be produced via petroleum based chemical synthesis or microbial fermentation. The preferred method is microbial fermentation due to use of renewable and sustainable feedstock, mild production conditions and low energy consumption (Reddy *et al*., 2015). Selection of an appropriate lactic acid producer for microbial fermentation can also result in the production of optically pure D- or L- isomers, which are required for specific applications (Grabar *et al*., 2006). Currently lactic acid production depends on expensive carbohydrates that compete with food resources (Abdel-Rahman, Tashiro and Sonomoto, 2013). Approximately 90% of commercially available lactic acid is produced by submerged fermentation of corn, with 70% of production cost due to corn feedstock prices (Abdel-Rahman, Tashiro and Sonomoto, 2013). Recently however, there has been investigation into production from inexpensive lignocellulosic waste, including corn stover (Hu *et al*., 2016; Jiang *et al*., 2016; Yi *et al*., 2016), oil palm empty fruit bunch, (Ye *et al*., 2014), rice straw (Kuo *et al*., 2015), hardwood (Hama *et al*., 2015), corncob residue (Bai *et al*., 2016), and sweet sorghum (Wang *et al.,* 2016). Production from these types of feedstocks involves overcoming current challenges associated with optimising fermentation conditions, in addition to those related to accessing carbon available within lignocellulose.

1.3.1. Fermentation mode

Key factors affecting lactic acid yields during fermentation include selection of the biocatalyst, fermentation temperature, pH and period, nutrient availability and substrate and product concentration (Ding and Tan, 2006; Hofvendahl and Hahn–Hägerdal, 2000; Ge *et* al., 2010; Upadhyaya, DeVeaux and Christopher, 2014; Abdel-Rahman and Sonomoto, 2016). Batch fermentation is the most widely used method for production, as highest conversion efficiencies and yields are achieved in comparison to continuous or fed-batch fermentation

(Abdel-Rahman, Tashiro and Sonomoto, 2013). Following batch fermentation all usable carbon is depleted, whereas in a continuous fermentation system residual carbon is always present. Continuous fermentation enables high dilution ratio and allows fermentation to be maintained for a long period of time (Komesu *et al*., 2017). However, when expensive feedstocks are involved, batch fermentation is preferred due to maximised lactic acid yield over greater volumetric production (John *et al*., 2007). As with all fermentation methods, there are limitations to batch fermentation, such as long fermentation periods and low cell densities (Abdel-Rahman, Tashiro and Sonomoto, 2013), and so investigation into optimum fermentation modes for specific feedstocks continues.

Currently, solid-state fermentation (SSF) is not performed industrially for lactic acid production, however there are a number of advantages associated with SSF, which could result in more efficient lactic acid production than submerged fermentation. SSF allows for higher final lactic acid concentrations, as lactic acid producers are less prone to inhibition as a result of high substrate concentrations at the start of fermentation (Singhania *et al*., 2009). Reduced requirement for instrumentation and equipment during SSF, also results in lower associated capital and operating costs (Martins *et al*., 2011), although ability to alter fermentation conditions is also reduced. Environmental benefits include less energy required for sterilisation and reduced susceptibility to bacterial contamination. Furthermore, SSF allows use of solid agro-industrial wastes as substrates in their natural form, and facilitates solid waste management, unlike submerged fermentation (Singhania *et al*., 2009).

1.3.2. Lactic acid bacteria

Selection of a suitable biocatalyst for the production of lactic acid depends primarily on the carbohydrate that is to be fermented, as a microorganisms metabolism differs depending on carbon source (Lunelli *et al*., 2010). Further desirable characteristics for industrial application include the requirement of minimal nitrogenous nutrients, production of high yields of stereo specific lactic acid at low pH and high temperature, and formation of minimal by-products (Srivastava, Narayanan and Roychoudhury, 2004).

Fermentative lactic acid production is currently driven by lactic acid bacteria (LAB), *Bacillus* strains, and some genetically modified bacteria, including *Escherichia coli* and *Corynebacterium* (Abdel-Rahman *et al*., 2013). LAB have a long history of industrial use. LAB are gram-positive bacteria that are facultatively anaerobic, non-motile and non-spore forming. Most LAB perform homofermentative fermentation of hexose sugars via the pentose phosphate pathway to produce lactic acid only. The alternative pathway, the phosphoketolase pathway is performed by heterofermentative LAB, and yields acetic acid,

ethanol and carbon dioxide as by-products (Abdel-Rahman, Tashiro and Sonomoto, 2013) (Fig. 1). Some LAB are facultatively heterofermentative, including *Lactobacillus planatarum,* which performs heterofermentation of hexose sugars under aerobic conditions (Castillo Martinez *et al*., 2013). By-product formation by heterofermentative bacteria reduces lactic acid yields and increases the costs of separation and purification (Guo *et al.,* 2014). As LAB have limited ability to synthesise B vitamins and amino acids, nutritionally rich media is required for growth, which results in further increases in production costs and hampers lactic acid recovery (Hofvendahl and Hahn–Hägerdal, 2000).

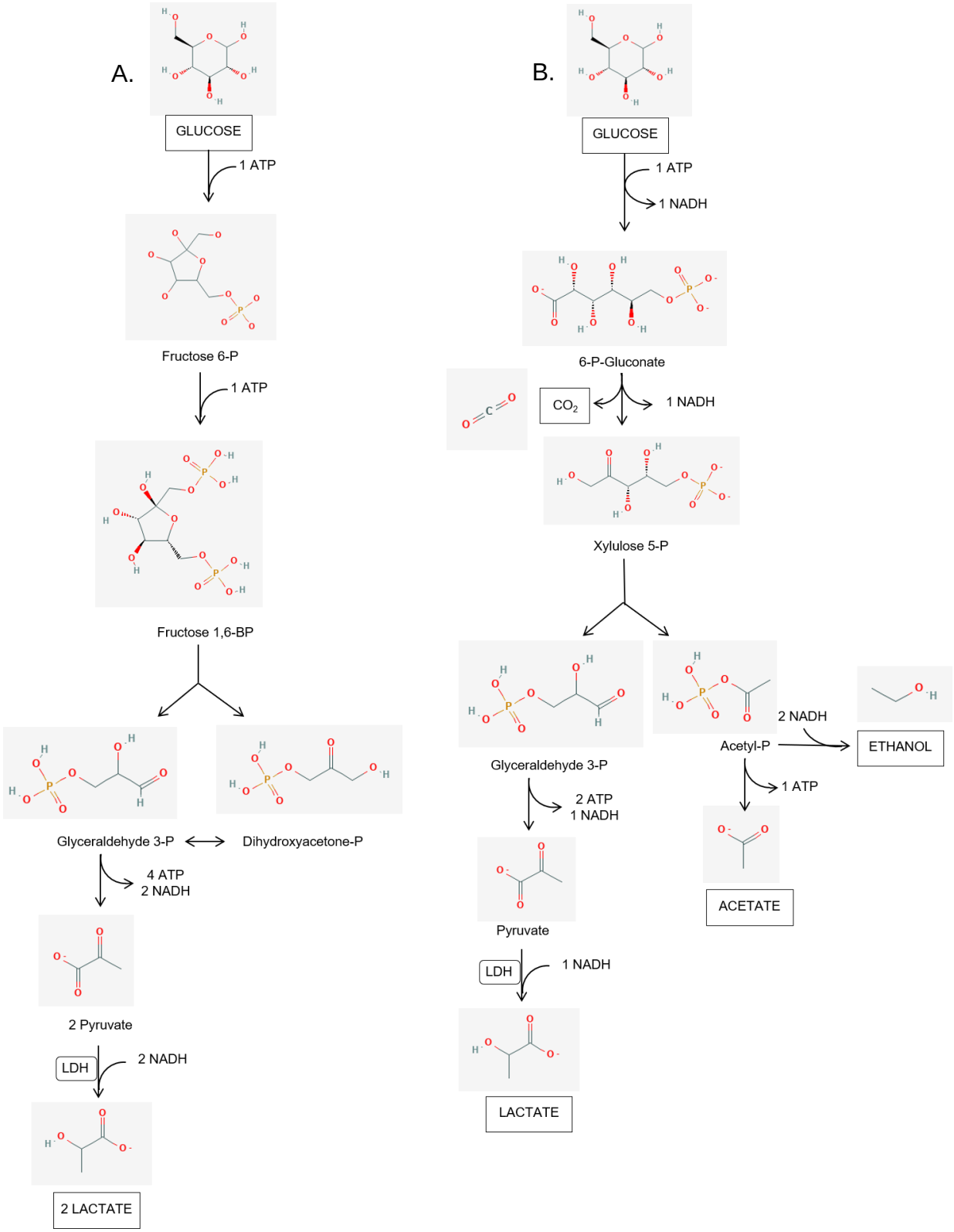


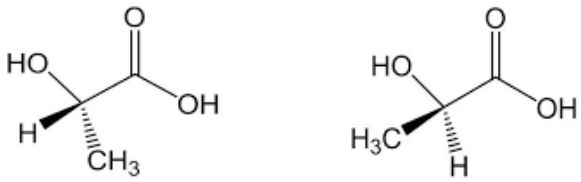
Figure 1. A schematic representation of (A.) homofermentation (pentose phosphate pathway) and (B.) heterofermentation (phosphoketolase pathway) of glucose to lactic acid with structural formulas shown (Adapted from Komesu *et al*., 2017).
P = phosphate, BP = biphosphate, and LDH = lactate dehydrogenase.

1.3.3. Separation and purification

Optically pure lactic acid isomers have greater value than a racemic mixture, as single isomers are required for specific applications (Komesu *et al*., 2017). L-lactic acid is preferred for applications in food and medicine, due to metabolic conversion of L-lactic acid in the body being faster than D-lactic acid (Komesu *et al*., 2017). It is also L-lactic acid that forms highly polymerised PLA, suitable for commercial use (Södergård and Stolt, 2002). Although many LAB naturally produce a single isomer, LAB that express two lactate dehydrogenase enzymes produce both L- and D- lactic acid isomers (Fig 2.). Some *Lactobacillus* species, produce the L-isomer, and following accumulation convert L-isomers into D-isomers, using lactate racemase, until equilibrium is reached and a racemic mixture is formed (Srivastava, Narayanan and Roychoudhury, 2004). Separation of racemic mixtures involves expensive high performance liquid chromatography techniques (Srivastava, Narayanan and Roychoudhury, 2004) and therefore LAB that produce single optical isomers are preferred for fermentation. *Lactobacillus* strains have the potential to be engineered for the selective production of a particular isomer (Benthin and Villadsen, 1995, Kyla-Nikkila *et al*., 2000). *L. plantarum* expresses both lactate dehydrogenase enzymes and lactate racemase, which is used as a rescue pathway for the production of D-lactic acid from L-lactic acid (Goffin *et al.,* 2005). When both the gene encoding D-LDH and the operon encoding lactate racemase were knocked out, production of D-lactic acid was completely inhibited, with the mutant exclusively producing L-lactic acid (Okano *et al*., 2018). However, growth rate of the double knockout mutant in D-lactic acid deficient media was reduced, as D-lactic acid is essential in the biosynthesis of peptidoglycan.

Between forty and seventy percent of operating and capital costs of lactic acid production are associated with the separation steps following fermentation (Wankat 2007). Common methods of separation include precipitation (Min *et al*., 2011, Kwak *et al*., 2012, Nokano *et al*., 2012), solvent extraction (Yankov *et al*., 2014; Alkaya *et al* 2009; Krzyzaniak *et al*., 2013), and membrane separation processes (Sikder *et al*., 2012; Pal and Day, 2013; Wang *et al*., 2014a). The number of downstream processing steps involved in separation and purification influences the price and quality of lactic acid (Idler *et al*. 2015). Lactic acid purity can be affected by several factors during fermentation, including temperature, pH, aeration, fermentation process and substrate (Ennahar *et* al., 2003; Tanaka *et* al., 2006; Tashiro *et* al., 2013). The cost of purification can be reduced when a pure substrate is used such as sucrose from sugarcane and sugar beet, however the high cost of such feedstocks make these unfeasible for use (Komesu *et al.,* 2017). Starchy materials like wheat and maize are advantageous as potential raw materials because these avoid inhibition of LAB as a result of high glucose concentrations (substrate inhibition) (Nakano *et al*., 2012). However,

physiochemical and/or enzymatic saccharification is required prior to their fermentation because most LAB are unable to utilise starchy materials directly (Narita *et al*., 2004). There have been successful examples of direct lactic acid production from starch by combination of efficient lactic acid producers and amylolytic enzymes (Narita *et al*., 2006; Okano *et al*. 2007). The α - amylase enzyme from *S. bovis* 148 degrades raw starch in the presence of a C-terminal starch-binding domain (Matsui *et al*., 2007). High yields of optically pure lactic acid were achieved from direct conversion of soluble starch, when pCUSαA from *S. bovis* was introduced into *L. lactis* strain IL 1403 (Okano *et al*., 2007). In addition, or as an alternative to engineered strains, the use of mixed strains in fermentation can provide useful combinations of metabolic pathways for the utilization of more complex materials for production of lactic acid (Cui *et al*., 2011; Kleerebezem and van Loosdrecht, 2007; Nancib *et al*., 2009; Taniguchi *et al*., 2004)



L-Lactic Acid D-Lactic Acid

Figure 2. A schematic representation of D - and L - lactic acid optical isomers showing their 3D orientations.

1.3.4. Effects of temperature and pH

Lactic acid production during fermentation results in increased pH, which has inhibitory effects on cellular metabolism and limits further lactic acid production (Grabar *et al*., 2006). pH can be controlled by extraction, adsorption or electrodialysis of lactic acid (Boontawan *et al*., 2011), although addition of a base, such as calcium carbonate, sodium hydroxide or ammonium hydroxide, is the most common method of pH control (Abdel-Rahman, Tashiro and Sonomoto, 2013). Several studies show that a pH below 5.7 is optimal for *lactobacillus* species (Hofvendahl and Hahn–Hägerdal, 2000) with optimal growth temperature ranging from 30 to 40 °C, although some thermophillic *Lactobacillus* strains grow well and have highly activated metabolism at around 45 °C (Dubernet *et al*., 2003). The mesophilic and neutrophilic nature of most LAB, increases the risk of contamination by non- lactic acid producing microbes and decreases fermentation efficiency (Abdel-Rahman *et al*., 2014). Furthermore, addition of neutralising agents for fermentation generates large amounts of gypsum during downstream processes (Abdel-Rahman and Sonomoto, 2016), which poses serious environmental problems and affects waste treatment (Vaidya *et al*., 2005). Temperate fermentation conditions also hamper simultaneous saccharification and fermentation of lignocellulosic biomass using hydrolytic enzymes, which require higher temperatures for optimum function (Abdel-Rahman, Tashiro and Sonomoto, 2013). *Enterococcus faecium* QU 50 was identified as a novel LA-producing strain that achieved efficient homofermentation (L-lactic acid yield, 1.0 g/g-consumed sugar) of different lignocellulose derived sugars at 50 °C at a controlled pH of 6.5 (Abdel-Rahman *et al*., 2014). However, further research is required to identify novel thermotolerant and acid tolerant strains or increase tolerance of existing strains in order to improve fermentation efficiency.

1.4. Lignocellulose structure

Further problems are encountered when a lignocellulosic feedstock is used for fermentation as fermentable sugars are not accessible due to the complex and recalcitrant structure of lignocellulose (Mohanty, Misra and Hinrichsen *et al*., 2000) (Fig. 3). Cellulose, hemicellulose and lignin form approximately 90% of lignocellulose dry weight (Balat, 2011; Yang *et al*., 2009), although exact structural and chemical composition of lignocellulose is a result of genetic and environmental factors, and how these factors interact (Demirbas, 2007). In general it can be assumed that lignocellulosic feedstocks contain approximately 40% of carbon bound as cellulose, 30% as lignin and 26% as hemicelluloses and other polysaccharides. Cellulose is a linear homopolymer composed of beta-linked D-glucose units (Mohanty, Misra and Hinrichsen *et al*., 2000). Cellulose polymers form bundles by intra- and intermolecular hydrogen bonding and these bundles aggregate into microfibrils. Linear covalent bonds and branching hydrogen bonds within microfibrils give cellulose its high

tensile strength and insolubility (Rubin, 2008). While cellulose is a uniform component of most types of lignocellulosic biomass, the proportions and composition of hemicelluloses and lignin differ between species (Fengel and Wegener, 1989; Sjöström, 1993).

Hemicellulose is a short, highly branched heterogenous polymer consisting of pentose (xylose and arabinose), hexose (galactose, glucose and mannose) and acid sugars (Saha, 2003). Hemicellulose of the plant family Pocaceae, which includes all temperate grasses and cereals, is comprised mainly of xylan-based structures with small amounts of xyloglucans and mixed linkage glucans (Scheller and Ulvskov, 2010). Although xylans are built on a backbone of xylose, they are a highly diverse group of polysaccharides. Xylans present in Poaceae have a high number of attached arabinose resuidues as well as glucuronic acids and 4-*O*-methyl glucuronosyl residues (Scheller and Ulvskov, 2010).

Lignin is considered as the glue that holds the lignocellulosic matrix together (Bajpai, 2006). It is primarily formed from three hydroxycinnamyl alchohols; p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Similarly to hemicellulose, lignin is a heterogenous polymer and includes a high diversity in series of linkages (Rubin, 2008). Lignin has been identified as a major deterrent to enzymatic and microbial hydrolysis of lignocellulose (Avgerinos and Wang, 1983) as lignin serves to protect cellulose and hemicellulose from any environmental stressors (He *et al*., 2017). By covalently linking to hemicellulose, lignin embeds into any spaces in the cell wall, which confers mechanical strength to the cell wall (Chabannes *et al*., 2001) and acts as a physical barrier to restrict access of cellulases to cellulose (Chang and Holtzapple, 2000). Protection of polysaccharides from enzymatic degradation by lignin is affected by degree and type of cross-linkage to polysaccharides, diversity of structures found in lignin composition and distribution of phenolic polymers throughout the cell wall (Laureano-Perez *et al.* 2005). Lignin content of the cell wall increases as the cell wall matures, which can significantly reduce pre-treatment efficiency (Hu and Ragauskas, 2012).

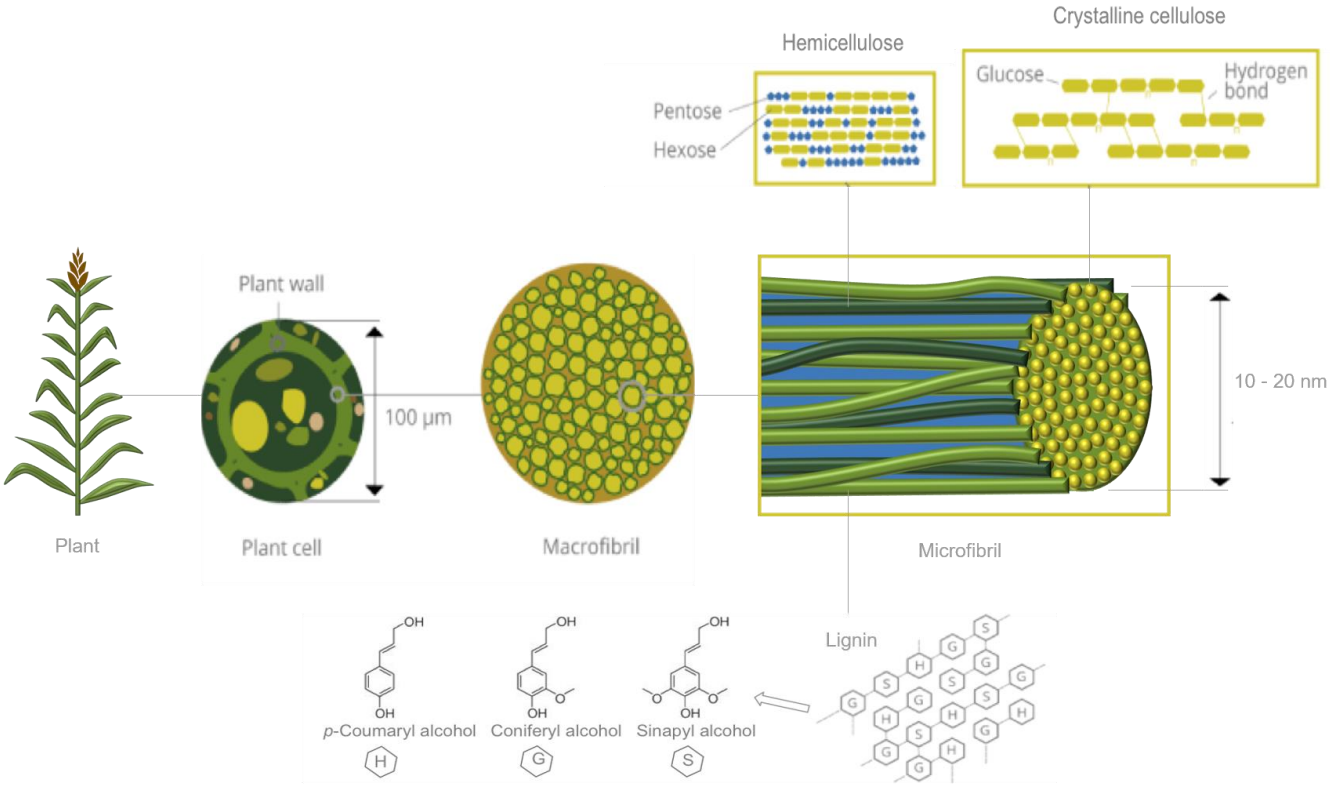


Figure 3. Structure of lignocellulose (Adapted from Streffer, 2014)

A schematic representation of the complex structure of lignocellulose. The molecular structure of cellulose, hemicellulose and lignin are shown. Hexagons represent lignin subunits; *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Cellulose microfibrils (light green) are surrounded by randomly organised hemicellulose (brown) and lignin fibrils (light green). Microfibrils are organised into macrofibrils that provide structural stability in the plant cell wall (Rubin, 2008).

1.5. Lignocellulose utilisation

Due to the complex structure of lignocellulosic biomass, pre-treatment and hydrolysis are required for high lactic acid production (Abdel-Rahman, Tashiro and Sonomoto, 2011). Pre-treatment aims to solubilise hemicellulose, lignin and crystalline cellulose (Van den Bosch *et al*., 2015), therefore making cellulose accessible for enzymatic hydrolysis, which yields fermentable sugars (Mosier *et al*., 2005). Pre-treatment includes physical (milling and grinding), chemical (alkali, dilute acid, oxidizing agents, and organic solvents), physicochemical (steam explosion, autohydrolysis, hydrothermolysis, and wet oxidation), and biological methods (lignin-hydrolysing Fungi, Bacteria and Archaea) (Abdel-Rahman, Tashiro and Onomoto, 2011; Kumar and Sharma, 2017). Highest sugar yields result from intensive

pre-treatment, however associated high temperatures, pressures and chemical loadings, require additional energy inputs and substantial capital and operating costs (Bensah and Mensah, 2013; Amin *et al*., 2017). Furthermore, it is not only fermentable sugars that are produced during pre-treatment. Some of the resulting by-products are known to inhibit the fermentation process by affecting cell growth, enzyme activities and lactic acid production (Abdel-Rahman and Sonomoto, 2016). These include phenolic compounds generated from lignin degradation, furan compounds generated from sugar degradation, aliphatic acids (acetic acid, formic acid and levulinic acid), inorganic ions and bio-alcohols (Zhang *et al*., 2016). Inhibitory effects can be reduced by detoxification using chemical additives or bio-abatement via microbial treatment (Larsson *et al*., 1999, Zhao *et al*., 2013). Product inhibition of cellulolytic enzymes by monosaccharides (glucose) and disaccharides (cellobiose), produced during pre-treatment and enzymatic hydrolysis, can be prevented by performing saccharification and fermentation simultaneously (Jönsson and Martin, 2016).

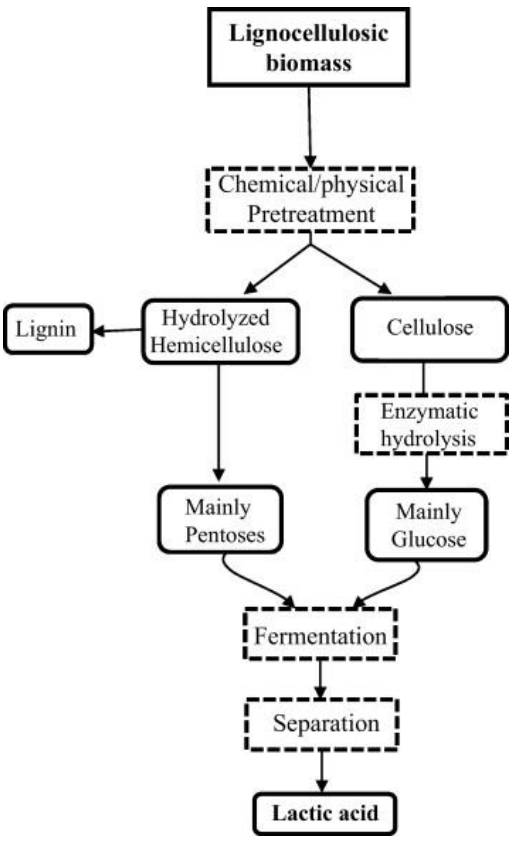


Figure 4. Conventional process for lactic acid production from lignocellulosic biomass. (Abdel-Rahman, Tashiro and Sonomoto, 2011)

Substrates and products are shown by a solid outline and processes by a dotted outline.

Hexose (mainly glucose) and pentose (mainly xylose) sugars are produced following the saccharification of cellulose and hemicellulose. Most lactic acid producers either cannot utilise xylose or demonstrate carbon catabolite repression (CCR), in which xylose is only consumed following glucose consumption (Abdel-Rahman and Sonomoto, 2016). This sequential sugar utilisation leaves most sugar unutilised, decreases the efficiency of fermentation and increases production costs (Wang *et al*., 2014b). Isolation of CCR negative strains (Reddy, Park and Wee, 2015; Abdel-Rahman *et* al., 2014; Abdel-Rahman *et* al., 2015) and genetic engineering approaches (Zhang *et al*., 2015) have been explored to overcome inefficient utilisation of mixed sugars. Yoshida *et al*. (2011) integrated the xylose assimilating pathway into *L. planatarum*, which enabled production of D-lactic acid from a mixture of 25 g L^{-1} xylose and 75 g L^{-1} glucose without CCR. A simpler strategy is through cultivation of mixed lactic acid producers. Co-culture of *L. rhamnosus*, which consumes glucose homofermentatively, and *L. brevis*, which consumes glucose and xylose heterofermantatively increased lactic acid yields during fermentation of NaOH pre-treated corn stover, by 18.6 % and 29.6%, compared with single cultures of *L. rhamnosus* and *L. brevis,* respectively (Cui, Li and Wan 2011). Co-cultivation generally achieves higher cell densities and improves lactic acid productivity (Nancib, Nancib and Boudrant, 2009), which is often affected by end product inhibition once lactic acid accumulates (Abdel-Rahman and Sonomoto, 2016). There is also evidence that cell immobilisation improves cell density and maintains long-term function of biocatalysts in addition to improving stability of the fermentation system (Li *et al*., 2015). Immobilisation of *Lactococcus lactis* increased LA productivity from 0.5 g/l/h in a free-cell system to 1.0 g/l/h in an immobilized cell system, using a Jerusalem artichoke hydrolysate substrate (Shi *et al*., 2012). Cell immobilisation is normally achieved by some form of physical entrapment and chemical adsorption (Shi *et al*., 2012), however cell immobilisation occurs naturally to some extent during solid state ensiling due to the non-motile nature of *Lactobacillus* species and absence of mixing in the system. Therefore similar effects on lactic acid productivity may be seen during solid-state fermentation as for cell immobilisation studies.

1.6. Ensiling for chemical production

Ensiling is a form of solid-state fermentation, which is commonly used within agriculture to preserve crops (Wilkinson, Bolsen and Lin, 2003). Silage is an essential part of a ruminant diet in most of the world, (Wilkinson, Bolsen and Lin, 2003) as ensiling allows year-round feeding of crops that have a short harvest window. During ensiling, freshly harvested wet biomass (usually shredded) is tightly packed into piles, which are covered with plastic sheeting to reduce oxygen intrusion (Gallagher *et al*., 2018). Endogenous LAB present ferment free sugars in the biomass to organic acids, which results in a decrease in pH to

around 4 (Van Soest, 1994; Duniere *et al*., 2017). Simultaneously, oxygen concentrations in the pile are depleted due to its use in microbial metabolism and the production of CO_2 and other fermentation gases, resulting in dilution and off-gassing (Gallagher *et al*., 2018). Reduced pH and oxygen concentration minimises further bacterial and fungal growth and prevents biomass degradation (Buxton, Muck and Harrison, 2003).

Ensiling has been investigated for the commercial production of ethanol, in which yeast inoculants were added to optimise fermentation towards ethanol production (Kitamoto *et* al., 2011, Gallagher *et al*., 2018). Potential exists for the production of other value added chemicals from biomass via optimisation of ensiling. As lactic acid is the major organic acid produced during the ensiling process (Duniere *et al*., 2017, Kung and Shaver, 2001) application of ensiling for the commercial production of lactic acid could be a cheaper alternative to current liquid-state lactic acid fermentations. Ensiling is not only less process-intensive but it bypasses a number of the previously discussed problems associated with liquid-state fermentations. Furthermore as ensiling relies on the endogenous microbial community in the biomass there is no requirement for sterile fermentation conditions. This avoids formation of furfural compounds, occurrence of the Maillard reaction and increased energy consumption associated with initial autoclaving of biomass (Abdel-Rahman, Tashiro and Sonomoto, 2013).

Silage fermentation is very dynamic and there are a number of factors that influence the biological process (Ambye-Jensen *et al*., 2014). These include ensiling conditions, initial biomass composition and selected additives. Although lactic acid is normally the major organic acid produced during ensiling, the presence of other endogenous bacteria can influence organic acid production. Enterobacteria are the principal competitors of LAB for available sugars in biomass during ensiling (Muck, 2010). Enterobacteria reduce nitrate to nitrite or nitrogen oxide, and so are the main source of gases produced during ensiling. Their principal fermentation product is acetic acid, which is also produced during heterofermentation by various LAB. Other organic acids produced include butyric acid and propionic acid (Kung and Shaver, 2001). Butyric acid concentrations depend on dry matter content of biomass as fermentation by Clostridia species results in butyric acid production and risk of Clostridial contamination increases as dry matter content decreases (Ambye-Jensen *et al*., 2014). Propionic acid is often only present in small concentrations (<0.2 – 0.3%) as most Propionibacteria are usually outcompeted (Kung and Shaver, 2001). The bacterial community present during ensiling can be manipulated via addition of chemicals and bacterial inoculums in order to control organic acid production (Cai *et al*, 1999; Yitbarek and Timir, 2014).

1.7. Ensiling as a pre-treatment

Ensiling of lignocellulosic material encounters the same problems regarding access to fermentable sugars, however there is evidence to suggest that in some cases ensiling can provide effect as a pre-treatment through degradation of hemicellulose. Dewar, McDonald and Whittenbury (1963) discovered the hydrolysis of hemicellulose during storage of perennial ryegrass, which was later supported following reduction in organic acids and pentose sugars associated with the hemicellulose structure (Morrison, 1979). Similar findings were reported during ensiling of wheat straw mixed with alfafa (Singh *et* al., 1996) and ensiling of orchardgrass (Yahaya *et al*., 2002). Ensiling of barley, triticale, wheat straws and cotton stalk significantly increased the conversion of hemicellulose and cellulose to sugars during subsequent enzyme hydrolysis, from 19-24% for untreated biomass to 24-34% for ensiled biomass (Chen, Sharma-Shivappa and Chen, 2007). Lignocellulose degradation during ensiling is highly dependent on type of biomass; the same silage treatment can result in biomass being almost unaffected while other biomass undergoes large structural changes and therefore high pre-treatment effect. Compared to conventional pre-treatment methods, ensiling requires a longer reaction time but due to ambient temperatures and pressures, requires less energy and lower capital costs (Chen, Sharma-Shivappa and Chen, 2007).

1.8. Consolidated Bioprocessing

Consolidated bioprocessing (CBP), in which pre-treatment, enzymatic hydrolysis and fermentation are performed in one step without enzyme addition, has been a major area of research (Lynd *et al*., 2005, ref required). CBP would reduce the requirement of physical and chemical inputs, offering a lower-energy, lower-cost route to fermentation of lignocellulose (Bhatia, Johri and Ahmad, 2012). However, engineering of a single microbe or design of a microbial consortia able to utilise insoluble lignocellulosic components and produce desired product at commercially attractive yields and titers remains challenging (den Haan *et al*., 2015, Minty *et al*., 2013, Peng, Gillmore and O'Malley, 2016). As there is already evidence that ensiling can provide effect as a pre-treatment, identification of the endogenous microbes responsible for lignocellulose degradation could provide a basis for CBP. Establishment of an efficient CBP system for lactic acid production from lignocellulose will not only transform the lactic acid industry but will provide a basis for the production of other value added products, including ethanol, from lignocellulosic biomass.

CBP using a microbial consortium as opposed to a single engineered microbe more closely reflects natural lignocellulose conversion systems and can result in extremely efficient substrate utilisation and increased product yields (Sabra *et al*., 2010). Most progress in consortia-based bioprocessing to date is based on a 'bottom-up' approach, where synthetic

communities of microorganisms have been constructed to achieve specific goals (Peng, Gillmore and O'Malley, 2016). Shahab *et al*., (2018) achieved co-cultivation of cellulolytic anaerobic fungus *Trichoderma reesei* with facultatively anaerobic LAB through a specially developed membrane reactor that enabled contrary oxygen requirements. The artificial cross-kingdom consortium produced 85.2% theoretical maximum of lactic acid from non-detoxified steam-pretreated beechwood. Although the study establishes potential of an engineered microbial consortium for CBP of lignocellulosic biomass, the requirement of steam pre-treatment and the complex fermentation set-up make application economically unfeasible. In order to effectively degrade non-pretreated lignocellulosic biomass, a larger microbial consortia is likely to be required, hence a 'top-down' CBP approach may be advantageous as these microbial systems display increased stability and resilience (Peng,Gilmore and O'Malley, 2016). The 'top-down' approach is also not limited by the fact that more than 99% of microorganisms have not yet been cultured or genomically characterised (Pace 1997; Peng,Gilmore and O'Malley, 2016). Production of biogas from anaerobic digestion is an example of 'top-down' application of natural consortia. Although there is limited knowledge of microbes involved in such consortia, recent advances in sequencing technology are allowing a greater understanding of microbial composition and diversity of natural consortia. In this study a 'top-down' approach has been applied for production of lactic acid based on the natural microbial consortia present during sorghum ensiling.

1.9. Sweet Sorghum as a feedstock

Sorghum (*Sorghum bicolor*) is a C4 crop that provides grain, sugar, and lignocellulosic resources (Li *et al*., 2013). A variety known as sweet sorghum is considered one of the most efficient crops to convert atmospheric CO_2 into sugar (Janssen *et al*., 2010). Forty percent of sweet sorghum dry weight is comprised of readily fermentable sugar, which makes sweet sorghum an attractive feedstock for fermentation (Himmel, Baker and Overend, 1994). There are a number of advantages associated with the growth of sweet sorghum. These include: adaptability to a wide range of environmental conditions (climate and soil conditions), low fertiliser requirement, high water-use efficiency compared to other crops (1/3 of sugarcane, ½ of corn) and short growth period of 3-5 months (Ratnavathi *et al*., 2011). These advantages allow sweet sorghum to be planted on marginal lands, which reduces competition with food crops (Matsakas and Christakopoulos, 2013). Sweet sorghum also provides the opportunity for production of food or feed from grain as well as bio-products from juice and lignocellulosic portions, which further eliminates food vs fuel related issues (Chiaramonti *et al*., 2002). Ensiling of sweet sorghum for animal feed is usually performed directly following harvest, however ensiling is also performed using sorghum stover (stalks

and leaves) following use of grain for human consumption (Mehra *et al*., 1989), or using bagasse (stalk residue) following juice extraction for syrup or ethanol production (Bernardes *et al*., 2015, Conway, Wilson and Conway, 2012). Ensiling of sweet sorghum for chemical production via consolidated bioprocessing would see all types of biomass utilised in one process. Free sugars in the juice would initially be fermented by LAB, followed by fermentation of sugars made available by the degradation of lignocellulose. Conversion of sweet sorghum to lactic acid would therefore result in no waste products and the generation of high value products from stover and bagasse, which currently have no other use apart from as animal feed.

2. Aims and Rationale

The aim of this project was to optimise the ensiling process for the production of lactic acid from sweet sorghum. The first objective was to establish optimum conditions for fermentation of free sugars to lactic acid, the second was to enable fermentation of the lignocellulosic biomass to lactic acid through a consortia based CBP approach. To achieve these objectives, ensiling conditions (time and temperature) and use of ensiling additives were explored. Additives used were $CaCO_3$ to control pH, *L. plantarum* inoculant to increase the LAB population and CTec3, a commercial enzyme package, for the hydrolysis of cellulose and hemicellulose.

After consideration of potential LAB to use as an inoculant, *L. plantarum* DSM20174 was selected due to previous reports of inoculation with *L. plantarum* having beneficial effects on silage quality as a result of lactic acid production (Ely, Sudweeks and Moon, 1981; Hu *et al*., 2009; Sifeeldein *et al*., 2018). For this reason *L. plantarum* is a common component of commercial silage inoculants (Khota *et* al., 2017; Matejčeková *et al*., 2018). *L. plantarum* is facultatively homofermentative and can grow in both aerobic and anaerobic environments and is able to ferment both pentose and hexose sugars (Morais *et al*., 2013). It is commonly isolated from plant material (Laskin, Sariaslani and Gadd 2010), including sweet sorghum (Daeschel, Mundt and McCarty, 1981). Therefore inoculation with *L. plantarum* avoids introduction of exogenous bacteria, which may have unpredicted effects on community dynamics or may be unable to survive under given environmental conditions. *L. plantarum* was selected due to its long history of use in industry (Teusink and Smid, 2006) and extensive knowledge of its physiology and genetics (Hui and Khachatourians, 1995). Furthermore an efficient transformation system has been established for *L. plantarum* (Bringel and Hubert, 1990; Teresa Alegre, Carmen Rodríguez and Mesas*,* 2004) so there is potential for further improvement of the ensiling system via introduction of beneficial genes from other bacteria.

A preliminary experiment in which *L. plantarum* DSM20174 was cultured in MRS media for 24 h at 27, 32, 37, 42 and 45 °C showed that *L. plantarum* reached highest OD at 37 °C, despite DSMZ recommendations of 30 °C. *L. plantarum* culture reached an OD of approximately 3.5 at 37 °C, which was significantly higher than for all other tested temperatures. As *L. plantarum* was to be used as an inoculant during ensiling experiments 37 °C was selected as the ensiling temperature for all initial ensiling experiments. A number of previous studies have showed optimum growth of *L. plantarum* strains and other *Lactobacillus* species at 37 °C during fermentation (Noori, Ebrahimi and Jafari *et al.* 2016;

Matejčeková *et al.* 2018; Pelikánová, Liptáková and Valík, 2015). As the highest optimum growth temperature of the *Saccharomyces* genus was demonstrated to be 32.3 °C (Salvadó *et al*., 2011), it is expected that enisling at 37 °C will favour the growth of *Lactobacillus* over endogenous yeast species.

3. Materials and Methods

3.1. Ensiling Process

N6G0 *S. bicolor* biomass (NexSteppe) was planted on 1st March 2017 and harvested from Rio Farms Field in Monte Alto, Texas on 5th June 2017. Biomass was transported to a walk-in freezer and frozen in 20 cm layers with regular turning. Frozen biomass was transported at -20 °C to Shell Technology Centre Houston, where it was stored at the same temperature until use. Prior to ensiling set-up, biomass was thawed at 4 °C for 15-20 h then transferred to room temperature for 1-2 h. Required biomass was weighed and additives in varying concentrations, based on experimental design (see section 3.3) were thoroughly mixed into biomass using gloved hands. Biomass initial pH was recorded and triplicate 1 ml exudate samples for High Performance Liquid Chromotography (HPLC) analysis obtained by pressing biomass using 20 ml syringes (BD Plastipak). Approximately 40-42 g biomass was tightly packed into 50 ml falcon tubes. Tubes were weighed and sealed with a rubber stopper (Fermenthaus) and three-piece airlock (True Brew). Biomass was ensiled in incubators at specified temperatures for specified time periods according to experimental design (see section 3.3). After ensiling, stoppers and airlocks were removed and individual tube weight recorded. Approximately 10 g biomass from each tube was sampled. Samples were well mixed and moisture content and pH measured (see section 3.5). Approximately 1 ml exudate samples for HPLC analysis were obtained using the same pressing method as for initial samples.

Ensiling was performed in triplicate replicates for each treatment, including sampling time during ensiling, due to destructive sampling. 1 x *L. plantarum* cell loading is equivalent to approximately 120 $x10^3$ cfu g^{-1} and 1 x *P. acidilactici* cell loading is equivalent to 310 $x10^3$ cfu g^{-1}. 1 day is equivalent to 24 h +/- 2 h and ensiling temperature +/- 1°C.

3.2. Preparation of LAB inoculum

3.2.1. Preparation of *Lactobacillus plantarum* inoculant

de Man, Rogosa and Sharpe (MRS) (Sigma-Aldrich) agar plates were inoculated with *L. plantarum* DSM20174 (DSMZ) from glycerol stocks and incubated at 37 °C for 24 h. MRS broth, was prepared (55 g MRS/ 1l dH_2O sterilised by autoclaving) and inoculated with *L. plantarum* from stock plates (approximately 1 plate / 600 ml broth). Cultures were incubated at 37 °C, 200 rpm for 20-24 h in baffled shake flasks (Fisher). After incubation, cell cultures from required number of shake flasks were combined and sampled to measure OD (600 nm). Volume of culture required for an accurate cell loading was calculated (see appendix

equation 1). Cell cultures were divided into 50 ml falcon tubes and centrifuged at 11985 *x g*, 15 °C for 10 min. Supernatants were discarded and cell pellets resuspended in dH_2O dependent on mass of biomass to which cells were added (1 ml dH_2O / 10 g biomass). Equivalent volumes of dH_2O were added to biomass to be ensiled without inoculum.

1 x *L. plantarum* cell loading ≈ 120 x 10^3 colony forming units (cfu) g^{-1}
≈ 50 ml of OD_{600} 3.75 culture for 500 g biomass

3.2.2. Preparation of *Pediococcus acidilactici* inoculant

MRS agar plates were inoculated with *P. acidilactici* ATCC25742 (ATCC) from glycerol stocks and incubated at 45 °C for 24 h. MRS broth was inoculated with *P. acidilactici* from plates (approximately 1 plate / 600 ml broth). Cultures were incubated at 45 °C, 200 rpm for 16-20 h in baffled shake flasks (Fisher). Then cell culture preparation was performed as described for *L. plantarum*.

1 x *P. acidilactici* cell loading ≈ 310 x 10^3 cfu g^{-1}
≈ 50 ml of OD_{600} 3.75 culture for 500 g biomass

3.3. Design of individual ensiling experiments

3.3.1. Ensiling of untreated biomass

Untreated biomass was ensiled at 37 °C for 1 day and end samples taken.

3.3.2. Ensiling with $CaCO_3$ addition and *L. plantarum* inoculation

Biomass only, biomass with 15 g l^{-1} $CaCO_3$ and biomass with 40 g l^{-1} $CaCO_3$ were ensiled, each with the following cell loadings: 0 x *L. plantarum*, 1 x *L. plantarum*, 3 x *L. plantarum* and 5 x *L. plantarum*. Ensiling was performed at 37 °C for 1 day and end samples taken.

3.3.3. Ensiling to determine effects of temperature

Biomass with 3 x *L. plantarum*, 40 g l^{-1} $CaCO_3$ was ensiled at 27, 32, 37, 42 and 45 °C for 5 days and end samples taken.

3.3.4. Ensiling with CTec3 addition, $CaCO_3$ addition and *L. plantarum* inoculation

Ensiling was performed at 37 °C or 45 °C for 9 days with the following additions: Biomass with 40 g l^{-1} $CaCO_3$, biomass with 40 g l^{-1} $CaCO_3$ and 3 g kg^{-1} CTec3, biomass with 40 g l^{-1}

$CaCO_3$ and 3 x *L. plantarum*, and biomass with 40 g l^{-1} $CaCO_3$, 3 g kg^{-1} CTec3 and 3 x *L. plantarum*. Sampling was performed after 1 day and 9 days.

3.3.5. Ensiling with *P. acidilactici* inoculation at 45 °C

Biomass with 40 g l^{-1} $CaCO_3$ and biomass with 3 x *P. acidilactici,* 40 g l^{-1} $CaCO_3$ were ensiled at 45 °C for 5 days. Sampling was performed after 1 day and 5 days.

3.3.6. Time course experiment

Biomass with 40 g l^{-1} $CaCO_3$ was ensiled at 45 °C for 3 days. Due to time frame limitations, biomass was ensiled in two batches to allow sampling to be performed at suitable intervals. Batch 1 was sampled after 3, 6, 9, 24, 30 and 48 h and batch 2 was sampled after 14, 18, 24, 48 and 72 h. Initial samples were taken for both batches. Results from different batches for the same time points (0, 24 and 48 h) were combined to give an overall mean (n = 6). Results from both batches were grouped in a single graph for continuous representation of changes in metabolite concentrations during the ensiling period.

3.4. Preparation and analysis of HPLC samples to identify ensiling products

1 ml samples were centrifuged at 21170 *x g*, 15 °C for 7 mins to pellet large particles. 0.8 ml of supernatant was transferred to 1 ml Costar® Spin-X centrifuge tubes (Sigma-Aldrich) and centrifuged as above to remove any large particles. 0.2 ml supernatant was added to HPLC vials (Agilent Technologies) with 0.8 ml 0.01 N H_2SO_4 for 5-fold dilution. Samples were analysed by HPLC on a Dioonex Ultimate 3000 HPLC system (Thermo Fisher Scientific). For quantification of ethanol, lactic and acetic acid, samples were analysed by the Aminex HPX-87 (H column) (Bio-Rad). For quantification of sugar components (glucose, sucrose and fructose) samples were analysed by the Aminex HPX-87P Column (P Column) (Bio-Rad). The H and P column are pre-packed HPLC columns, in hydrogen form with 9 µm particle size, 8% cross linkage and pH ranges of 1-9. Sample analysis was performed 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 min. Concentration of ethanol, lactic acid, acetic acid and sugar components in samples were determined by comparison to known concentration standards of 1, 5, 10, 15 and 20 g l^{-1}.

3.5. Measurement of moisture content

Approximately 4-5 g of biomass was weighed into a 50 ml falcon tube and exact weight recorded. Samples and two empty falcon tubes (control) were dried at approximately 62 °C in a vacuum oven for a minimum of 12 hours. Samples were re-weighed post drying and moisture content determined (see appendix equation 2).

3.6. Acid hydrolysis to determine cellulose content of biomass

Approximately 4 g of biomass was mixed with 100 – 125 ml dH2O and then filtered through a 0.32 mm glass fibre filter circle using a vacuum. Filter paper with residue was transferred to an empty conical and dried at approximately 62 °C in a vacuum oven for a minimum of 12 hours. After drying, caps were returned to falcon tubes and samples were allowed to cool. Dried residues were scraped from filter paper using a spatula and filter papers discarded. Samples were ball milled for 30 s at a frequency of 30 Hz to form a uniform powder. Ball milling was performed using Mixer Mill MM400 (Retsch®) following manufacturer's recommendations. 0.1 g of sample was combined with 0.54 ml 70% H_2SO_4 in 20 ml pressure tubes with caps. Tubes were incubated in 30 °C water bath for 60 mins, vortexing for 15 s after 15 mins and 45 mins incubation. Tubes were removed from water bath and 14.46 ml dH_2O added to dilute acid to approximately 3.6% weight. Samples and 15 ml of sugar recovery standard (4% H_2SO_4, dH_2O, 2.4% w/v glucose and 1.2% w/v xylose) in pressure tubes were autoclaved at 110 – 121 °C for 90 mins. Immediately after autoclaving, tubes were transferred to a Laminar Flow hood until samples reached room temperature. Samples were then filtered through 0.32 mm glass fibre filter circles and diluted for HPLC analysis.

3.7. Statistical analysis

3.7.1. Ensiling with $CaCO_3$ addition and *L. plantarum* inoculation

For each product concentration (ethanol, acetic acid and lactic acid), an ordinary two-way analysis of variance (ANOVA) with a post-hoc Tukey's multiple comparisons test (Tukey 1949) was performed for comparison between cell loadings at the same $CaCO_3$ concentration and for comparison of $CaCO_3$ concentrations at the same cell loading. The Tukey test enables simultaneous pairwise comparison of all means and identifies any difference between two means that was greater than the expected standard error (Witte and Witte, 2009).

Association between ethanol and lactic acid concentrations, and between pH and lactic acid concentration was determined by linear regression and Pearsons correlation.

3.7.2. Ensiling to determine effects of temperature

An ordinary two-way ANOVA with a post-hoc Dunnett's multiple comparisons test (Dunnett, 1955) was performed to determine if product concentrations at different ensiling temperatures were statistically different from at 37 °C.

3.7.3. Ensiling with CTec3 addition, $CaCO_3$ addition and *L. plantarum* inoculation

In order to firstly determine effects of temperature and additives only, time was excluded as a factor and statistical analysis performed separately for each time point. For each product concentration (ethanol, acetic acid and lactic acid), an ordinary two-way ANOVA with a post-hoc Sidak's multiple comparisons test (Sidak, 1967) was performed to compare results for the same additives between ensiling temperatures and to compare different additives within the same ensiling temperature. The Sidak test is used as an alternative to the Tukey test for comparison of a selected set of means.

To determine interactive effects of time, temperature and additives on lactic acid concentration, an ordinary three-way ANOVA with post-hoc Tukey's comparison test was performed.

An ordinary one-way ANOVA with a post-hoc Dunnett's multiple comparisons test was performed to determine if cellulose content was statistically different from initial cellulose content of biomass. An ordinary two-way ANOVA with a post-hoc Sidak's multiple comparisons test was performed for comparison of % cellulose degradation with CTec3 addition and without CTec3 addition.

3.7.4. Ensiling with *P. acidilactici* inoculation at 45 °C

For each product concentration (ethanol, acetic acid and lactic acid), an ordinary two-way ANOVA with a post-hoc Tukey's multiple comparisons test was performed for comparison of ensiling times and for comparison of *Pediococcus* inoculation with uninoculated biomass.

4. Results

4.1. Ensiling to determine natural metabolite consumption and production

Results of ensiling at 37 °C without any modifications to the ensiling system show that the endogenous microbial community in the biomass favours production of ethanol over lactic acid and acetic acid after 1 day (Fig. 5). Although some lactic acid and acetic acid is produced (< 3 mmol g^{-1}), modifications are required to optimise the system to favour lactic acid production, by altering factors that will affect growth of the endogenous microbial community.

Approximately 1.6 g of total free sugars (sucrose, glucose and fructose) were available per gram of wet pre-ensiled biomass. This is equivalent to approximately 1.5 mmol sucrose and 5.7 mmol combined glucose and fructose available per gram of wet pre-ensiled biomass. Hence maximum theoretical lactic acid yield based on measured free sugar concentrations is 17.4 mmol g^{-1}.

All available sugars (sucrose, glucose and fructose) were consumed after 1 day (24 hours) ensiling (Fig. 5), therefore this was the chosen duration for subsequent ensiling experiments.

During ensiling there was a decrease in pH from mean initial pH of 5.2 to a mean end pH of 3.0, due to the production of organic acids, including lactic acid and acetic acid. The acidic pH may have limited further organic acid production and therefore favoured ethanol production, hence pH control was a key factor considered for increased lactic acid production.

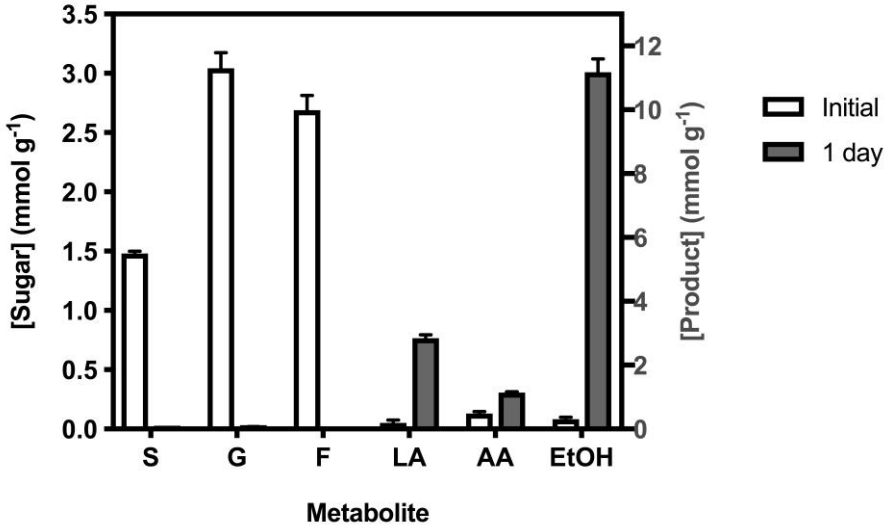


Figure 5. Metabolite concentrations from initial biomass and untreated biomass ensiled at 37 °C for 1 day.

N6G60 *Sorghum bicolor* biomass was ensiled at 37 °C without additives. Initial metabolite concentrations are represented by white bars and metabolite concentrations following 1 day ensiling are represented by grey bars. Bars represent the mean of n=3 independent replicates with standard error shown. Sugar concentrations; sucrose (S), glucose (G) and fructose (F), are shown on the left y axis. Product concentrations; lactic acid (LA), acetic acid (AA) and ethanol (EtOH) are shown on the right y axis. Following 1 day ensiling, concentrations of S, G and F were all below 0.07 mmol g^{-1} (bars not visible on graph).

4.2. Ensiling with $CaCO_3$ and *L. plantarum* addition

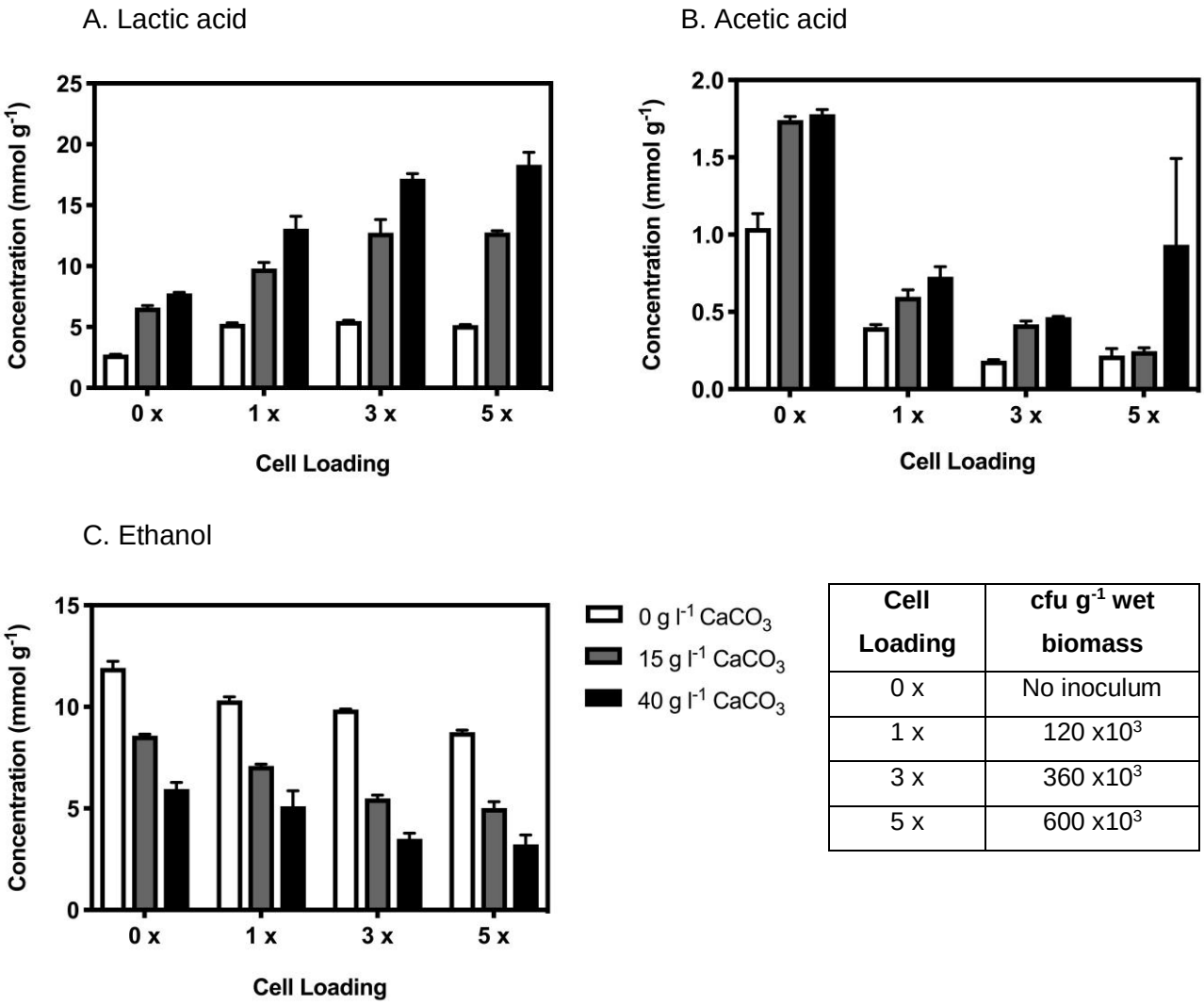
$CaCO_3$, at two different concentrations, was added to biomass prior to ensiling as a method of buffering the system to prevent a drop in pH. *L. plantarum* inoculum, at various cell loadings, was added in combination with $CaCO_3$ to determine the treatment that produced highest lactic acid yields.

Performance of a two-way ANOVA followed by Tukey's multiple comparisons test showed that cell loading significantly affected mean lactic acid concentration (Fig. 6) ($P < 0.0001$). At 0 g l^{-1} $CaCO_3$ addition there was a significant increase in final mean lactic acid concentrations for 3 x and 5 x cell loading compared to biomass without inoculum (0 x cell loading) ($P = 0.0089$ and $P = 0.0239$, respectively). This showed that increasing the presence of *L. plantarum* in the biomass increased lactic acid production, although the decrease in pH limited lactic acid production to a maximum of approximately 5 mmol g^{-1}. At 15 g l^{-1} and 40 g l^{-1} $CaCO_3$ addition there was a significant increase in mean lactic acid production as cell loading increased, up until 3 x cell loading. Between 3 x and 5 x cell loading there was no significant increase in mean lactic acid production. Therefore the lower cell loading of 3 x *L. plantarum* was selected for use in subsequent ensiling experiments.

There was a significant interaction between cell loading and $CaCO_3$ concentration ($P < 0.0001$). For all cell loadings, increasing $CaCO_3$ concentration increased lactic acid concentrations and decreased ethanol concentrations, excluding biomass without inoculum, in which mean lactic acid concentration at 15 g l^{-1} $CaCO_3$ was not significantly different from that at 40 g l^{-1} $CaCO_3$ (Fig. 6). In the absence of cell loading, addition of 40 g l^{-1} $CaCO_3$ increased lactic acid concentration by 2.8 x compared to lactic acid concentration without $CaCO_3$. With 5 x cell loading, addition of 40 g l^{-1} $CaCO_3$ increased lactic acid concentration by 2.6 x compared to lactic acid concentration without $CaCO_3$.

For all conditions acetic acid concentrations were minimal when compared to lactic acid and ethanol concentrations. There was no significant correlation between acetic acid concentration and ethanol or lactic acid concentration. Acetic acid concentration was significantly higher without inoculum than for all other cell loadings regardless of $CaCO_3$ addition. In the absence of inoculum, $CaCO_3$ addition of 15 g l^{-1} ($P = 0.0175$) and 40 g l^{-1} ($P = 0.0121$) significantly increased acetic acid concentration compared to biomass without $CaCO_3$. At all other cell loadings results suggest that acetic acid concentration was not limited by reduced pH.

The large standard error bar for mean acetic acid concentration with 5 x cell loading and 40 g l^{-1} $CaCO_3$ (Fig. 6) is due to a single sample having a higher acetic acid concentration (2.1 mmol g^{-1}) than the other samples (0.4 mmol g-1). This may be the result of a sampling error, variation in initial biomass or uneven distribution of $CaCO_3$. For the same sample, ethanol concentrations were not any higher than for the other samples with the same treatment, which suggests high acetic acid production was not due to a shift towards heterofermentation.



Cell Loading	cfu g^{-1} wet biomass
0 x	No inoculum
1 x	120 $x10^3$
3 x	360 $x10^3$
5 x	600 $x10^3$

Figure 6. Effect of calcium carbonate concentration and *L. plantarum* cell loading on (A.) lactic acid, (B.) acetic acid and (C.) ethanol concentrations from 1 day ensiled biomass.

N6G60 *Sorghum bicolor* biomass was ensiled for 1 day at 37 °C. Biomass with 0 g l^{-1} $CaCO_3$ addition is represented by white bars, 15 g l^{-1} $CaCO_3$ addition by grey bars and 40 g l^{-1} $CaCO_3$ addition by black bars. Equivalent approximate number of cfu g^{-1} wet biomass for each cell loading is shown in the table. Bars represent the mean of n = 3 independent replicates with standard error shown.

Performance of a two-way ANOVA showed that cell loading significantly affected mean ethanol concentration ($P < 0.0001$) (Fig. 6). There is significant correlation ($P < 0.0001$) between ethanol concentration and lactic acid concentration (Fig. 7). As lactic acid concentration increases, as a result of $CaCO_3$ addition, ethanol concentration decreases.

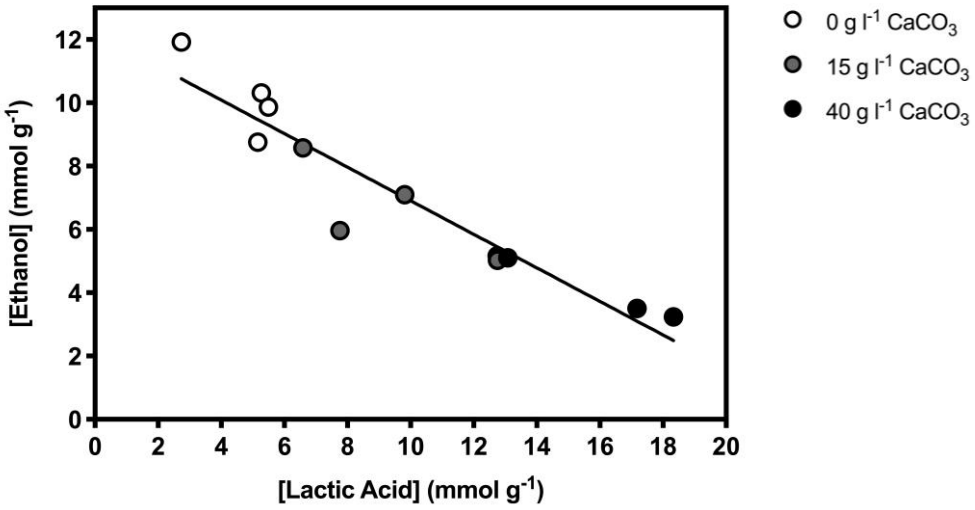


Figure 7. The relationship between ethanol concentration and lactic acid concentration from 1 day ensiled biomass with calcium carbonate addition at various concentrations

N6G60 *Sorghum bicolor* biomass was ensiled for 1 day at 37 °C. Biomass with 0 g l^{-1} $CaCO_3$ addition is represented by white circles, 15 g l^{-1} $CaCO_3$ addition by grey circles and 40 g l^{-1} $CaCO_3$ addition by black circles. Circles represent the mean of n = 3 independent replicates with linear regression shown ($y = - 0.53 x + 12.21$, $r^2 = 0.90$).

A decrease in pH occurred from initial pH of 5.4 to a pH of 3.5 after 1 day ensiling without $CaCO_3$ for all biomass with *L. plantarum* addition at any cell loading (Fig. 8). This decrease in pH was a result of lactic acid produced by additional cells (approximately 5 mmol g^{-1} for all cell loadings). Compared to other treatments, lactic acid concentrations without $CaCO_3$ addition was low, due to lactic acid production being limited by the decreased pH. Addition of 40 g l^{-1} $CaCO_3$ increased end pH from 3.5 to 4.8, which allowed increased production of lactic acid. Although there was still a small decrease in pH, 40 g l^{-1} $CaCO_3$ maintained the pH at approximately 4.8, regardless of increased lactic acid production with increased cell loading. There was no significant increase in lactic acid production between 3 x and 5 x cell loading, but the results show that this is not due to insufficient $CaCO_3$ concentrations to maintain the pH of biomass during ensiling, as pH remained at 4.8.

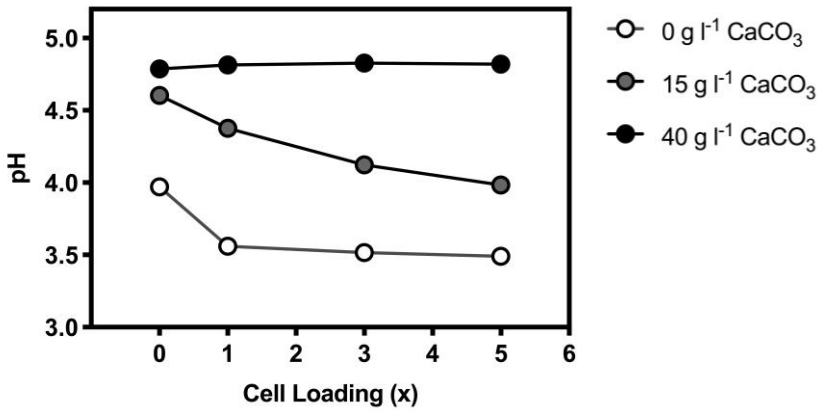


Figure 8. Effect of *L. plantarum* cell loading and calcium carbonate concentration on pH of 1 day ensiled biomass

N6G60 *Sorghum bicolor* biomass was ensiled for 1 day at 37 °C. Biomass with 0 g l^{-1} $CaCO_3$ addition is represented by white circles, 15 g l^{-1} $CaCO_3$ addition by light grey circles and 40 g l^{-1} $CaCO_3$ addition by dark grey circles. Cell loading of 1 is equivalent to approximately 120 $x10^3$ cells g^{-1} wet biomass. Circles represent the mean of n = 3 independent replicates.

There is a significant overall correlation between pH and lactic acid concentration (P = 0.022) (Fig. 9). Results show this correlation is affected by $CaCO_3$ addition, although the interaction between lactic acid concentration and pH is complex. High lactic acid concentrations (> 6 mmol g^{-1}) only occur when pH is maintained by $CaCO_3$ addition, therefore the effect of increased lactic acid on pH of biomass, or vice-versa, cannot be determined. The pH of biomass with 40 g l^{-1} $CaCO_3$ addition is higher than the pH of biomass with 15 g l^{-1} $CaCO_3$ addition at similar lactic acid concentrations. For example, when lactic acid concentration is 12.8 mmol g^{-1} pH of biomass with 15 g l^{-1} $CaCO_3$ is approximately 4.0, but when lactic acid concentration is 13.1 mmol g^{-1} pH of biomass with 40 g l^{-1} $CaCO_3$ is approximately 4.8. Rather than the small increase in lactic acid concentration having no effect on pH or resulting in a decrease in pH, pH increased because $CaCO_3$ successfully buffered the system. Highest lactic acid concentrations were achieved from ensiling when 40 g l^{-1} $CaCO_3$ was added, as lactic acid production was no longer inhibited by low pH. 40 g l^{-1} $CaCO_3$ was therefore used in all subsequent ensiling experiments as a base case condition.

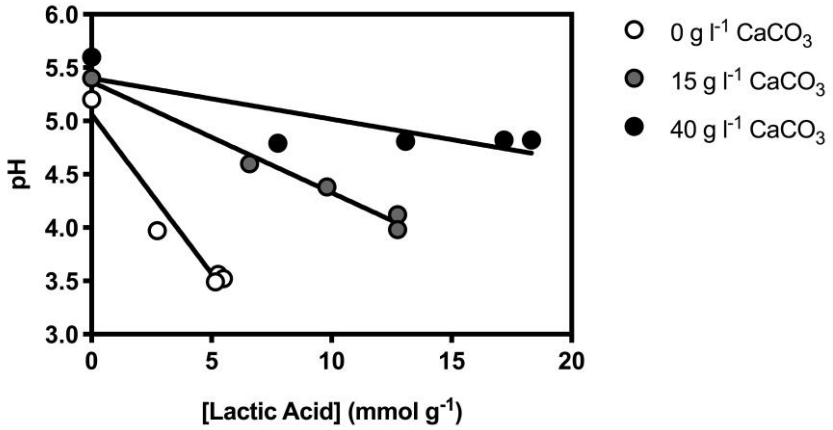


Figure 9. Effect of calcium carbonate concentration on lactic acid concentration and pH of 1 day ensiled biomass

N6G60 *Sorghum bicolor* biomass was ensiled for 1 day at 37 °C. Biomass with 0 g l^{-1} $CaCO_3$ addition is represented by white circles, 15 g l^{-1} $CaCO_3$ addition by grey circles and 40 g l^{-1} $CaCO_3$ addition by black circles. pH values for 0 mmol g^{-1} lactic acid are initial pH of biomass with $CaCO_3$ addition prior to ensiling. Circles represent the mean of n = 3 independent replicates with linear regressions shown (for 0 g l^{-1} $CaCO_3$ y = - 0.30 x + 5.06, r^2 = 0.95; for 15 g l^{-1} $CaCO_3$ y = - 0.10 x + 5.37, r^2 = 0.99; for 40 g l^{-1} $CaCO_3$ y = - 0.04 x + 5.40, r^2 = 0.67).

4.3. Ensiling to determine effects of temperature

As temperature is a key factor affecting microbial growth, ensiling was performed at a range of temperatures from 27-45 °C, with inoculation of *L. plantarum*, to determine how different temperatures influenced lactic acid production.

Performance of a two-way ANOVA followed by Dunnet's multiple comparisons test showed that temperature significantly affected lactic acid production ($P < 0.0001$). Lactic acid production was increased at 27, 32, 42 and 45 °C compared to 37 °C (Fig. 10). Ensiling at 37 °C may have produced the least lactic acid due to increased competition of *L. plantarum* with other mesophillic microbes at this temperature, including yeasts responsible for ethanol production. The highest concentration of lactic acid was produced at 45 °C, with a mean of 19.75 mmol lactic acid produced per g wet biomass. *L. plantarum* cannot grow at 45 °C (demonstrated by inoculation in MRS and incubation at 45 °C, 200 rpm for 72 hours) therefore high lactic acid concentration must have been due to alternative lactic acid bacteria, which were thermotolerant or thermophillic.

Change in ensiling temperature did not significantly affect acetic acid production or ethanol production except at 27 °C and 45 °C when ethanol concentrations were significantly lower ($P < 0.0001$) than ethanol concentrations at 37 °C. It is likely that at 45 °C ethanol producing microbes (yeasts) could not grow and so were outcompeted by thermotolerant and thermophillic lactic acid producers.

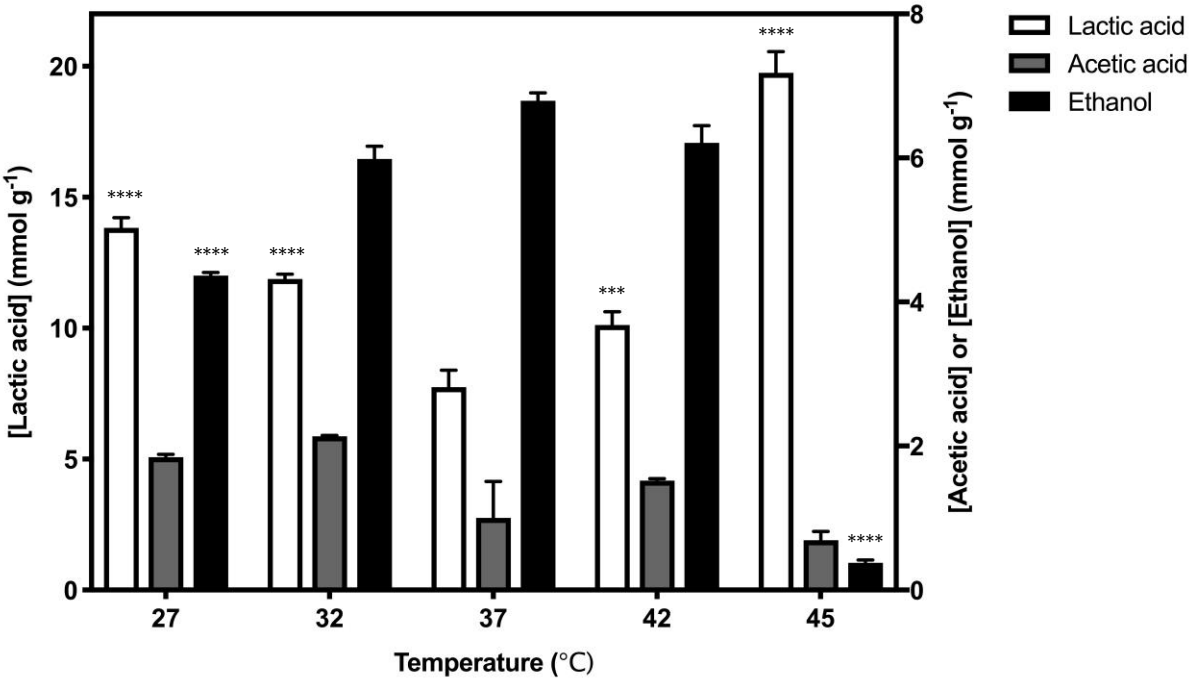


Figure 10. Effect of temperature on lactic acid, acetic acid and ethanol concentrations from biomass ensiled with *L. plantarum* inoculation

N6G60 *Sorghum bicolor* biomass was ensiled for 5 days (+/- 2 hours) at 27, 32, 37, 42 and 45 °C with 40 g L^{-1} $CaCO_3$ addition and inoculation with *L. plantarum* at approximately 360 $x10^3$ cells g^{-1} wet biomass. Lactic acid is represented by white bars, acetic acid by grey bars and ethanol by black bars. Lactic acid concentration is shown on the left axis and acetic acid and ethanol concentrations are shown on the right axis. Bars represent the mean of n=3 independent replicates with standard error shown. *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

4.4. Ensiling with *L. plantarum* inoculation and CTec3 addition at 37 °C and 45 °C

CTec3 is a cellulase and hemicellulose complex, produced by Novozymes, for the conversion of pretreated lignocellulosic materials to fermentable sugars. Ensiling was performed with addition of CTec3 to determine if CTec3 could increase lactic acid production by making more sugars available for fermentation, and to determine if ensiling had any effect as a pre-treatment method. *L. plantarum* inoculant was also added to selected treatments to determine if *L. plantarum* affected lactic acid production at different ensiling temperatures and times, and if there was any interaction between *L. plantarum* and CTec3 addition.

Performance of a two way ANOVA followed by Sidak's multiple comparisons test showed that temperature significantly affected mean lactic acid production after 1 day and 9 days ensiling ($P < 0.0001$ and $P = 0.0049$, respectively) (Fig. 11). One day ensiling at 45 °C significantly reduced mean lactic acid production compared to 37 °C when biomass was ensiled with *L. plantarum* addition ($P < 0.0001$), CTec3 addition ($P < 0.0001$) or combined *L. plantarum* and CTec3 addition ($P = 0.01$). This may be because *L. plantarum* cannot grow at 45 °C and so lactic acid production is expected to be higher at 37 °C than at 45 °C, although it is unclear why there was a decrease at 45 °C with addition of CTec3 alone.

After 9 days there was an increase in lactic acid production at 45 °C compared to 37 °C for all treatments. However, addition of CTec3 and *L. plantarum* was the only treatment that showed a significant increase ($P = 0.0411$). Ensiling at 45 °C virtually eliminated all ethanol production at 45 °C and reduced acetic acid production for all treatments.

The results demonstrate that at 45 °C lactic acid production is not due to *L. plantarum* activity as there was no significant difference in mean lactic acid production with or without *L. plantarum* inoculation. There was a significant increase in lactic acid production when *L. plantarum* was inoculated at 37 °C after 1 day but after 9 days ensiling lactic acid concentrations were similar with and without inoculation.

Performance of a 3 way ANOVA followed by Tukey's multiple comparisons test showed that ensiling time significantly affected lactic acid production at both 37 °C and 45 °C ($P < 0.0001$). Generally, increasing ensiling time to 9 days increased lactic acid production at 37 °C. This was not true for biomass ensiled with *L. plantarum* addition as there was no significant increase in lactic acid concentration between 1 day and 9 day ensiling at 37 °C. This is possibly because addition of *L. plantarum* cells at exponential phase accelerated lactic acid production so that after 1 day ensiling maximum lactic acid concentration had

already been achieved and therefore extending ensiling time had no further effect on lactic acid production.

At 45 °C increasing ensiling time to 9 days increased lactic acid production for all samples independent of additives in the biomass. Furthermore any significant differences in lactic acid production observed between additives after 1 day ensiling were eliminated after 9 days ensiling at both 37 °C and 45 °C. Therefore, it can be assumed that temperature and ensiling time had a greater influence on the microbial community during ensiling than any of the additives investigated. Results suggest that additives (particularly *L. plantarum)* influence the rate of lactic acid production but do not affect total lactic acid yield.

There was a significant interaction between ensiling temperature and time on lactic acid concentration ($P < 0.0001$). This may be due to growth or lactic acid production by thermotolerant and thermophillic LAB species present at 45 °C occurring at a slower rate e than for mesophillic bacteria present at 37 °C°C. Therefore increasing both temperature and ensiling time had a synergistic effect on lactic acid production.

There was a significant difference in lactic acid concentration between 37 °C and 45 °C with CTec3 addition after 1 day ensiling ($P < 0.0001$). Ensiling with CTec3 addition at 37 °C for 1 day made no significant difference to lactic acid concentration compared to ensiling without additives, whereas ensiling with CTec3 addition at 45 °C for 1 day resulted in a significantly lower lactic acid concentration than ensiling without additives ($P = 0.0006$). This suggests that at 45 °C CTec3 may have some form of inhibitory effect on bacteria. After 9 days ensiling, CTec3 addition made no significant difference to lactic acid concentrations at 37°C or at 45°C when compared to lactic acid concentrations without CTec3. However, CTec3 did significantly increase acetic acid concentrations after 9 days ensiling, at 37 °C and 45 °C, compared to ensiling without or with other additives. As 37 °C generally resulted in higher acetic acid concentrations it is not possible to determine if the significant difference between acetic acid concentrations at different temperatures is due to the effect that temperature has on CTec3 function or temperature alone. When considering effect on ethanol concentration CTec3 resulted in a significant increase after 9 days ensiling at 37 °C.

The large error bar for mean lactic acid concentration of biomass ensiled at 45 °C for 9 days with *L. plantarum* inoculation is due to one sample being significantly lower (5.4 mmol g^{-1}) than the others (15.6 mmol g^{-1} and 14.6 mmol g^{-1}) (Fig. 11).

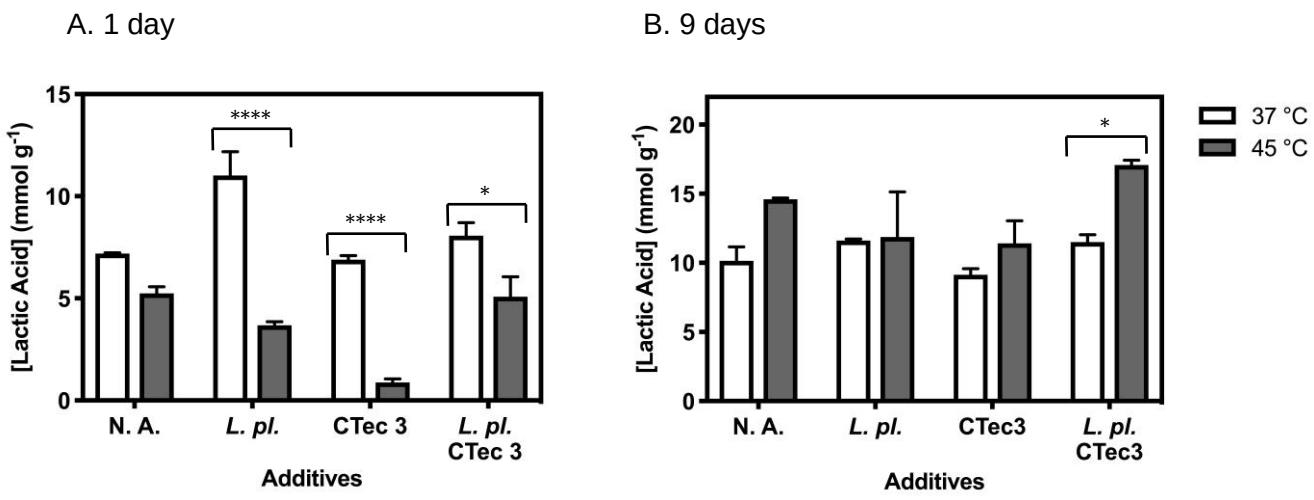


Figure 11. Effects of ensiling additives and ensiling temperature on lactic acid concentrations from (A.) 1 day and (B.) 9 day ensiled biomass

N6G60 *Sorghum bicolor* biomass with 40 g l^{-1} $CaCO_3$ addition was ensiled at 37 °C or 45 °C for 1 day and 9 days (+/- 2 hours). Biomass ensiled at 37 °C is represented by white bars and 45 °C by grey bars. CTec3 was added at a concentration of 3 g CTec3 kg^{-1} wet biomass. *L. plantarum* (*L. pl.*) was added at a cell loading of approximately 360 $x10^3$ cells g^{-1} wet biomass. N. A. denotes biomass with no additives. Bars represent the mean lactic acid concentration of n=3 independent replicates with standard error shown. * indicates $P < 0.05$, **** indicates $P < 0.0001$.

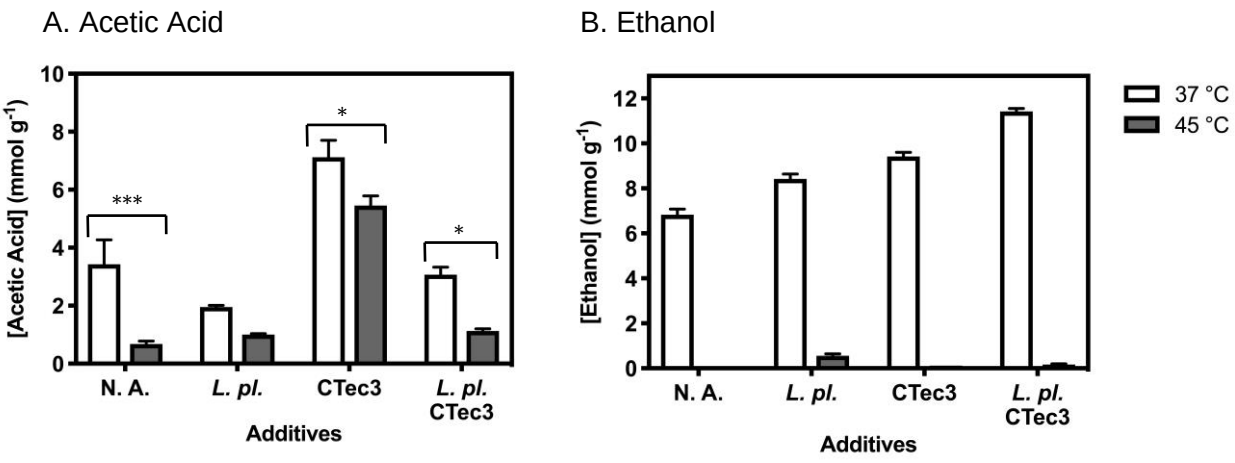


Figure 12. Individual and combined effects of CTec3 cellulosic enzyme package addition, *L. plantarum* cell addition and ensiling temperature on (A.) acetic acid and (B.) ethanol concentrations from 9 day ensiled biomass

N6G60 *Sorghum bicolor* biomass with 40 g l^{-1} $CaCO_3$ addition was ensiled at 37 °C or 45 °C for 9 days (+/- 2 hours). Biomass ensiled at 37 °C is represented by white bars and 45 °C by grey bars. CTec3 was added at a concentration of 3 g CTec3 kg^{-1} wet biomass. *L. plantarum* (*L. pl*) was added at a cell loading of approximately 360 $x10^3$ cells g^{-1} wet biomass. N.A. denotes biomass with no additives. Bars represent the mean product concentration of n=3 independent replicates with standard error shown. * indicates $P < 0.05$, *** indicates $P < 0.001$.

Cellulose degradation was a better measure of CTec3 performance than ensiling product concentrations. Performance of a one way ANOVA followed by Dunnet's multiple comparisons test showed that when compared to initial cellulose content, biomass ensiled without CTec3 at 37 °C was the only treatment that did not show a significant decrease in mean cellulose content. Addition of CTec3 made no significant difference to % cellulose degradation at 45 °C after 9 days ensiling (both with or without *L. plantarum* inoculation), probably due to cellulose degradation already occurring naturally after 9 days ensiling at 45 °C. Addition of CTec3 significantly increased % cellulose degradation for biomass ensiled at 37 °C for 9 days both with *L. plantarum* ($P = 0.0082$) and without *L. plantarum* ($P = 0.0007$) compared to biomass ensiled with no CTec3 under the same conditions. However, there was no significant difference in % cellulose degradation with or without CTec3 for any biomass ensiled at 37 °C for 1 day. Temperature made no significant difference to % cellulose degradation following 9 days ensiling with CTec3, despite 45 °C being nearer to the optimum temperature of CTec3 (50-55 °C) (Novozymes).

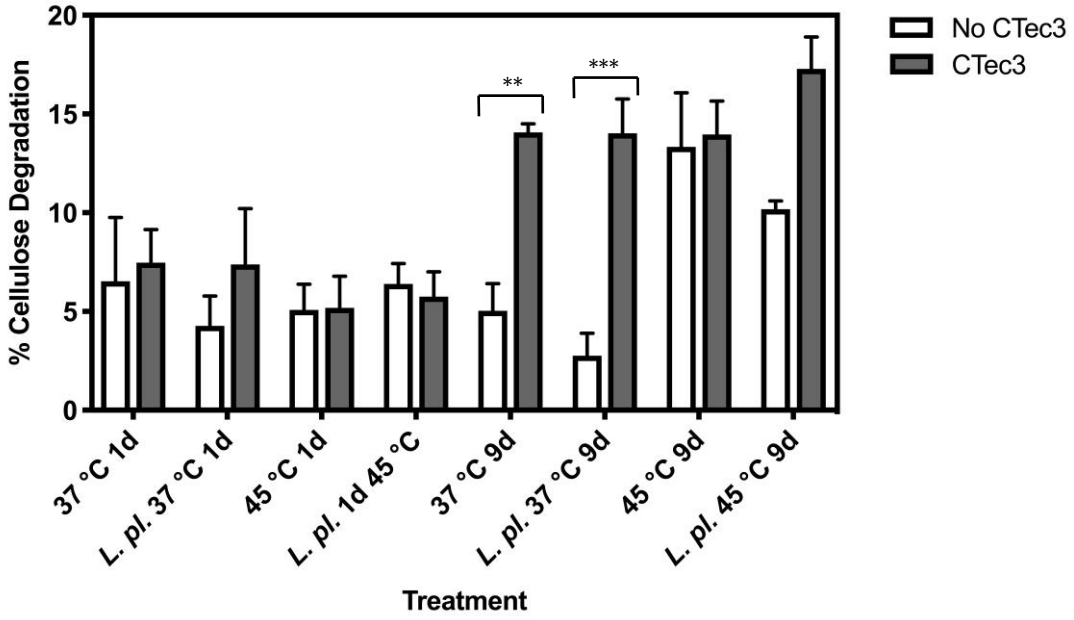


Figure 13. Effect of CTec3 cellulosic enzyme package addition on cellulose content of ensiled biomass with various treatments

N6G60 *Sorghum bicolor* biomass with 40 g l^{-1} $CaCO_3$ was ensiled at 37 °C or 45 °C for 1 day and 9 days. Biomass ensiled with no CTec3 addition is represented by white bars and biomass with CTec3 addition (3 g CTec3 kg^{-1} wet biomass) by grey bars. '*L. pl.* 37 °C 1d' represents inoculation of *L. plantarum* at a cell loading of approximately 360 $x10^3$ cells g^{-1} wet biomass, ensiling temperature of 37 °C and ensiling period of 1 day. Bars represent the mean % cellulose degradation of n=3 independent replicates with standard error shown. Percentage cellulose degradation was calculated by expressing decrease in cellulose content as a percentage of initial cellulose content (approximately 40% total wet biomass weight). ** indicates $P < 0.01$, *** indicates $P < 0.001$.

4.5. Ensiling with *P. acidilactici* at 45 °C

A preliminary metagenomics study of biomass ensiled at 45 °C identified *Pediococcus* species as the major genus. Therefore additional *Pediococcus* cells were added to biomass to determine if lactic acid production could be increased further. *P. acidilactici* was selected as it is a thermophillic and homofermentative LAB that has been previously isolated from silage (Cai *et al*., 1999). However addition of *P. acidilactici* cells during ensiling at 45 °C did not increase lactic acid production.

Performance of a two-way ANOVA followed by a post-hoc Tukey's multiple comparisons test showed that inoculation with *Pediococcus* significantly decreased lactic acid concentrations following both 1 day and 5 days ensiling ($P = 0.0012$ and $P = 0.0051$, respectively). Ethanol production during ensiling at 45 °C was minimal (0.13 - 0.19 mmol g^{-1}) and acetic acid concentrations were relatively low, however for both 1 and 5 days, acetic acid concentrations were significantly higher with *Pediococcus* addition ($P < 0.0001$ and $P < 0.0001$, respectively) (Fig. 14).

There was no significant difference in lactic acid concentrations after 1 day or 5 days ensiling at 45 °C. Lactic acid reached a similar concentration after 1 day ensiling as it did after 9 days ensiling under the same conditions in the previous experiment. Since after 1 day ensiling lactic acid concentration had already reached close to its previous maximum it was to be expected that no more lactic acid would be produced following 5 days ensiling.

A. Lactic acid B. Acetic acid

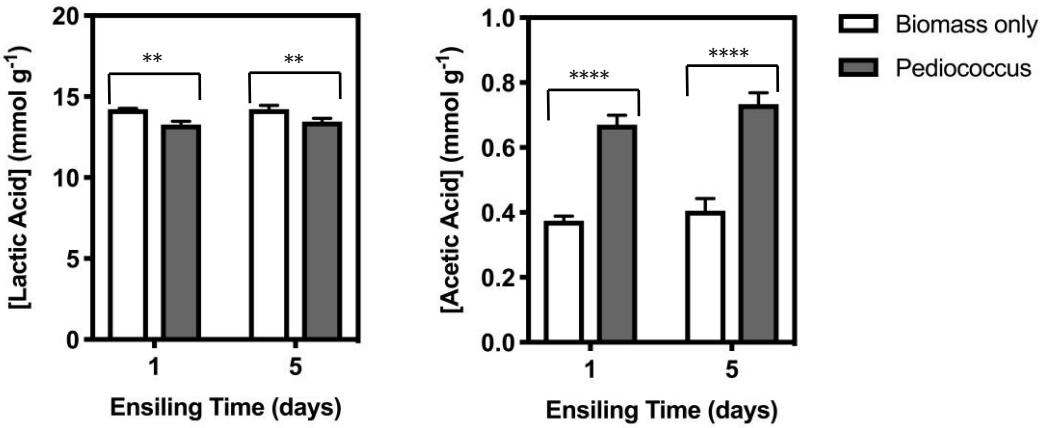


Figure 14. Effect of *P. acidilactici* addition on A. lactic acid and B. acetic acid concentration from biomass ensiled at 45 °C for 1 day and 5 days.

N6G60 *Sorghum bicolor* biomass with 40 g l^{-1} $CaCO_3$ addition was ensiled at 45 °C for 1 day and 5 days (+/- 2 hours). Biomass ensiled without inoculation is represented by white bars and with *P. acidilactici* inoculation by grey bars. *P. acidilactici* was added at a cell loading of approximately 310 x10^3 cells g^{-1} wet biomass. Bars represent the mean lactic acid concentration of n=3 independent replicates with standard error shown. ** indicates $P < 0.01$, *** indicates $P < 0.001$.

4.6. Time course ensiling at 45 °C

Although results for 4.4 showed that 9 days ensiling always increased lactic acid production compared to 1 day ensiling, there was no sampling performed at any intermediate time point. To better characterise the ensiling process under determined optimum conditions, including minimum ensiling time to achieve maximum lactic acid yields from free sugars, a time course experiment was performed at 45 °C.

Due to possible short–term freezer failure, storage of biomass was not maintained at 20 °C and some defrosting of biomass occurred. This affected initial sugar concentrations in the biomass. Sugar concentrations of initial biomass measured in the first ensiling experiment performed showed total free sugar concentration of approximately 1.6 g g^{-1} biomass, composed of 32.9% sucrose, 35.6% glucose and 31.5% fructose. In comparison, sugar concentrations measured from initial biomass in the current time course experiment showed total free sugar concentration of approximately 1.4 g g^{-1} biomass, composed of 2.9% sucrose, 48.9% glucose and 48.2% fructose. The 0.2 g g^{-1} difference in total sugar concentration may have been a result of natural variation between batches of biomass, although decrease in total sugar content could have been due to changes in storage temperature. The decrease in sucrose percentage by 30% and associated increase in glucose and fructose percentages are a result of changes in storage temperature. Within the time period when biomass reached room temperature sucrose will have degraded into glucose and fructose due to activity of an enzyme called invertase, which is naturally present in sweet sorghum (Njokweni, Ibraheem and Ndimba, 2016). Previous studies have confirmed degradation of sucrose and decrease in total sugar concentrations of biomass due to increased storage temperatures and times (Skrede, 1983; Lingle *et al*., 2012).

Results show that glucose was fermented before sucrose. Fermentation of glucose and fructose occurred at a similar rate, however initial fermentation of fructose was approximately 8 hours after initial fermentation of glucose (Fig. 15). Therefore glucose was depleted approximately 12 hours before sucrose was depleted. Preferential utilisation of glucose over fructose has been reported previously in fermentations with LAB but the reasons for it are unclear (Erten, 1999). After 60 hours ensiling all sugars were depleted. This is considerably slower than ensiling at 37 °C, in which all sugars were depleted after 1 day ensiling. However, it should be considered that ensiling at 37 °C was performed without $CaCO_3$ addition.

Lactic acid concentration reaches its maximum after approximately 48 hours enisling, despite a small amount of fructose still being present. Compared to ensiling at 37 °C fermentation at

45 °C occurred at a slower rate. Although in this experiment lactic acid production reached a maximum after 48 hours, in the previous experiment lactic acid reached a similar average concentration of 14.2 mmol g^{-1} after 1 day ensiling and showed no further increase following another 4 days ensiling. In contrast, in experiment 4.4 average lactic acid concentration after 1 day ensiling at 45 °C was 5.25 mmol g^{-1}, approximately half of lactic acid concentration reached after 1 day in the current experiment and considerably far from the maximum. Despite the same conditions and additives between experiments, rate of lactic acid production showed considerable variation. There was even considerable variation between batches in the same experiment, which can be seen by the large error bars for all metabolites after 24 hours ensiling when lactic acid concentrations from two separate batches of biomass were combined.

Addition of 40 g l^{-1} $CaCO_3$ to biomass increased the initial pH of silage to 6.0. During ensiling, pH decreased to approximately 4.9 at which it remained stable due to the buffering effect of 40 g/L $CaCO_3$. Decrease in pH followed increases in lactic acid concentration over time.

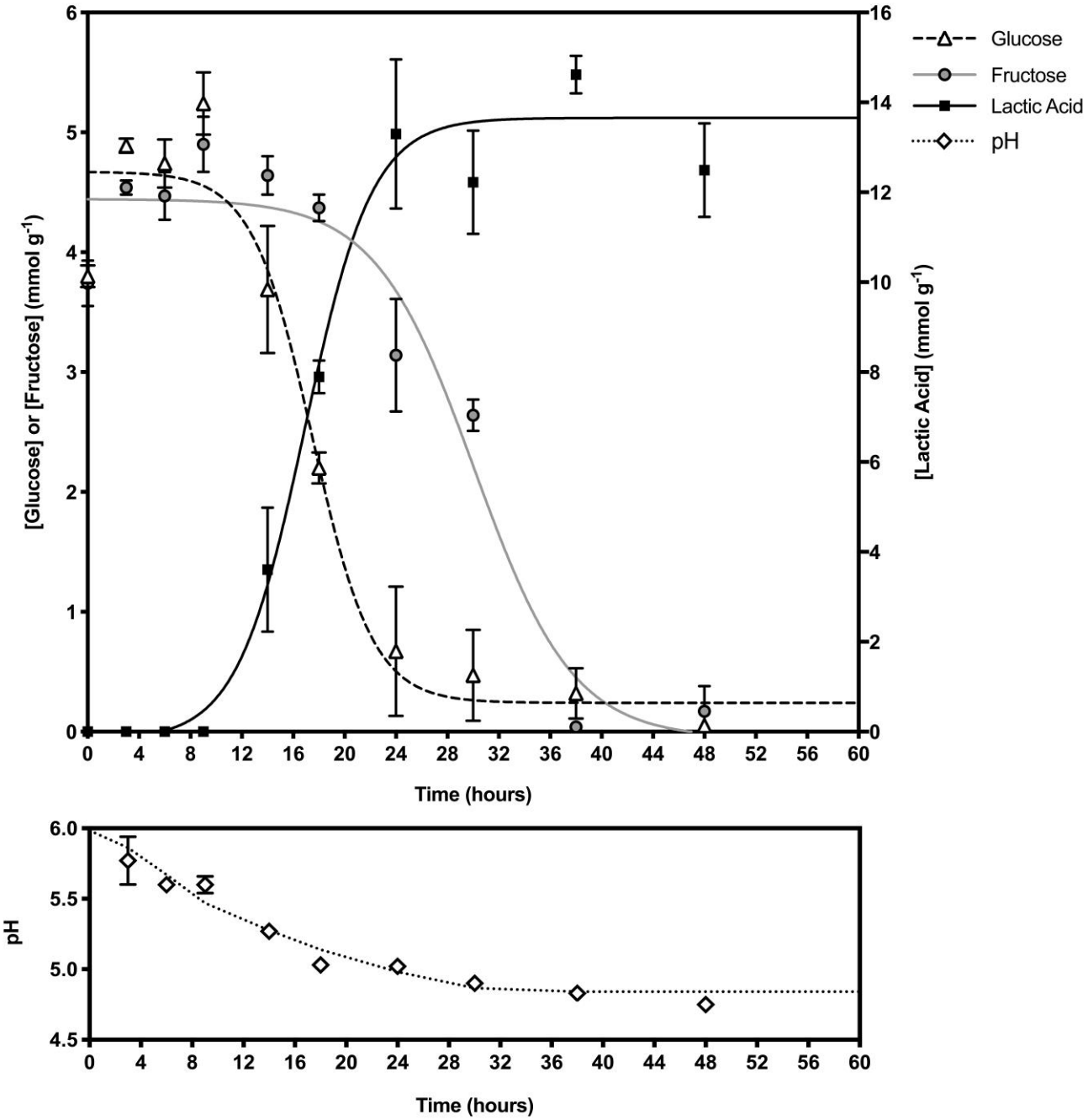


Figure 15. Metabolite concentrations and pH over time from biomass ensiled at 45 °C for 3 days.

N6G60 *Sorghum bicolor* biomass with 40 g l^{-1} $CaCO_3$ addition was ensiled at 45 °C for 3 days (72 hours). Metabolite concentrations are shown for up to 60 hours. Sampling was performed at 0, 3, 6, 9, 14, 18, 24, 30, 38, 48 and 72 hr from 2 ensiled biomass batches. 24 hr and 48 hr time points represent the mean of n =6 independent replicates from 2 batches. All other time points represent the mean of n=3 independent replicates with standard error shown. Sugar concentrations (glucose and fructose) are shown on the left y axis and product concentration (lactic acid) is shown on the right y axis. Data is fit to sigmoidal dose-responsive non-linear model (for glucose r^2 = 0.97; for fructose r^2 = 0.96; for lactic acid r^2 =

0.99). Sucrose, acetic acid and ethanol concentrations were negligible. pH at each time point is shown in the lower graph. For pH, data is fit to smooth 2nd order polynomial curve (r^2 = 0.98).

5. Discussion

5.1. Ensiling product yields and microbial community composition of silage

Most ensiling processes are driven by lactic acid fermentation, which results in highest quality silage, due to lowest dry matter and energy loss from the crop (Kung and Shaver, 2001). Generally, lactic acid fermentation naturally dominates during ensiling, however in the current study; ensiling of sweet sorghum at 37 °C without additives favoured ethanol fermentation. When ensiling product concentrations were converted from mmol g^{-1} of wet biomass to g l^{-1} of expressed liquid from biomass, ethanol concentration was approximately double lactic acid concentration. In a previous study, ensiling of sweet sorghum in 18.9 l buckets at 30 °C gave considerably different results. After 24 hours, approximate concentrations were 3 g l^{-1} ethanol and 8 g l^{-1} lactic acid. After 2 weeks this increased to 5 g l^{-1} ethanol and 23 g l^{-1} lactic acid (Gallagher *et al*., 2018). The difference in relative ethanol and lactic acid concentrations between studies is likely to be due to a number of factors affecting microbial community composition.

Sweet sorghum used in both studies was of the same variety and harvested from the same field at the same growth stage, however silage set-up was performed immediately in the study by Gallagher *et al*., whereas in the current study biomass was frozen and then silage set-up performed in the laboratory. Other key differences between the studies include ensiling temperature (30 °C vs. 37 °C) and scale of ensiling (8.9 L buckets vs. 50 ml falcon tubes).

It is likely that in the current study, the microbial community of biomass ensiled at 37 °C comprised a greater proportion of yeasts than LAB, which were responsible for observed high ethanol concentrations. Some species of yeasts are strictly aerobic and therefore rapid removal of air normally prevents their growth, in addition to the growth of aerobic moulds and bacteria in the silage (Pahlow *et al*., 2003; Liu *et al*., 2014). It is possible that during transport and storage sweet sorghum was exposed to oxygen, which increased growth of yeasts. Mills and Kung (2002) reported increases in the number of yeasts and moulds in chopped barley forage that had been exposed to air in a forage wagon for 24 h prior to silo filling. Packing of silage may have also affected oxygen levels, as if falcon tubes were not packed tightly enough then it may have taken longer for an anaerobic environment to be established.

Following depletion of oxygen, ethanol production can occur via fermentation of glucose by anaerobic yeast (Equation 1) and enterobacteria (Equations 2) and via heterofermentation of glucose by LAB (Rooke and Hatfield, 2003). It is likely that ethanol was produced by a

combination of these fermentation pathways as ratios of lactic acid, acetic acid and ethanol cannot be linked to a single fermentation reaction. Previous metagenomic analysis identified several genera of yeasts present in sweet sorghum silage after 1 day at 30 °C (Gallagher *et al*., 2018). These were; *Candida, Saccharomyces, Cladosporium, Issatchenki, Papiliotrema* and *Hannaella*. *Candida* was the most dominant genus in ensiled biomass and in pre-ensiled biomass*. Candida* can assimilate lactic acid in addition to glucose for fermentation to alcohol. Other lactic acid assimilating yeasts include *Saccharomyces*, *Cryptococcus*, and *Pichia* species, which are commonly isolated from maize and sorghum silage (Middelhoven, de Jong and de Winter, 1990). Therefore lactic acid concentrations may be directly linked to ethanol concentrations if lactic acid is fermented to ethanol by such yeasts during current ensiling.

Yeast:

Glucose → 2 Ethanol + 2 CO_2

Equation 1. Simplified equation for anaerobic fermentation of glucose to ethanol by yeast species (Rooke and Hatfield, 2003)

Enterobacteria:

2 Glucose → 2 Lactate + Acetate + Ethanol + 2 CO_2

Equation 2. Simplified equation for fermentation of glucose to ethanol by enterobacteria species (Rooke and Hatfield, 2003)

The strong negative correlation between lactic acid concentration and ethanol concentration (Section 4.2) suggests that there is direct competition for available sugars or required nutrients in the biomass between ethanol producing microbes and lactic acid producing microbes. Therefore ensiling system modifications designed to limit growth of ethanol producing microbes should indirectly or directly increase lactic acid concentrations.

Although it can be assumed that strategies to decrease yeast populations will enable increased growth of LAB, there is evidence of commensalism between yeasts and LAB. During dough fermentation, CO_2 production and O_2 consumption by *S. cerevisiae* played a role in stimulating the growth of *Lactobacillus sanfranciscensis* (Sieuwerts, Bron and Smid, 2018). As many LAB are stimulated by CO_2 (Stevens *et al*., 2008) and inhibited by the presence of reactive oxygen species produced in an aerobic environment (An *et al*., 2011*)*, presence of yeast may contribute to LAB growth during initial stages of ensiling.

Yeasts also directly stimulate LAB growth by release of stimulatory compounds, including riboflavin (Megee *et al*., 1972). A study by Sieuwerts, Bron and Smid (2018) later showed that *L. sanfranciscensis* and *L. plantarum* were stimulated by a factor secreted by *S. cerevisiae,* during growth on chemically defined medium. This commensalism between LAB and yeast is just one example of the many complex interactions that may exist in the silage microbial community and indeed any natural microbial consortia. Such interactions make it difficult to predict the effect of ensiling system modifications on product yields.

5.2. pH control

Fermentation by LAB and other microbes produced organic acids, which decreased the pH of silage. After 1 day ensiling at 37 °C the pH had dropped to approximately 3. Unlike LAB, yeasts are not inhibited by low pH and are able to grow and multiply within the pH range of 3 to 8 (McDonald *et al*., 1991). Therefore yeasts will have outcompeted LAB once pH dropped below a certain threshold. It was previously demonstrated that *L. plantarum* growth stopped when internal cellular pH of 4.6 – 4.8 was reached, with external pH limit dependent on growth medium. When *L. plantarum* was cultured in MRS with hydrochloric acid as the acidulant, internal pH approached 4.5 when external pH neared 3 (McDonald, Fleming and Hassan, 1990). In line with this study, results from 1 day ensiling at 37 °C suggest that growth of endogenous LAB and therefore lactic acid production may have stopped when external pH reached 3. To enable maximum growth and lactic acid production by LAB, $CaCO_3$ was added to buffer the system so that pH remained suitable for the LAB present. Fermentation of brewer's spent grain hydrolysate, with 20 g l^{-1} $CaCO_3$ addition increased total lactic acid yield by 13% in *L. fermentum* and 17% in *L. rhamnosus* compared to fermentation without $CaCO_3$ (Pejin *et al*., 2015). Similar increases in lactate productivity by *Lactococcus lactis* were observed following addition of up to 30 g l^{-1} $CaCO_3$ during hydrolysed sago starch fermentation (Bujang, Sujang and Adeni, 2004). Based on effective $CaCO_3$ concentrations used previously, biomass was ensiled with 15 g l^{-1} and 40 g l^{-1} $CaCO_3$ addition in initial attempts to control pH of silage.

As has been demonstrated in previous fermentations, addition of $CaCO_3$ increased lactic acid concentrations from ensiled biomass. 40 g l^{-1} $CaCO_3$ buffered the system so that pH remained stable at approximately 4.8, despite production of lactic acid and other organic acids during ensiling. The increased pH prevented inhibition of growth and lactic acid production by LAB. 40 g l^{-1} was a more suitable concentration than 15 g l^{-1} $CaCO_3$ as at 15 g l^{-1} there was still a decrease in pH, from an average of 4.6 to an average of 4 as cell loading increased. In the presence of inoculum of any size, 40 g l^{-1} $CaCO_3$ increased lactic acid concentrations significantly more than 15 g l^{-1} $CaCO_3$. In the absence of inoculum there was

no significant difference in lactic acid concentrations between 15 g l^{-1} $CaCO_3$ addition and 40 g l^{-1} $CaCO_3$. This may be because lactic acid production was limited by *L. plantarum* cell number and not by decrease in pH. Without inoculum, lactic acid production was minimal and so any minor change in pH could be successfully buffered by 15 g l^{-1} or 40 g l^{-1} $CaCO_3$. Even without inoculum, addition of 40 g l^{-1} $CaCO_3$ resulted in a 2.8 x increase in lactic acid production. In subsequent experiments 40 g l^{-1} $CaCO_3$ was sufficient to successfully buffer pH of silage (data not shown).

Initial pH of biomass with 40 g l^{-1} $CaCO_3$ was 5.6 in Section 4.2, whereas in the time course experiment the initial pH of biomass with 40 g l^{-1} $CaCO_3$ was 6.0 (Section 4.6). This difference in initial pH is likely due to natural variation in the pre-ensiled biomass (moisture content also differed between samples). During the time course ensiling, pH decreased to approximately 4.9, at which it remained stable. This was similar to 4.2, in which the pH decreased to approximately 4.8. As expected, decrease in pH followed lactic acid production during ensiling, however a small initial decrease occurred before lactic acid production occurred, which suggests production of other organic acids by LAB and other microbes. Growth of these microbes may have been due to ensiling temperature and anaerobic conditions not yet being reached.

5.3. Inoculation with LAB

Inoculation with *L. plantarum* resulted in significant increases in lactic acid production during ensiling at 37 °C, both with and without $CaCO_3$ addition. The decreases in acetic acid concentration that occurred suggest that inoculation with *L. plantarum* increased competition, which prevented growth of endogenous bacteria responsible for acetic acid production. The significant interaction between cell loading and $CaCO_3$ concentration on lactic acid concentration is due to $CaCO_3$ altering silage pH so that additional *L. plantarum* cells are able to continue lactic acid production. Initial cell loading size for *L. plantarum* was based on the most common inoculation size recommended of 1 x 10^5 cfu per gram of wet forage. There was a significant increase in lactic acid concentration between 1 x and 3 x cell loading with 40 g l^{-1} $CaCO_3$, which suggests that 1 x cell loading was not sufficient to effectively dominate the fermentation. However, there was no significant difference in lactic acid concentration between 3 x and 5 x cell loading, likely due to *L. plantarum* reaching its maximum cell density within the biomass under the particular ensiling conditions. 3 x cell loading (3.6 x 10^5 cfu g^{-1} biomass) was selected for use in subsequent ensiling experiments as a smaller inoculation size is economically favourable. Despite these considerations it was later determined that similar lactic acid concentrations could be achieved at 37 °C without any inoculation, following an extended ensiling period of 9 days. Subsequent results also

suggest that increasing ensiling temperature to 45 °C may further improve yield and increase selectivity towards lactic acid production, irrespective of *L. plantarum* inoculation. Therefore, although addition of *L. plantarum* increases the rate of lactic acid production, extending ensiling time or increasing ensiling temperature is likely to be more practical and economical than inoculation.

Following identification of *Pediococcus* as the dominant genus at 45 °C inoculation with *P. acidilactici* was performed in attempt to increase lactic acid concentrations further. As *P. acidilactici* is obligately homofermentative, inoculation with *P. acidilactici* provides greater potential than *L. plantarum* inoculation, however there was no increase in lactic acid production after 1 day or 5 days ensiling at 45 °C with *P. acidilactici* inoculation. This was likely because *Pediococcus* cells were already present at their maximum cell density in the biomass under the given conditions and so adding more cells had no effect on the system. In fact, significantly less lactic acid (approximately 1 mmol g^{-1} less) was produced with *P. acidilactici* addition. This may have been due to contamination of *P. acidilactici* cell cultures added to the biomass, which competed with endogenous *Pediococcus* species. Small but significant increases in acetic acid production suggest contamination by heterofermentative LAB or other acetic acid producing species. Alternatively the endogenous *Pediococcus* bacteria responsible for lactic acid production may have not been *P. acidilactici*, but another species. Therefore introduction or further addition of *P. acidilactici* cells may have affected the dynamics of microbial growth, and subtly shifted community metabolism away from lactic acid production.

5.4. Ensiling temperature and time

Ensiling temperature and time had a greater influence on product yields than inoculation with LAB or CTec3 addition. Increasing ensiling temperature to 45 °C significantly improved selectivity towards lactic acid production. When biomass was ensiled at 45 °C with 40 g l^{-1} $CaCO_3$ only, both ethanol and acetic acid production were virtually eliminated. This may have been due to a reduction in the growth of mesophillic microorganisms that are responsible for production of alternative fermentation products and the decreased likelihood of contamination by non-endogenous species. It is likely that fewer microbial species endogenous to silage would have been able to tolerate the elevated temperature. Previous evidence suggests that microbial communities in high-temperature environments are often dominated by a few types of microorganisms, and are significantly less diverse than those in lower temperature environments (Li *et al*., 2015). This was supported by preliminary metagenomic data, which showed that when considering relative taxon abundance, bacterial diversity was considerably lower for biomass ensiled at 45 °C than at 37 °C. The decreased

diversity corresponded with dominance of the *Pediococcus* genus, which are homofermentatative LAB and so would have been responsible for resulting high lactic acid concentrations.

Ensiling at elevated temperature may have benefits regarding optical purity of lactic acid, as during fermentation of non-sterile kitchen waste; increasing fermentation temperature from 35 °C to 45 °C increased the ratio of L- lactic acid:D - lactic acid from 60:40 to 83:17 (Zhang et al., 2008). The same study also demonstrated that pH and fermentation time can also have an effect on optical purity. As optical purity is a key factor in determining the value of lactic acid for commercial application, it is worthwhile evaluating the effect that various ensiling conditions had on optical purity in addition to yield of lactic acid.

Manipulation of ensiling temperature will undoubtedly be more difficult at commercial-scale than at laboratory scale, however rapid rises in temperature in the early stage of ensiling often naturally result in farm-scale silage reaching temperatures over 45 °C (Barnett, 1954). Silage temperature is affected by ambient temperature (Borreani *et al*., 2018) and type of silo used (Moisio and Heikonen, 1994). Therefore there is potential for manipulation of ensiling temperature at commercial scale based on location and season at which growth and ensiling are performed and based on silo design.

Between experiments, there was considerable variation in the time required for maximum lactic acid yields to be achieved and for all sugar to be consumed, for the same ensiling treatments and conditions. For example, in Section 4.3. mean lactic acid concentration of 19.8 mmol g^{-1} was reached after 1 day ensiling at 45 °C with 40 g l^{-1} $CaCO_3$ and 3 x *L. plantarum* cell loading. Whereas in Section 4.4. for the same treatment, mean lactic acid concentration only reached 3.7 mmol g^{-1} after 1 day ensiling, with significant increases observed following 9 days ensiling. These results suggest that a longer time period is generally required for maximum lactic acid concentration to be reached during ensiling with 40 g l^{-1} at 45 °C than ensiling at 37 °C. Performance of a time course experiment showed that maximum lactic acid yields were reached after approximately 30 hours ensiling, however fructose was not completely utilised until 48 hours. Ensiling time required for maximum lactic acid production is highly dependent on microbial composition in silage and is likely to be greatly influenced by ensiling scale; therefore it is difficult to make any recommendations as to optimal ensiling period without further research. On a commercial scale, optimal ensiling period should be based on continuous measurements of lactic acid concentration, to determine when lactic acid concentration remains stable and so has reached its maximum.

5.5. Cellulose degradation and effect of CTec3

Although maximum theoretical yield was calculated (Section 4.1), it is important to consider that this is based on measured free sugar content of the particular batch of biomass. Initial free sugar concentration is likely to be variable between batches of biomass harvested from the same field due to the influence of environmental factors and potential differences in storage time and conditions. As maximum theoretical yield is based on free sugar content, it does not include any lactic acid produced from the fermentation of lignocellulose-derived sugars. It is not possible to determine the proportion of ensiling products that were produced from lignocellulose-derived sugars. Lignocellulose utilisation can only be inferred from high product yields and significant cellulose degradation.

Ensiling at 45 °C for 9 days produced similar effects on cellulose degradation as were seen for ensiling at 37 °C for 9 days with CTec3 addition. Therefore ensiling at elevated temperature may offer a more economical alternative to CTec3 addition for the degradation of cellulose. Despite 45 °C being nearer to the optimum temperature of CTec3 (50-55 °C), temperature made no significant difference to perecentage cellulose degradation following 9 days ensiling with CTec3. This was likely to be due to the majority of hemicellulose, lignin and crystalline cellulose remaining insoluble without effective pre-treatment. 45 °C may have increased the rate of cellulose degradation by CTec3, however after 9 days ensiling all available hemicellulose and cellulose were still degraded.

Previous studies identified ensiling as a form of pre-treatment. Chen, Sharma-Shivappa and Chen (2007) effectively evaluated the effect of pre-treatment by performing enzymatic hydrolysis for 72 hours following pre-treatment or no pre-treatment and measuring cellulose degradation. However, as the current ensiling method reflects simultaneous pre-treatment and enzymatic hydrolysis, it is difficult to demonstrate if ensiling functions as a pre-treatment. Based on the assumption that 1 day was sufficient for CTec3 to degrade available cellulose, results suggest that ensiling may have acted as a form of pre-treatment. If 1 day ensiling results were considered as cellulose degradation without pre-treatment and the following 8 days ensiling was considered as potential pre-treatment, then increased cellulose degradation suggests that 8 days ensiling may have resulted in solubilisation of a proportion of lignocellulose. However, effectiveness of ensiling as pre-treatment under these conditions requires further investigation.

Despite a significant increase in cellulose degradation at 45 °C and a decrease in other measured ensiling products at 45 °C compared to 37 °C, increases in lactic acid concentration were not proportional. This suggests that additional carbon resulting from

cellulose degradation, and carbon no longer being utilised for acetic acid and ethanol production, were being utilised by the microbial community for alternative purposes that were yet to be investigated. Nevertheless, results suggest that ensiling at 45 °C provides greater potential than ensiling at lower temperatures in terms of lignocellulose degradation and lactic acid yield and selectivity.

5.6. Dry matter recovery

Moisture content of biomass is a determining factor for both growth rate and minimum pH tolerance of fermenting microorganisms. Sweet sorghum is typically harvested below moisture contents of 65% to minimise leachate production (Grebrehanna *et al*., 2014), however average moisture content of pre-ensiled biomass (Section 4.1) was 73.4%. At lower dry matter content, microorganism growth rate is generally higher, and pH tolerance lower. Therefore dry matter content of harvested biomass may have improved lactic acid production rates and increased time taken to reach terminal pH (Buxton, Muck and Harrison, 2003). Although ensiling is an effective method of biomass preservation, average forage dry matter loss is still 6.2% following ensiling, although this can range from 0 – 28.6% (Goeser, Heuer and Crump, 2015). Following 1 day ensiling at 37 °C without additives there was a significant decrease in dry matter content from 26.6% to 21.1% ($P = 0.0063$). Dry matter loss after 1 day ensiling at 45 °C was 0.91%, compared to 5.5% at 37 °C. This may have been a result of increased fermentation by LAB at 45 °C, as dry matter recovery is usually worse in yeast-dominated fermentations than in LAB dominated fermentations. The minimal dry matter loss, which occurred during ensiling for lactic acid production, is a desirable property of silage that is to be used for animal feed.

5.7. Silage as animal feed

Application of silage as animal feed, following extraction of lactic acid for commercial use, would improve economic viability of the process and result in minimal waste production. Although dry matter content of silage may be suited for animal feed, there are a number of other properties that determine suitability of silage as animal feed, including nutritional quality and digestibility (Huhtanen, 1998). Undesirable microorganisms or metabolites may be detrimental to nutritional quality of silage. Others may be harmful to livestock or affect the safety of milk and other animal products for human consumption (Driehuis *et al*., 2018). Butyric acid has detrimental effects on milk yield and fertility of cattle (Andersson, 1988), however butyric acid is commonly produced during ensiling due to contamination by Clostridia (Kung and Shaver, 2001). Therefore gas chromatography should be carried out to confirm absence of butyric acid and other undesirable metabolites in silage.

5.8. Ensiling scale

Despite similar total free sugar concentrations in initial biomass, rate of sugar consumption and acid or ethanol production was faster during ensiling in the current study than was reported in the previous study by Gallagher *et al*., (2018). In the current study all sugars were consumed following 1 day ensiling at 37 °C without additives. This was not the case for biomass ensiled at 30 °C in 8.9 L buckets, in which approximately 35% of sugar still remained after 48 hours ensiling. Slower sugar consumption may have been due to the lower temperature slowing microbial growth, and/or the effects of ensiling at a larger scale. Although previously, small-scale laboratory silos have been found to be representative of the fermentation products of farm-scale silos (Naoki and Yuji, 2008) there are still a number of factors to consider during commercial scale up. Larger scale silos will experience a greater depth and time dependent oxygen gradient (Kraut-Cohen *et al*., 2016). Therefore it will take longer to establish an anaerobic environment and microbial communities are likely to differ depending on spatial position within the silo. Furthermore, silage temperature is likely to be affected by commercial scale up as temperature is an environmental variable that is determined by exothermic fermentation reactions and the insulative properties of silage (Gallagher *et al*., 2018).

5.9. Result variability

Preliminary metagenomic data suggests that contamination occurred in the sample ensiled at 45 °C for 9 days, for which lactic acid concentrations were significantly lower than other samples of the same treatment. Bacteria belonging to the *Bacillus* genus were present in the particular sample, which were not found to be present in any other ensiled biomass. As *Bacillus* are common soil bacteria it is likely that soil present in the pre-ensiled biomass sample resulted in growth of bacteria that are not normally endogenous to sweet sorghum biomass. Therefore a proportion of carbon will have been directed away from lactic acid fermentation for fermentation by *Bacillus* species. The growth of *Bacillus* species is a prime example of the variation that can occur during ensiling.

Gallagher *et al*. identified a number of factors that may influence the *in-situ* microbial composition of ensiled material. Even for a single hybrid grown in one field, as used in this study, microbial composition is influenced by the heterogeneity of soil properties; localised crop pathogens, epiphytes and endophytes (Shakya *et al*., 2013) and pesticide residues. Due to these potential sources of variability, experimental design was based on a single factor method rather than a Design of Experiment approach. Although measures were taken to minimise sources of variability between experiments, results may still have been affected by difference during experimental set-up. For example; variability in ambient temperature

during silage preparation, differences in growth of inoculant and variation in storage conditions of biomass.

Due to the considerable degree of variation in results between ensiling experiments conclusions were drawn based on comparisons within the same experiment only. The highest concentration of lactic acid from silage recorded during this study was 19.75 mmol g^{-1}, which was achieved following 1 day ensiling at 45 °C with addition of 40 g l^{-1} $CaCO_3$ and *L. plantarum* inoculation, although *L. plantarum* was determined later to not be an affecting factor. In subsequent ensiling experiments ensiling at 45 °C produced higher lactic acid concentrations than 37 °C following a sufficient ensiling period, but lactic acid concentration was still considerably lower than 19.75 mmol g^{-1}. The greatest degree of variability was demonstrated by relative lactic acid concentrations at 37 °C and 45 °C for biomass ensiled with 40 g l^{-1} $CaCO_3$ and *L. plantarum* inoculation. Results in Section 4.3. show that lactic acid concentration at 45 °C exceeded 37 °C after 1 day ensiling, whereas results in Section 4.4. show that lactic acid concentration at 37 °C exceeded 45 °C after 1 day ensiling, although after 9 days ensiling this was no longer the case. These results demonstrate that due to temporal variability in microbial composition during ensiling, different conclusions can be drawn as to the effects of the same factor between ensiling experiments. Therefore experiments that produced key findings should be repeated over longer ensiling periods to ensure validity of results. Following confirmation of key findings and reproduction of high lactic acid yields, with different batches of biomass and varying experimental set-up, commercial scale up can begin to be considered.

5.10. Recommendations for lactic acid production via ensiling

Based on results of the series of ensiling experiments performed in this study, key recommendations for the optimisation of ensiling for lactic acid production from sweet sorghum are the addition of 40 g l^{-1} $CaCO_3$ and an ensiling temperature of 45 °C, to improve lactic acid yields and selectivity, and enable potential lignocellulose degradation. From an economic viewpoint addition of $CaCO_3$ is more viable than other additives, which were investigated (LAB and CTec3) during the study and an ensiling temperature of 45 °C may be achieved with little modification of current silos in sub-tropical climates. However, optical purity and methods of separation and purification of lactic acid from silage should first be evaluated before commercial scale-up is considered.

6. Conclusion

Sweet sorghum provides potential as a feedstock for the simultaneous conversion of free sugars and lignocellulosic biomass to value-added products. Through a series of lab-scale ensiling experiments, this study identified suitable conditions and additives for the production of lactic acid from sweet sorghum biomass. Optimisation of the ensiling system resulted in a shift from initial ethanol dominated fermentation to lactic acid dominated fermentation. Under selected ensiling conditions, lactic acid yields neared or exceeded theoretical maximum from free sugars in biomass (17.4 mmol g^{-1}). Evidence of lignocellulose degradation under these conditions demonstrates the potential of ensiling as a top-down, consortia-based CBP system for the conversion of lignocellulose to lactic acid and other value-added products.

The project identified addition of $CaCO_3$ as an effective method for increasing lactic acid production during ensiling, by buffering silage pH. Addition of 40 g l^{-1} $CaCO_3$ demonstrated potential to increase lactic acid yields by 3.6 x compared to ensiling without $CaCO_3$. Ensiling temperature and time were demonstrated to be key factors in determining ensiling product yields from sweet sorghum. 45 °C improved lactic acid selectivity compared to other investigated ensiling temperatures. However, due to the degree of variability between ensiling experiments, further investigation is required before accurate conclusions can be drawn as to the effects of ensiling temperature and time on lactic acid yields. Metagenomics based approaches will improve our knowledge of the silage microbial community, which will help to determine optimal conditions for maximum lactic acid production and may enable further lignocellulose degradation.

Although this study demonstrates the potential for production of lactic acid via ensiling of sweet sorghum, observed variability related to biomass composition and ensiling scale is likely to affect commercial application. Further investigation at lab-scale is required to enable validation of conclusions drawn from individual ensiling experiments, and to reproduce achieved high lactic acid yields (19.8 mmol g^{-1}), before commercial application can be considered.

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9. Appendix

$$\text{Culture volume required} = \frac{(\text{Biomass (g)} / 500\ \text{g}) \times (3.75 / \text{Measured } OD_{600} \text{ of culture}) \times 50\ \text{ml} \times \text{Required cell loading}}{}$$

Equation 1. Equation to calculate culture volume required for desired LAB cell loading

$$\text{Total solids (\%)} = \frac{\text{Sample mass post-drying (g)} + \text{Average mass loss of controls (g)}}{\text{Sample mass pre-drying (g)}} \times 100$$

Moisture content (%) = 100 – Total solids

Equation 2. Equation to calculate pre- and post- ensiled sample moisture content