Evaluating the impact of byproducts generated by an industrially relevant crop pretreatment on lignocellulosic ethanol yields

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1. Abstract

Lignocellulosic ethanol is an emerging biofuel industry close to implementation in the market; however refinements are still needed to make the technology profitable. Shell has developed a system by which high yields of ethanol can be obtained from lignocellulose, however reaching profitability is limited by inhibition of enzymatic saccharification of the lignocellulose and subsequent fermentation. A better understanding of inhibitors of cellulase enzymes and the GM-strain of *S. cerevisiae* is needed to troubleshoot the technology and the biological systems involved. In this work Central Composite design demonstrated that ethanol, lactic acid and acetic acid act to synergistically inhibit fermentation, allowing a model of their effect to be produced, but only ethanol inhibited saccharification. Solid Phase Extraction was used to discriminate molecules, showing that non-polar components of the biomass strongly inhibit saccharification final yield, but worked alongside polar acids to achieve inhibition in fermentation. Propagation strategies were also tested to determine how conditioning impacted the results.

Table of Contents

Table of Figures

2. Introduction

2.1. Fossil fuels

Energy is a vital commodity. According to the International Energy Association (IEA), in 2013 alone, 13541 MToe (41.868 peta-joules) of energy was supplied worldwide (IEA, 2015). The increased demand for technological commodities, the growing economic and industrial market in developing countries (Raupach *et al.*, 2007) and rapid urbanization to cope with high population density (Creutziga *et al.*, 2015) adds to a growing energy demand. Much of this energy has been supplied from the burning of fossil fuels, however despite high energy yields there are two key concerns: the limited supply of fossil fuels and the environmental damage of fossil fuel combustion.

Fossil fuels are combustible material derived from organic matter, produced under high heat and pressure over millions of years (McKendr, 2002). Fossil fuels are found in the form of coal, natural gas and crude oil, the latter of which is often converted into more useful compounds by processing, including fractional distillation and catalysis (Corma, 2014). However, fossil fuels are consumed at a rate greater than new reserves can be discovered. Even new technologies that increase the availability of fossil fuels in the short term, such as fracking, are unable to overcome long-term limitations (Shafiee *et al.*, 2009) (Nicoletti *et al.*, 2015).

While the burning of fossil fuels yields large amounts of energy, the chemical byproducts generated are known to have adverse effects (Apergisa, 2012). The pollutants produced from fossil fuels include carbon dioxide, unburnt hydrocarbons, sulfur dioxide and fluorogases (Solomon *et al.*, 2007), with the non-carbon species often originating from contamination (Van Hamme *et al.*, 2003). Many of these pollutants have been long removed from the atmosphere, and therefore increasing their concentration can disrupt the existing balance (Farzin *et al.*, 1996).

Among the pollutants produced from the combustion of fossil fuels, some are associated with a group of molecules called the greenhouse gases (GHG). GHG such as CO_2 are responsible for maintaining the temperature of the earth by regulating the amount of outgoing radiation leaving the Earth's atmosphere (Solomon *et al.*, 2007). Models suggests that complete absence of CO_2 would result in a decrease of 34.8 ˚C in global temperature from the average temperature of 16 ˚C (Lacis *et al.*, 2010). On the other hand, increases in the concentration of GHG can further reduce the amount of outgoing radiation, therefore increasing global temperatures.

Since 1850 the concentration of CO_2 and other GHG in the atmosphere has risen sharply, from a low of 54 million metric tonnes (MMT) of carbon, to a high of 9449 MMT in 2011 (Boden et al., 2015). Accompanying this increase, the global temperature has also gradually increased in the region of ~1.2^{o}C from 1880 to 2010 (Hansen *et al.*, 2010). Transport is responsible for up to 28% of energy used worldwide in 2015 (EIA, 2016) and for 26% (2,039.3 MMT CO_2 eq.) of all CO_2 produced in 2014 (EPA, 2016). In spite of the realities of climate change, the significant energy demand of modern society has resulted in an increasing dependency on fuels. To cope with the energy demand, while responding to the environmental damage, researchers in academia and industry are focused upon producing alternatives to fossil fuels.

2.2. Biofuels

Biofuels are compounds derived primarily from plant biomass, and are an important energy source, as their combustion is considered carbon neutral (Moshi *et al.*, 2014). The principal advantage of biofuels for atmospheric carbon balance is that, during growth of the plant biomass CO_2 is absorbed in order to produce carbohydrates, which then either polymerize or are used as substrates to produce essential metabolites, such as fatty acids; the carbohydrates are then converted to a more desirable fuel by fermentation. Combustion releases only the recently stored atmospheric CO_2; there is no net change in the atmospheric CO_2 concentration. (Peterson *et al.*, 1998). Biofuels include ethanol, biodiesel (FAME) and biogas (Guo *et al.*, 2015).

Biofuels are currently designated in terms of generations, with first generation (1G) being the earliest to produce, and currently implemented source. 1G biofuels are derived from sugar and starch-rich agricultural crops such as sugar cane, *Saccharum officinarum*, or corn, *Zea mays* (Goldemberg *et al.*, 2009)(Cherubini *et al.* 2009)(US Department of Agriculture, 2016). 1G ethanol is developed from the fermentation of glucose monomers often using the yeast strain *Saccharomyces cerevisiae*, following the saccharification of starch when necessary (Naik *et al.*, 2010) (Olsson *et al.* 1996). *S. cerevisiae* is used due to the robust nature of the cell with respect to inhibitors (Tomás-Pejó *et al.* 2015). Alternatively, biodiesel is another major 1G fuel, referring to methyl esters and other alkyl esters generated by catalytic transesterification of vegetable and animal oils (Hoekman *et al.*, 2012) such as palm oil from *Elaeis guineensis* (Johari et al., 2015).

1G biofuels are important in industry, as there is an existing infrastructure in which the biofuels are blended with fossil fuels. Currently, car petrol engines are designed to tolerate up to 10% ethanol blending with petrol (Office of Energy Efficiency and Renewable Energy, 2016), and cars containing flexifuel engines can use petrol containing up to 83% ethanol (Office of Energy Efficiency and Renewable Energy, 2016); however high ethanol blending reduces petroleum engine performance (Al-Hasan, 2003). The stability of biodiesel blends in petroleum diesel engines is still uncertain, therefore Europe, a major market, limits on-road vehicles to B7, 7% biodiesel to diesel blend, in accordance with EN 590 (Kampman *et al.*, 2013)(César *et al.*, 2015).

Blending ethanol and biodiesel with conventional fossil fuels can reduce the production of secondary pollutants with a greater GHG effect than CO_2, such as improving complete combustion of carbon monoxide and small hydrocarbons to the less potent CO_2 (Yüksel *et al.*, 2004) (Wang *et al.*, 2000). Furthermore using high ethanol blends such as E85 can reduce emissions of nitrogen oxides by up to 90% (Madigan *et al.* 2012).

Application of biofuels can vary depending upon the country. In Brazil, “bioethanol” (actually, simply “ethanol”; the “bio” prefix is added to differentiate from fossil hydrocarbons in fuel) is a major energy source. There is a standard blending of 25% in regular petrol engines, with flexifuel

engines installed in vehicles to tolerate up to 100% ethanol (Dias De Oliveira *et al*., 2005) (Janssen *et al*., 2011). Comparatively, Europe's current target is that 7% of all energy will be from 1G biofuels by 2020, a reduction from an initial goal of 10% (Hill, 2016). The USA has a strong ethanol blending market, with E10, a 10% ethanol blend with petrol, accounting for 95% of the gasoline sold in 2016 (Office of Energy Efficiency and Renewable Energy, 2016).

While a significant market, 1G biofuels are not implemented equally worldwide (Kampman *et al.*, 2013) (Office of Energy Efficiency and Renewable Energy, 2016). While in some cases political reasons may motivate biofuel implementation, in others legitimate concerns about 1G biofuel production have halted further implementation (Vertès *et al.* 2006). In order to produce 1G ethanol, crops selected must contain large supplies of glucose or starch, which creates a conflict of interest. Crops used for 1G biofuels are defined as food crops due to the ingrained value of the sugars, starch or fatty acids yielded, and the ethics of using food crops for fuel production is heavily debated. The financial result of the debate over food vs fuel is competition that raises prices and reduces available land, risking a supply shortage such as Brazil's ethanol supply crisis of 1989 (Boretti, 2012). The current profits of 1G ethanol have also been shown to be volatile, dependent upon petrol prices, cost of crops and other external factors outside of control of the manufacturer (Irwin, 2016).

Environmental concerns have been raised about producing 1G biofuels, as the GHG effect from producing food crops for biofuels may offset the improvements made from biofuel combustion (Luo *et al.*, 2008)(EASAC, 2012). This concern is due to the poor robustness of many agricultural crops during growth, which necessitates the use of large amounts of fertilizers and pesticides (Hill, et al. 2006). Fertilizers contain high levels of nitrogen, vital for amino acid production, but the application of excess nitrogen to crops is not necessarily translated into higher yields because the excess nitrogenous fertilizer is not stored in the soil, but metabolized by bacteria to produce nitrous oxide (Smeets *et al.* 2009). The GHG effect of nitrous oxide is

almost 300 times per pound greater than CO_2, and so the small amounts released may have a drastic effect (Gelfand *et al.*, 2011) (Oates *et al.*, 2016).

It is also important to consider the GHG effect from changes to soil carbon and soil methane, both of which can be disturbed by the seasonal cultivation of land and subsequent harvesting procedure, meaning crop growth on new land could result in indirect GHG production (Cherubini, 2010) (Rutz *et al.*, 2007). Furthermore, crops needed in other industries will be forced to move to a new area to be cultivated, which can lead to deforestation and further changes in soil carbon (Havlík *et al.*, 2011). As a result of these concerns, the EASAC published a report in 2012 which suggested that biodiesel achieves no reductions in greenhouse gas in its current state (EASAC, 2012).

To achieve the desired environmental and economic improvements to so that biofuels can function as an alternative to fossil fuels, future generations of biofuels are currently being studied (Cherubini et al., 2009).

Second generation (2G), or lignocellulosic, biofuels are based around the premise of using all plant biomass to produce biofuels. Unlike the 1G biofuel industry, the 2G biofuel industry has largely been limited in pilot plant scale as research continues to improve yield (McIntosh *et al.* 2016)(Saha *et al.* 2015)(Shekiro III *et al*., 2014). 2G biofuels are produced from the fermentation of lignocellulose, therefore refers to ethanol production. Ongoing research is focused on how to ferment the lignocellulosic biomass most efficiently (Carriquiry *et al*., 2011) (Aro 2016).

Third generation (3G) biofuels are a feedstock focused advancement that uses algae and other aquatic autotrophic organisms to produce a variety of compounds themselves from carbon dioxide alongside biomass suitable for fermentation (Behera *et al.*, 2015)(Ahmad *et al.*, 2011). There is a significant interest in applying this technology to produce higher yields of oils for biodiesel production than conventional plants (Rawat *et al.*, 2013) (Chisti, 2007). Chemicals produced could even be converted to conventional fuels such as gasoline (Wang *et al.*, 2016)

(Xia *et al.*, 2015). Fourth generation (4G) biofuels, or advanced biofuels, use synthetic biology to encode new biological pathways, generating desired fuel molecules by manipulating the chemical reactions of the host cell (Mukhopadhyay 2015) (Aro 2016). While still in early stages of experimentation, 4G biofuels may also provide methods for generation of conventional fuel products, including propane (Kallio *et al.* 2014) and various diesel molecules (Howard *et al*., 2013)

2.3. Second generation biofuels

The majority of plant biomass in the world is not suitable for consumption, existing either as agricultural residues or unused plant matter from forestry and wood processing (Antizar-Ladislao *et al.*, 2008). The low value of unconsumed biomass means that it would be a less competitive source of biofuels compared to food crops, however limitations in using the unconsumed plant biomass are present due to the polysaccharide lignocellulose.

Lignocellulose is a component of plant cell walls which is made up of cellulose, hemicellulose and lignin. These three polymers interact to achieve a rigid polysaccharide complex that acts to protect the plant cell from degradation (Rubin, 2008). Figure 1 shows a schematic representation of lignocellulose and its three polymers. Of the three lignocellulose polymers, cellulose and hemicellulose contain potentially fermentable sugars.

Cellulose is a highly polymerized homo-polysaccharide and main constituent of lignocellulose, composed of glucose monomers linked by unbranched β-1,4-glucosidic bonds. The strands of cellulose are then linked to one another in parallel crystalline structures producing a supramolecular fiber structure (Zhang *et al.*, 2004)(Li *et al.*, 2010). Pure cellulose can be catabolized by enzymes produced by many organisms, however the other components of lignocellulose can be more challenging.

Unlike cellulose, hemicellulose polymers are composed of a broad range of sugars, which include glucose and xylose but may also include arabinose, galactose and mannose, and

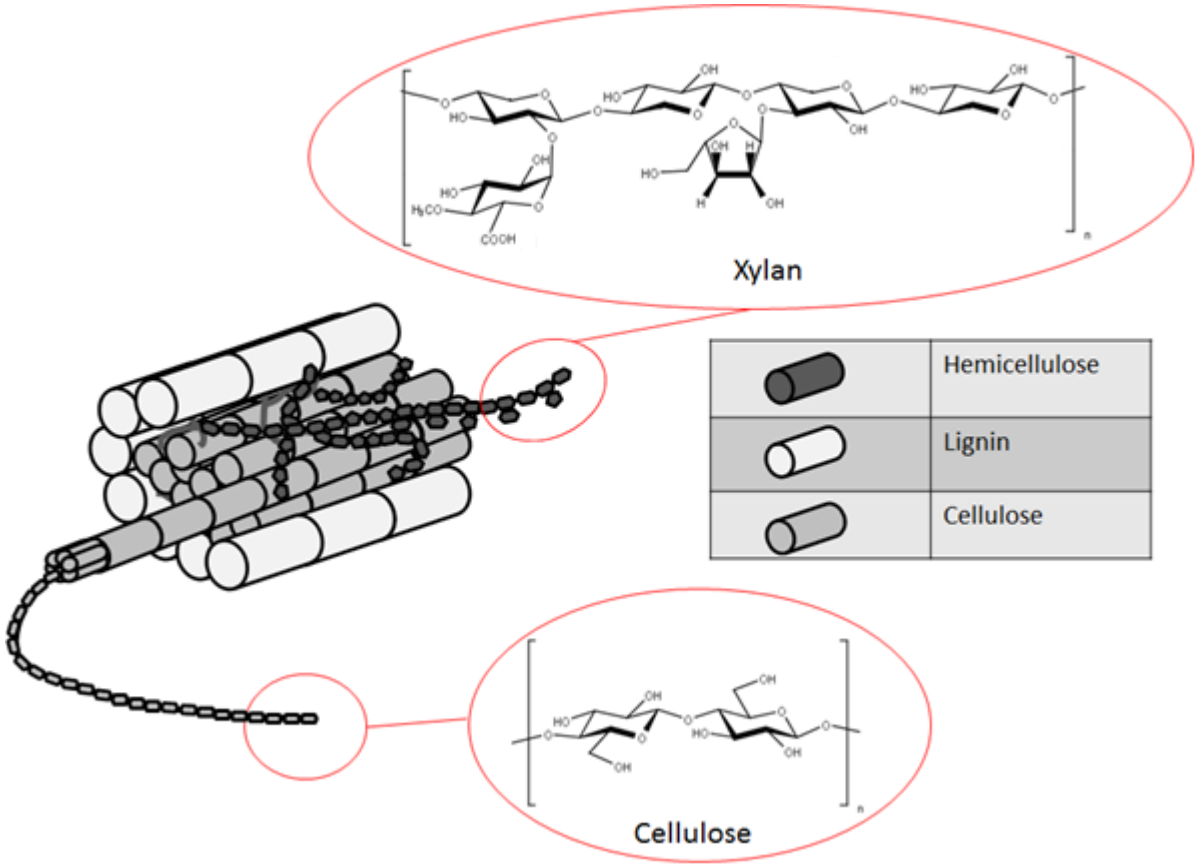


Figure 1: A cartoon representation of lignocellulose.

Shown are the polymer lignin, interacting with polysaccharides hemicellulose and lignin. Two molecular groups are shown, representing the structure of cellulose and a generic structure of xylan, one of the components of hemicellulose. Cellulose is composed of repeating units of α-D-glucose bound by β-1,4,glucosidic bonds. Conversely xylan contains not only bonds between the α-D-xylose monomers, but also additional components branching from the main chain. These components include the α-L-arabinofuranose and 4-O-methyl glucuronic acid, as displayed in the example above, however can also include other groups such as acetate.

therefore hemicellulose is non-homogenous (Saha, 2003). “Hemicellulose” is therefore a broad classification that describes multiple types of polysaccharide, including xylan and glucomannan. Hemicellulose structure shows similar diversity, as bonds between monomers can be both alpha and beta glucosidic, and shows examples of branches (Dutta *et al.*, 2012). Sugars in hemicellulose may also be acetylated and the polymers can contain uronic acids, such as 4-*O*-methyl glucuronic acid (Tramice *et al.*, 2009).

Finally, lignin is a polymer made from cross-linked phenolic rings synthesized as part of the phenylpropanoid pathway, therefore it is not a polysaccharide and does not yield sugars (Stewart *et al.*, 2009).

The complex structure of lignocellulose limits fermentation by organisms currently used in the biofuel industry (Cragg *et al.* 2015). Lignocellulosic biomass is therefore largely used as animal feed, as some vertebrates are able to degrade the lignocellulose due to microbes of their stomach (Bayané *et al.* 2011). In order to generate energy from lignocellulose, the lignocellulose may instead be burnt, however this is an inefficient mechanism to obtain energy that risks release of toxic GHG, such as wood ash and nitric oxides (Rosillo-Callem *et al*. 2007). Current biofuel research looks into enabling cells to ferment the sugars within lignocellulose to ethanol.

Lignocellulosic biomass available for 2G ethanol production can be divided into agricultural residues, energy crops, and forest residues. Agricultural residues refer to the lignocellulosic plant biomass from sugar or starch rich crops which are produced as the waste from another procedure (Carriquiry, *et al*. 2011). Forest residues on the other hand describe the lignocellulosic biomass which is obtained as a byproduct from logging and wood processing mills (Carriquiry, *et al*. 2011). “Energy crops” are crops that are cultured specifically for biofuel production and often lack value outside of this purpose. Energy crops are better able to grow in unfavorable conditions, such as drought or nutrient deficiency, and energy crops can also increase ethanol production per hectare (Antizar-Ladislao *et al.*, 2008). The improved tolerance of energy crops to negative growth conditions could also enable energy crops to be grown on marginal agricultural

land, which lacks the nutrients required for food crop cultivation (Balan *et al.*, 2013)(Ra *et al.* 2012),

When cultured on agricultural land, the improved robustness of energy crops is important in reducing the use of fertilizers in biofuel production, thereby reducing the amount of nitrogen added to the soil which can be fixated to nitric oxides. The low nitrogen requirement of energy crops is due to high nitrogen use efficiency, such as 104.9 kg kg^{-1} for napiergrass (*Pennisetum purpureum* schumach), meaning a greater proportion of soil nitrogen is fixed into useful amino acids for the crop (Ra *et al.* 2012). Energy crops include sweet and fiber sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), miscanthus (*Miscanthus sinensis*) and switch grass (*Panicum virgatum* L.) (Koçar, *et al.* 2013).

Perennial forage crops are of particular interest as energy crops, and include monocots such as sorghum (*Sorghum bicolor*), switch grass (*Panicum virgatum*) or indiangrass (*Sorghastrum nutans*) (Carriquiry, *et al.* 2011). Perennial forage crops can be grown over longer periods of time than traditional crops, such as corn, enabling a large agricultural period in which carbon can be amassed in biomass. Perennial forage crops are associated with significant lignocellulosic biomass production compared to other energy crops, such as napiergrass (52 t ha^{-1} y^{-1}) compared to sugar cane (13.5 t ha^{-1} y^{-1}) (Mullet*, et al.* 2014) (Sharwood *et al.*, 2016) (Ra *et al.* 2012).

In order to ferment lignocellulose using microbes from the biofuel industry, the polysaccharide must first be saccharified before the fermenting cells are introduced (Aro, 2016). However, while saccharification of starch simply requires addition of enzymes (Naik *et al.*, 2010), it is a greater challenge to complete saccharification of lignocellulose due to its complex molecular structure. Lignocellulose saccharification is therefore completed by pretreatment, followed by enzymatic saccharification. Pretreatment is a process by which lignocellulose is depolymerized by a series of chemical reactions (Jönsson *et al.* 2016).

Pretreatment functions to enable cellulose and hemicellulose saccharification, by disrupting the interactions between cellulose and the polymers lignin and hemicellulose to increase access to enzymes which can degrade cellulose. The ester linkages that connect the different polymers of lignocellulose together must therefore be broken. Such linkages are formed from oxidized sugars, side chains of hemicellulose and phenols (Iiyama *et al.*, 1990). With the polymers separated, it is then important to disrupt hydrogen bonds between the cellulose fibers in order to reduce crystallinity, increasing both the porosity and surface area of cellulose to further increase enzyme access (Fig. 2B) (Li *et al.*, 2010). The biomass generated by pretreatment is referred to in this report as “Delignified Biomass” (DLB), representing the removal of lignin from the polysaccharide produced.

Pretreatment can be achieved by physical, chemical and biological reactions, each with their own advantages and disadvantages (Kumar *et al.*, 2009). Pretreatment methods include dilute acid pretreatment, steam explosion pretreatment, alkaline pretreatment, wet oxidation and organosolvent pretreatment (Jönsson *et al.* 2016) (Hu *et al*. 2012). In this report the focus will be on acid pretreatment due to its relevance in Shell’s current procedures.

Dilute acid pretreatment is an actively studied method, associated with cellulose exposure, alongside production of xylose as the conditions degrade xylan polysaccharides. The dilute acids used for pretreatment can include sulfuric acid, nitric acid and phosphoric acid. The acid acts as a catalyst to reduce lignocellulose recalcitrance through hydrolysis of ether bonds (Zheng *et al.*, 2013) (Zheng *et al.*, 2009). However, dilute acid pretreatment is a slow procedure and therefore must be performed at a high temperature range in order to accelerate the rate of lignocellulose degradation, 160-180^{o}C (Noparat *et al*., 2015). High temperatures can result in sugar degradation and the production of side products from lignin decomposition (Modig *et al.*, 2003) (Berlin et al. 2006).

Enzymatic saccharification using cellulase enzymes follows pretreatment, facilitating the breakdown of the cellulose contained within the DLB, releasing glucose monomers at potentially

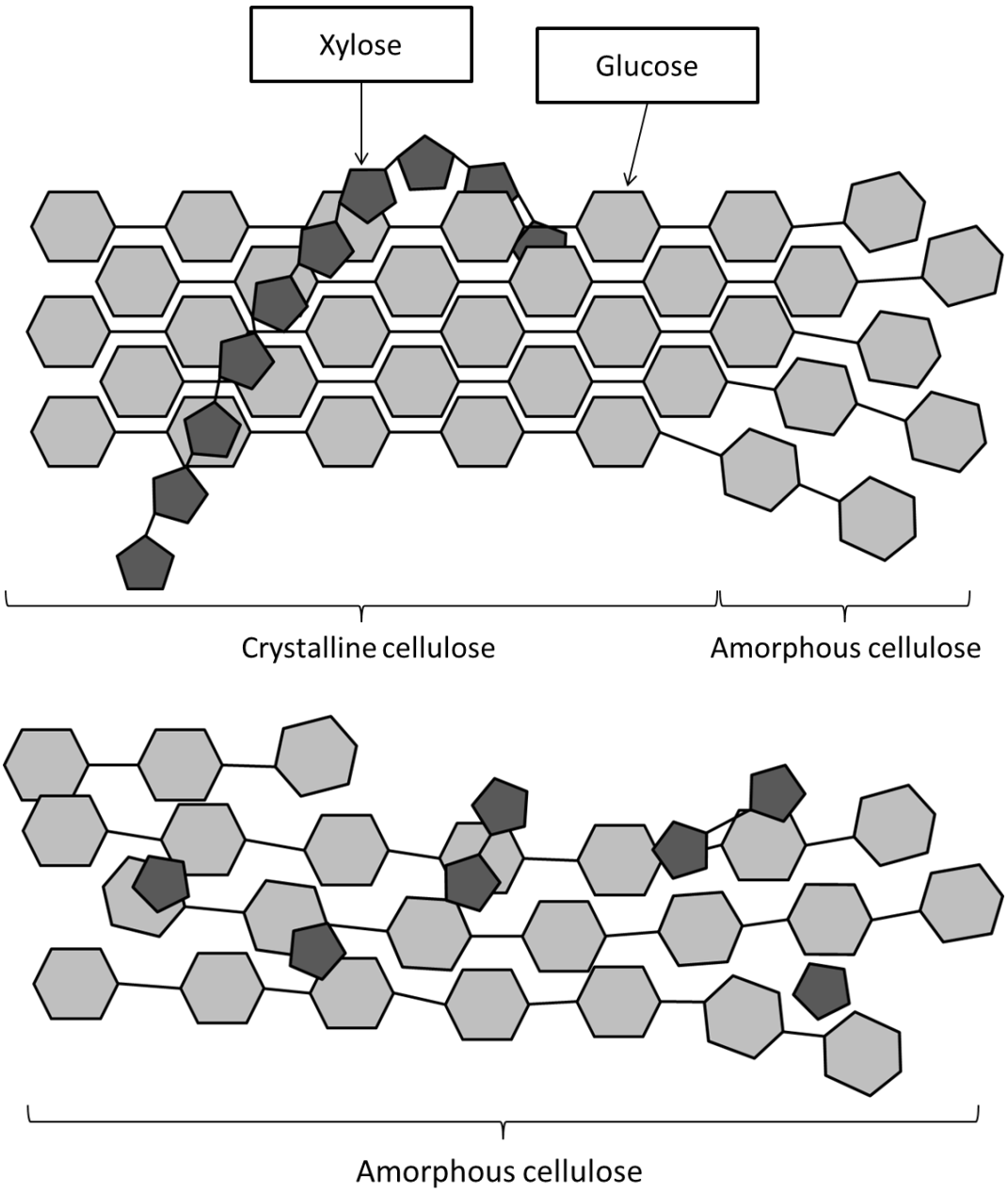


Figure 2 Schematic representation of the structure of cellulose and xylose both before and after pretreatment.

(A) Structure of cellulose and xylan in lignocellulose prior to pretreatment. Light grey hexagons represent glucose molecules in cellulose. Dark grey pentagons represent the pentose xylose sugars in xylan. (B) Structure of cellulose and xylan following pretreatment. Xylan degraded to xylose and crystaline cellulose structure disrupted, enabling enzymatic saccharificaiton.

high yields. Cellulase enzymes must be capable of hydrolyzing the 1,4-β-glycosidic bonds between glucose monomers, and are divided into 3 categories: cellobiohydrolases, endo-1,4-β-D-glucanases and 1,4-β-D-glucosidases (Ulson de Souza *et al.* 2013). It is also important to note that cellulase enzymes are largely suited to amorphous regions of cellulose, in which fibers are randomly distributed, as the enzymes cannot access the bonds in highly crystalline regions, in which fibers are closely bunched together by hydrogen bonding, (Fig. 2A)(Zoglowek *et al.* 2015), necessitating the pretreatment stages to reduce crystallinity of the cellulose polymer (Fig. 2B). Saccharification of the DLB generates the "hydrolysate," a sugar rich slurry.

In order to generate ethanol from the hydrolysate, fermentation of the sugars is required. *S. cerevisiae*, the major organism in the 1G biofuel industry has continued to be applied in 2G ethanol production (Mcintosh *et al.* 2016). *S. cerevisiae* used in biofuel production are associated with high yields of ethanol, which can be challenging to replicate in other organisms better suited to lignocellulosic ethanol production, such as *Thermoanaerobacterium saccharolyticum* or *Scheffersomyces stipites* (Papini *et al.* 2012)(Shaw *et al.* 2008). While the robust nature of *S. cerevisiae* causes it to be selected in many cases for 2G ethanol production (Hahn-Hägerda *et al.*, 2007), it faces limitations in generating high yields from hydrolysate. Limitations occur when producing 2G ethanol because *S. cerevisiae* must take into account multiple addition variables, including fermentation of pentose sugars (Ha *et al.* 2011) and the presence of new compounds produced during pretreatment (Cray *et al.*, 2015).

The new pressures derived from the hydrolysate conditions results in incomplete fermentation which currently limits 2G ethanol production. Overcoming the limitations of hydrolysate is a major factor in Shell's current methodology.

2.4. Shell's methodology for lignocellulosic fermentation

Over the last decade Shell has been researching methods of lignocellulosic ethanol production. Shell's current procedure is described in both Figure 3 and Figure 4, with the current focus to

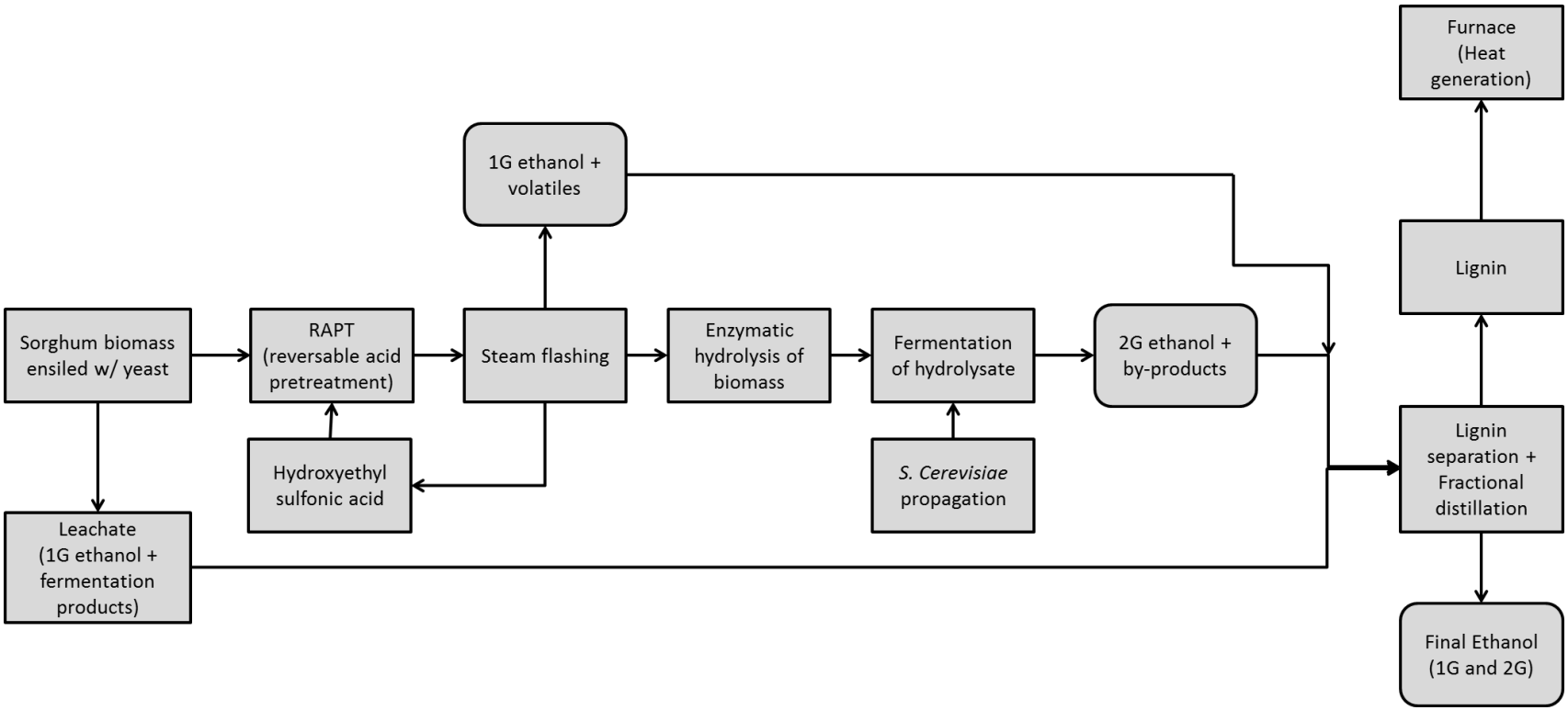


Figure 3: Schematic representation of the Silenus procedure

This schematic details the interconnectivity between ensiling (generation of 1G ethanol), RAPT (cellulose and xylose production), Enzymatic hydrolysis (glucose and xylose production) and fermentation (Production of 2G ethanol).

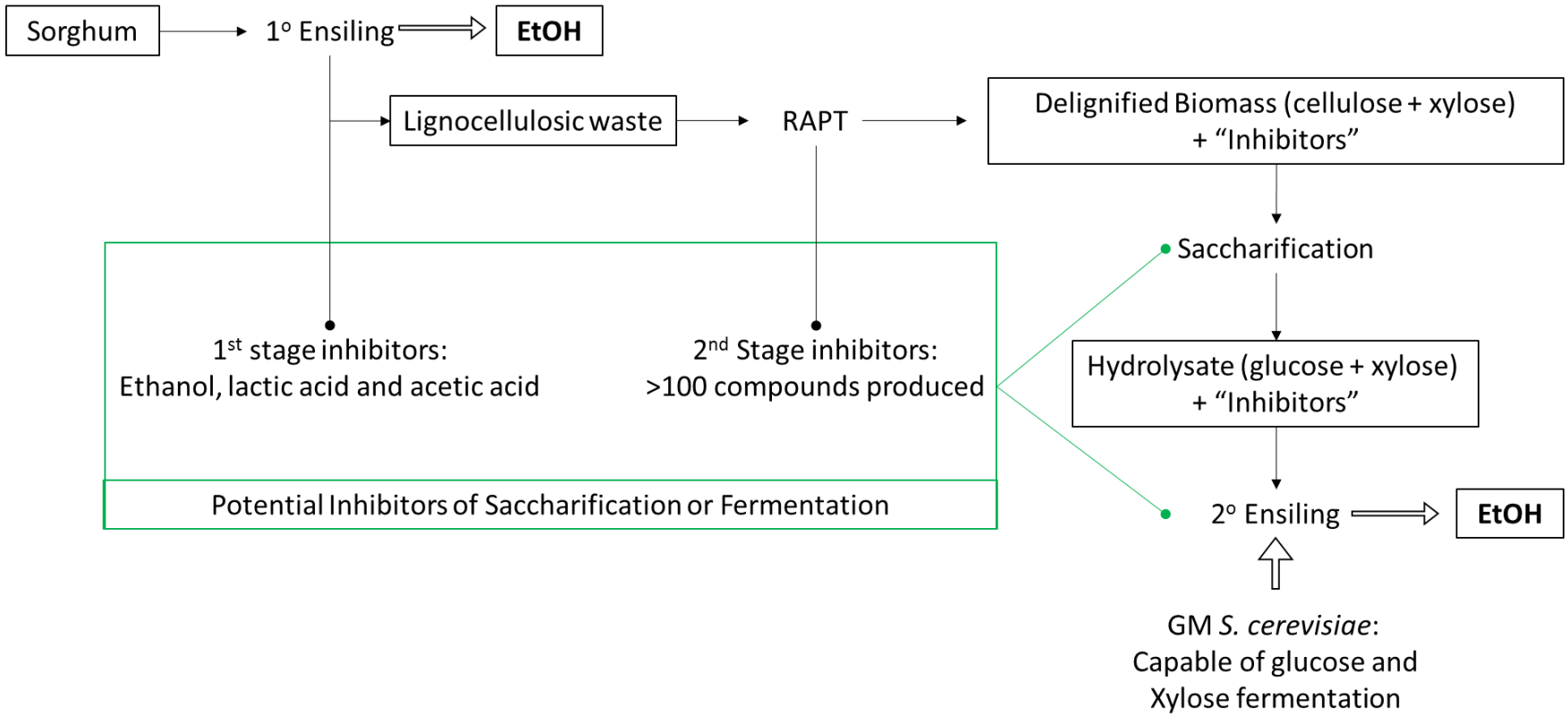


Figure 4: *Schematic representation of Sorghum fermentation following the Silenus procedure*

A schematic representation of the conversion of sorghum biomass to ethanol in the Silenus procedure, taking into account the compounds and inhibitors obtained.

produce high levels of both 1G and 2G ethanol from an energy crop harvest, in a procedure termed the “Silenus procedure”.

The Silenus procedure is based on generating two ensiling stages. The first ensiling stage, in which a harvested crop is ensiled with ethanol-fermenting yeast, produces 1G ethanol from free glucose monomers. Following production of 1G ethanol, the lignocellulose is then subjected to concentrated acid pretreatment, degrading lignocellulose to produce cellulose, xylan and xylose. The lignocellulose is then brought to a standardized moisture content and undergoes saccharification with cellulose enzymes to yield glucose and xylose. The glucose and xylose are then fermented in a second ensiling stage by a genetically modified *S. cerevisiae* strain.

2.4.1 Crop selection

Shell currently uses the crop sweet sorghum (*Sorghum bicolor* (L.)) for 2G ethanol production. Sorghum crops are a member of the grass family and are a robust plant capable of C_4 photosynthesis (Paterson, *et al.* 2009). The ability to complete C_4 photosynthesis increases efficiency of photosynthesis, which is vital for growth of sorghum in regions such as the northeast Africa, as C_4 photosynthesis allows drought tolerance which is essential for growth in dry regions. Resistance to hot, dry conditions also make sorghum ideal for growth in the Southern states of the US. In addition select cultivars of sorghum can tolerate periods of waterlogging from sudden rainfall to show only small reductions in biomass (Promkhambut *et al.* 2010). Sorghum is superior to other crops, such as sugar cane, in respect to weather tolerances. It is not limited by dry, tropical conditions (Everingham *et al.*, 2002). Furthermore, sorghum is supported by government funding, another disadvantage facing sugar cane (US Department of Agriculture, 2015)(US Department of Agriculture, 2014). .

Sorghum yields both free sugars and lignocellulose offering two different sources of metabolite for ethanol production, with the lignocellulose enabling 2G biofuel production. The free sugar also allows fermentation during the initial storage and ensiling, as opposed to needing enzymes

to breakdown starch (Shoemaker *et al.* 2010), as would be required for corn (Naik *et al.*, 2010). The ability to tolerate drier climates can be useful beyond the risk of drought, as the crop is more likely to tolerate land undesirable for food crop growth, reducing direct competition with food crop cultivation.

Sorghum is currently grown in the USA, Africa and China. Sorghum can be used in order to obtain the syrup sorghum molasses from milling and evaporating the juice (Kuepper, 1992), for crop residues to use as animal feed, or is fermented for alcoholic beverages such as Baijiu. This creates some competition over the use of this crop as a fuel source instead of its application for food products. Due to the differing application of sorghum between countries, there are different suggestions for how sorghum can be used for ethanol production depending on the source country in question (Gnansounou *et al.* 2005). In the USA application of sorghum is limited to animal feed, therefore has low value compared to other crops. Application of sorghum as a bioethanol crop in Africa may be more challenging where it is a major source of food, and where 55% of sorghum cultivation areas were located in 2001 (Belton *et al.* 2003), as it has a greater comparative value for the African community.

Shell proposes that sorghum has the potential as an energy crop in the United States, China and India. As previously discussed, the majority of world sorghum production is actually completed in Africa, where sorghum could be considered a food crop and therefore would face competition as a source of ethanol.

Shell allows sorghum to be grown until the flowering period, at which point it is monitored until it reaches the so-called “Soft Dough” stage of growth, approximately 15 to 25 days after flowering when the seed is soft to the touch (Espinoza *et al.*, 2014). At this point the sorghum is harvested either by hand or with machine, and undergoes primary ensiling.

2.4.2. Primary ensiling

Ensiling is a method traditionally used to retain crops during storage, so that there is minimal waste of material, tightly compacting the plant biomass in an anaerobic environment with bacteria to lower pH and preserve the lignocellulose in the crop (Rosillo-Callem *et al.*, 2008). The Shell ensiling methodology differs from traditional ensiling, in which anaerobic bacteria are used, by consciously introducing yeast cells not to preserve the crop, but to generate ethanol and other volatile compounds. Acid may also be added in this stage in order to reduce plant respiration and enhance yeast fermentation. If required, the antibiotic lactrol can also be introduced to remove contaminating microbes (Radtke *et al.* 2015).

Once ethanol has been produced by primary ensiling, the lignocellulose is separated from the ethanol. Two methods have been proposed to achieve this. In one iteration, a superheated steam dryer is applied to the lignocellulosic biomass in order to remove high levels of volatile molecules from the solid. Alternatively, in the second iteration, the liquid phase can be extracted by draining the soluble products to an oxygen deprived lagoon, a large body of solution, where the ethanol can be captured separately from the liquid phase by fractional distillation at a later date. The use of a lagoon is a cheaper, but less efficient method to separate volatiles from the lignocellulosic biomass, therefore while the majority of ethanol is removed by using a lagoon, some ethanol and fermentative products are retained. The lignocellulosic biomass is therefore described as wet biomass (Radtke *et al.* 2015).

Ensiling overcome one of the major problems limitations of 2G ethanol production from lignocellulose-containing energy crops, that contaminating organisms will respire the free sugars of the energy crop during storage and transport to a bioethanol plant, producing inhibitors of fermentation and saccharification. The use of yeast in the ensiling procedure therefore not only obtains yields of 1G ethanol immediately, but also allows storage of the lignocellulosic biomass without the risk of producing inhibitors or affecting yield. Evidence shows that the lignocellulosic

biomass and products of ensiling can be stored for up to 700 days without significant loss of material, following primary ensiling (Radtke *et al.* 2015).

2.4.3. Reversible Acid Pretreatment

Shell's current procedure uses a lagoon to separate lignocellulosic biomass from fermentative products of the yeast cells due to the cost of SSD. The lignocellulose is then transported to a pilot plant during which time it is subject to Reversible Acid Pretreatment (RAPT). RAPT is a variation of the acid pretreatment method, in which concentrated α-hydroxyethane sulfonic acid (HESA) is introduced. As a concentrated acid is used, lower temperatures, 125 ˚C, can be used to achieve lignocellulose depolymerization compared to dilute acid pretreatment, 160-180 ˚C (Noparat *et al*., 2015). Under moderate temperatures of 125 ˚C, HESA can break down the bonds of the lignocellulose and reduce the crystallinity of the cellulose. The lowered temperature also reduces the concentrations of the degradation products of lignin and decomposed sugars (Weider and Blackbourn 2016).

Theoretically, the RAPT method should be more expensive than dilute acid pretreatment, as the acid is more concentrated and must be removed, not neutralized, from the resultant DLB slurry. Removal of acid has limited previous concentrated acid pretreatment methods, making the procedure too expensive (Singh *et al.*, *2015*) (Kumar *et al.* 2009). RAPT enables collection of concentrated acid due to an additional step following pre-treatment in which increased heat and pressure volatize the acid, causing it to evaporate and largely be removed from the biomass. The collected HESA can then be recycled (Weider and Blackbourn 2016).

The produced DLB slurry contains high levels of amorphous cellulose and xylan. In addition, acid pretreatment can further degrade the xylan in order to produce high yields of the monomer xylose. In RAPT the concentration of xylose is as high as 25 g l^{-1}; therefore RAPT generates high concentrations of the xylose in addition to cellulose. The DLB slurry will also contain products of primary ensiling, such as ethanol, alongside lignin and compounds produced from

lignin decomposition, however some of these compounds are predicted to be volatized and removed alongside HESA during vaporization, with ethanol being of great interest (Weider and Blackbourn 2016).

The ability to vaporize HESA in order to collect the acid creates a cycle in which HESA can be used multiple times, and therefore the volume HESA to be purchased is reduced. Removing HESA from the DLB will also have positive consequences downstream compared to dilute acid pretreatment, as acids when neutralized produce inhibitory compounds such as gypsum. The greatest improvement of the RAPT procedure compared to dilute acid pretreatment is the reduction of the concentration of byproducts produced during pretreatment (Weider and Blackbourn 2016). RAPT also avoids the lignin depolymerization seen at the temperatures used in dilute acid pretreatment, as depolymerized lignin is associated with damaging reaction vessels by sticking to surfaces, as well as changing the activity of cellulase enzymes (Rahikainen *et al.*, 2013).

2.4.4. Saccharification of cellulose using commercial enzyme package

The pretreatment of lignocellulosic biomass by RAPT produces a DLB slurry rich in cellulose, ~46% of remaining solid, as well as xylan, 20% of remaining solid. In order to obtain monosaccharides from cellulose and xylan for ethanol production, saccharification enzymes must be used. Shell has not produced an internal method of producing cellulase enzymes, instead using commercial enzymes for cellulose saccharification.

Commercial enzymes for cellulose saccharification include Novozyme's Cellic Ctec and Dupont's Accellerase (Ju *et al.*, 2014). An advantage of using commercial enzymes is that they can be purchased in large amounts suitable for large scale saccharification. Efforts have also been made to produce enzymes for hemicellulose breakdown, such as Novozyme's Htec2, in order to support the role of cellulose degrading enzymes (Joe *et al.* 2015). More often a mixture

of cellulase and hemicellulase is applied in order to maximize sugar production, as is the case for modern iterations of Ctec2 (Hu *et al.*, 2015).

Saccharification of the pre-treated biomass is completed using the enzyme mixture Ctec3, which contains cellulases, hemicellulase and β-glucosidases. Ctec3 is a commercial enzyme blend provided by the Novozymes LLC, and has the ability both to degrade cellulose and degrade hemicellulose. The exact combinations of enzymes has not been revealed to Shell, however a partnership has been formed between Novozyme and Shell so that research is communicated with one another, enabling future generations of the enzyme package to be developed to Shell's requirements. Research in the lab has demonstrated high yields of glucose, up to 80%, can be achieved using 5% Ctec3 to hydrolyse pretreated biomass.

A limitation in using saccharification enzymes is that the moisture content of the DLB slurry is directly correlated to yields. For the lignocellulosic biomass to receive funding from government support systems, the moisture must be limited to a high UDS during pretreatment. The DLB slurry produced therefore contains 11% undissolved solid. To improve yields the undissolved solids in the DLB slurry is lowered to 10%. However to achieve this, products of primary ensiling, secondary ensiling or volatization must be added back to the DLB. This introduces additional compounds to the DLB.

Saccharification must be completed at a temperature of 53 ˚C for optimal enzyme performance. This creates a need for heating which is supplied by combustion of lignin. Furthermore, stirring of the biomass is required to mix the enzyme into the DLB slurry at equal consistency. The energy requirements in combination with the cost of enzyme makes saccharification an expensive stage of 2G ethanol production. To compensate for this expense, a projected yield of 85% glucose must be reached.

2.4.5. Fermentation of sugars using commercial *S. cerevisiae* GM strain

Once glucose and xylose sugars are made accessible by saccharification, a fermentative organism must then be used in order to produce ethanol. Fermentation is achieved in the Silenus project by the use of a commercially available, genetically modified *S. cerevisiae* strain C5fuel, provided by Mascoma. Fermentation of the saccharified slurry, termed hydrolysate, is a secondary ensiling stage in which the 2G ethanol is produced.

C5fuel is capable of the fermentation of xylose alongside glucose. It is important for C5fuel to be able to ferment xylose due to the high concentration contained within lignocellulose. Pentoses, such as xylose, are vital for gaining high yields of lignocellulosic ethanol, as they make up 30% of the sugars available in lignocellulose. The abundance of xylose means that even complete consumption of other sugars would only result in a 70% ethanol yield, which would not produce profits large enough to overcome the costs of pretreatment and enzymatic saccharification (Ha *et al.*, 2011). Furthermore, xylose can make to 40% of sugars in some hardwood plants (Casey *et al.* 2010). As degradation of lignocellulose releases large amounts of xylose, it becomes increasingly important to introduce an organism that can metabolize this sugar. One of the major limitations that faced lignocellulosic ethanol production was that consumption of xylose for ethanol production is a process that is largely limited to select bacteria, such as *Thermoanaerobacterium saccharolyticum*, which are unable to produce the yields demanded by industry without substantial genetic modification (Shaw *et al.* 2008).

Due to the demand for xylose fermentation, numerous experiments have been undertaken in order to generate *S. cerevisiae* strains capable of rapidly converting xylose to ethanol. Of the many studies completed to achieve xylose fermentation, efforts have been largely focused on introducing bacterial xylose isomerase (Brat *et al.*, 2009) or fungal xylose isomerase enzymes (Zhou *et al.* 2012). Another method being researched is the expression of xylose reductase that utilizes the same co-enzyme as xylulose dehydrogenase (Ha *et al.* 2011). C5fuel is believed to

utilize a bacterial xylose isomerase strain to achieve fermentation of 20 g l^{-1} xylose within 24 hours, overcoming one of the major enzyme limitations to the RAPT procedure.

Glucose and xylose compose the majority of sugars in the sweet sorghum hydrolysate, therefore complete fermentation of these metabolites should lead to an ethanol yield of >85% of total sugars. However, C5fuel has the capacity to produce the byproduct glycerol during stress, or divert sugars towards biomass production. Together, these alternative routes of sugar metabolism have limited ethanol yields to 80% at 48 hours.

C5fuel cells are provided to Shell at small volumes which are not suitable for direct use in fermentation. In order to cultivate the volume of cells required for fermentation propagation is used. Propagation is a respiration step in which yeast biomass is rapidly grown. The Shell procedure also takes into account the role of conditioning, introducing stress to the cell during propagation in order to produce future generations of the cell line adapted to the aforementioned stress.

2.4.6 The limitations of the RAPT procedure

While the Silenus procedure is an effective method to generate high yields of sugars, 80%, and 2G ethanol, 80%, it is unable to recuperate the costs of both the chemicals and organisms used to make a significant profit. It is important to understand the limitations of saccharification and fermentation to improve yields and therefore enable the RAPT procedure to generate profit.

Inhibition is the major obstacle facing saccharification and fermentation. Inhibitors are generated at two key points in over the course of lignocellulosic ethanol production: during primary ensiling and during RAPT. The exact identity of the compounds that act as inhibitors, however, is unknown.

During primary ensiling three key compounds are produced: ethanol, lactic acid and acetic acid. The presence of free sugars in sorghum enables production of 1G ethanol in order to improve

overall ethanol production. However, ethanol itself has been shown to be an inhibitor of wild type *S. cerevisiae*, meaning that primary ensiling may limit secondary ensiling (Adeboye, 2014). Ethanol is associated with a large number of unfavourable effects, such as reducing the cell viability, changing permeability of membranes to ions, altering vacuole morphology and even causing cell death (Brown *et al.*, 1981). One notable effect of ethanol is to disrupt protein structure, such as the glycolytic enzymes and can even trigger their subsequent denaturation (Ma *et al.*, 2010). Together ethanol can cause both cell death and glycolysis inhibition, which would reduce the rate of ethanol production and could lead to a reduced final yield.

Acetic acid and lactic acid are the products of contamination by microbes such as *Acetobacter* and *lactobactus*, (Hofvendahl *et al.* 2000) however primary ensiling with yeast limits the concentration of organic acids. Nevertheless lactic acid and acetic acid are found in excess of 6 g l^{-1}in lignocellulosic biomass post ensiling. Wild type *S. cerevisiae* is inhibited when in the presence of both acetic acid and lactic acid. *Narendranath et al.* found that both organic acids acted with high statistical significance ($p<0.001$) to achieve a negative synergistic inhibition, able to reduce growth of the cell, glucose consumption and ethanol production at greater levels in combination than they would alone. (Narendranath *et al*., 2001). The synergistic interactions between these two compounds are a formidable concern as resistance genes in the genetically modified C5fuel that lessen the inhibition of a compound in isolation can become masked when both compounds are present.

Cellulase inhibition, on the other hand, is subject to change depending on the origin of the enzyme. Different cellulases show differing levels of inhibition, and some cases inhibition recorded require high levels of compounds not seen in the DLB slurry, such as lactic acid concentration exceeding 30 g l^{-1} before inhibition is noted, and 90 g l^{1} before inhibition becomes substantial (Iyer and Lee, 1999).

Chemical pretreatment is associated with production of a broad range of undesired components as the chemical react with lignin, degrade free sugars and release compounds such as uronic acid from hemicellulose. The extent of this range of compounds can vary depending on the pretreatment method used.

It is very difficult to analyze the compounds present in DLB following RAPT pretreatment. This is due to the significant overlap of peaks in analytical techniques such as NMR and mass spectrometry, making data illegible, particularly for non-polar compounds. Looking into the literature, phenolic acids and phenolic aldehydes have been identified as being produced by dilute acid pretreatment, and while reduced in concentration, phenolic acids and aldehydes are predicted to be retained when RAPT pretreatment is used instead (Almeida *et al.*, 2007)(Weider and Blackbourn 2016).

More than 20 phenolic compounds produced from lignin decomposition have been identified following dilute acid pretreatment or steam pretreatment (Almeida *et al.*, 2007). The concentration of phenolic acids can be as low as 2 mg l^{-1} (catechol) or as high as 146 mg l^{-1} (Hibbert's ketone) in spruce hydrolysate, with total phenolics at 3.1 g l^{-1}. However, these numbers can vary between differing pretreatment methods applied and the source of plant biomass (Almeida *et al.*, 2007). While the low concentrations of phenolics could suggest limited inhibition of *S. cerevisiae* strain C5fuel or Ctec3, one such species, coniferyl aldehyde can be toxic to wild type *S. cerevisiae* at concentrations as low as 1 mM (Adeboye *et al.* 2014).

The effect of phenolic compounds on cellulases has been variable; the impact on Ctec3 could be positive or negative. The phenolic compound vanillin has been shown to achieve a reversible, non-competitive inhibition of cellulase at concentrations of 5 g l^{-1} (Li *et al.* 2014), higher than phenolics expected to be produced. On the other hand it is also argued that some phenolic groups, such as syringic acid, may aid cellulosic degradation by activating cellulase, with syringic acid inhibitory at 0.5 g l^{-1}, yet appears to activate cellulase at 2–4 g l^{-1} (Zhao *et al.*

2014). This suggests phenolic compounds could have a broad range of effects going forwards, and an understanding of such activities could be vital in reaching a balance between saccharification and fermentation optimization.

Other compounds that may be produced during RAPT include organic acids and aldehydes, such as furfural, and uronic acid. In addition to lignin degradation, compounds can be derived from degradation of sugar molecules, or unbranching from xylan during pretreatment. (Almeida *et al.*, 2007).

Overall, primary ensiling generates a small range of compounds at high concentrations (Radtke *et al*. 2015), whereas RAPT pretreatment produces a broad variety of compounds at lower overall concentrations (Weider and Blackbourn, 2016). Determining the role that the byproducts of ensiling and RAPT pretreatment have on fermentation and hydrolysis is important as it will enable the methodology to be evaluated. As Silenus optimization continues, changes to the conditions of RAPT pretreatment or ensiling will impact the concentration of all compounds produced. An understanding of the inhibitors produced in Silenus would allow predictions to be made about the impact of increasing or decreasing their concentration, preventing potentially wasteful tests being made on fermentation and saccharification. Furthermore, information on the role of chemical byproducts will enable modifications to be made on Ctec3 and C5fuel, reducing the impact of an inhibitor.

It is especially important to overcome limitations at small-scale which prevent desired yields from being reached. When saccharification and fermentation are completed at larger-scales, new concerns are introduced to further lower yields, such as uneven heat distribution compared to small-scale. Fermentation can also be hindered by poor propagation, as a result of the Crabtree effect, limiting cell growth due to a respirofermentive metabolism, which is more pronounced at high sugar concentration (Rosas-Lemus *et al.* 2014)(Gómez-Pastor *et al.* 2011)(Vieira *et al.*, 2013). If limitations of fermentation and saccharification are tackled at small

scale it will reduce the need for troubleshooting once the procedure is scaled up, a much more expensive option.

C5fuel and Ctec3 are still currently undergoing modification, with research focused on increasing the resistance to the inhibitory compounds within the DLB. In order to improve saccharification and fermentation, however, an understanding must be made of the most relevant inhibitors to the current generations of Ctec3 and C5fuel within the Silenus project.

2.5. Aims of the project

This project will investigate the hypothesis that inhibitors of fermentation and saccharification are a limitation to the production of 2G ethanol and experiments will be performed to validate the hypothesis that components of primary ensiling and RAPT processing have a negative impact on the kinetics of fermentation and saccharification. A constraint of this work is that the experiments completed must be close to the conditions that will be seen in industry. This so that the effect of compounds is understood in relation to the complete procedure.

The first aim of the project, therefore, is to understand whether inhibitors impact the activity of the Ctec3 enzymes, by evaluating how the concentration of enzyme impacts the saccharification of DLB. Looking into how rate and yield of glucose production is impacted both by increasing the concentration of Ctec3 and reducing the concentration of components in the DLB could give insight into how inhibitors effect final yields and kinetics. Determining how inhibitors impact yields could give insight into how to improve the Silenus procedure to generate more glucose for fermentation.

It is important to evaluate the two stages at which inhibitors could be introduced into the pre-treatment process: primary ensiling and RAPT. While multiple organic acids can be produced during storage, it is important to evaluate the role of ethanol, lactic acid and acetic acid of saccharification. Ethanol, lactic acid and acetic acid are the three compounds produced during

primary ensiling, therefore determining their impact on saccharification gives insight as to whether the ensiling procedure used in Silenus could adversely affect glucose yield. The concentration of the products of primary ensiling can be controlled by crop selection or altering of Silenus methodology, and any inhibitory effects will important to take into account when changing the methodology.

The role of RAPT in saccharification performance is a second key stage at which inhibitors may be introduced. As RAPT alters the production of by-products compared other dilute acid pretreatment, making determination of exact inhibitors challenging, efforts will focus on broadly identifying the chemical nature of compounds that are responsible for inhibition of saccharification. To understand how the products of RAPT impact saccharification, experiments will discriminate the role of non-polar and polar components on enzyme kinetics. There will be a focus on the inhibitory nature of non-polar, potentially phenolic products of lignin decomposition.

Once the role that primary ensiling and RAPT have on saccharification is known, experiments will be repeated to understand the role of primary ensiling and RAPT on fermentation. Attempts will be made during these experiments to find any compounds that inhibit both saccharification and fermentation. If possible, reducing the concentration of the compounds through changes to the methodology may increase final yields of ethanol.

Independent of saccharification, a study which focuses on C5fuel propagation and culturing will be completed with respect to a known growth inhibitor of wild type *S. cerevisiae*, furfural. This is to determine whether culturing of the C5fuel strain impacts the inhibitory activity of components contained within the hydrolysate.

3. Results

3.1. Enzymatic saccharification

3.1.1. Confirmation of max digestibility with 40% Ctec3 dose

Enzymatic hydrolysis is a saccharification process used to obtain glucose from the breakdown of cellulosic fibers, producing free sugars for fermentation. The ability of enzyme inhibitors to reduce the yields and kinetics of saccharification is a concern for the lignocellulose-derived biofuel industry. The enzyme solution Ctec3 was introduced to break down the bonds of cellulose, alongside some remaining hemicellulose, in order to obtain glucose and xylose. In practice the enzyme dose for this process is 5% w/w relative to the cellulose content in the DLB. The low 5% dose was selected in order to maintain a balance between saccharification efficiency and cost. In this experiment the dosing was increased to 40% to prove that greater yields can be gained by increasing the relative concentration of enzyme with respect to inhibitors, alongside determining the feasibility of reaching a high, near maximum, digestion of cellulose.

Glucose yield can be calculated to determine saccharification ability of Ctec3. Glucose yield is calculated from the total cellulose content of the biomass sample, taking into account the initial glucose levels before addition of Ctec3, as well as the dosing concentration of Ctec3 itself. A measurement of the amount of Ctec3 added is needed in order to obtain an approximation of sugars added from the stabilizing solution. For this reason, time 0 h is presented as having a 0% yield.

By comparing the yield and the rate of hydrolysis at 5% Ctec3 dosing with the 40% dose, a significant improvement of 14% glucose yield was observed with an increased concentration of Ctec3. Looking at initial yields, the expectation would be for rate of glucose production to be 8 fold greater for the 40% dose than the 5% dose, however the yield of the 40% dose at 2 hours is

50% glucose production, whereas it is 18% for the 5% dose, less than a 3 fold increase in rate (Fig 5A). A potential reason for the percentage yield being lower than anticipated is inhibition of Ctec3. The correlation between increased concentration of cellulase enzymes and an improved glucose yield suggests a significant impact of inhibitors of Ctec3 activity on final glucose yield. As scaling up the two-step procedure of hydrolysis and fermentation would result in a reduced yield in comparison to the flask-based experiments, it is important to understand where improvements to the yield at the current scale can be made so to overcome these reductions.

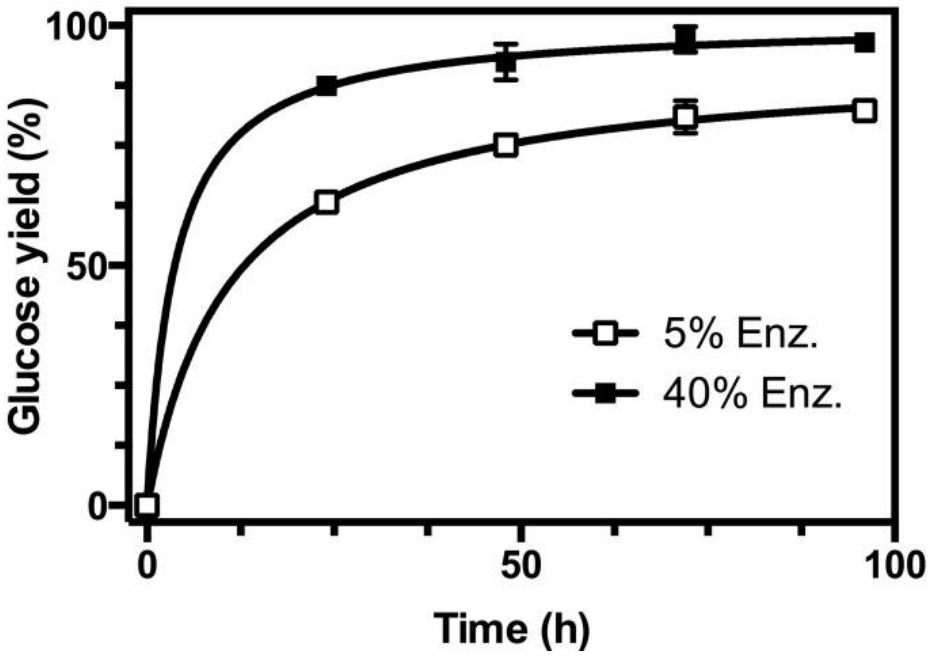
In the experiment it was found that the final yield of glucose at 96 hours for the max digest run was 96.5% ± 0% from cellulose degradation, when the biomass was at a UDS of 9%. This is a near maximum digestion of cellulose. The lost 3.5% glucose represents the cellulose that could not be hydrolyzed by enzymes. Comparatively, the glucose yield at 96 hours with 5% enzyme dosing was found to be 82.3% ± 1.5%, with material at 8.4% UDS. The disparity between these results demonstrates the substantial losses in glucose yield that are found in the process of enzymatic hydrolysis; characterized by the difference in final yield of approximately 12% glucose.

In both pretreated sorghum biomass treated with a 40% Ctec3 dose and pretreated biomass with a 5% Ctec3 dose, the breakdown of xylan to xylose was shown to be a minor product. Xylan breakdown achieved a limited increase in the concentration of xylose in the range of 2-4 g l^{-1}. At a Ctec3 dose of 40% the final xylose yield is that of 35.4% ± 2.2% (fig. 5B). Comparatively a 5% Ctec3 dose results in a 29.5% ± 0.7% xylose yield at 96 hours. While this is an improvement of 6%, it is only a minor product, with yields still only around a third of the full potential of xylan breakdown. This data suggests that while significant improvements to xylose production during the stage of enzymatic hydrolysis can be achieved, the yields are still much lower than desired, and cannot reach 87% simply by increasing the concentration of the enzyme. Due to being a minor product, with a substantially lower percentage yield compared to

glucose, studies into the effects of inhibitors will not focus on xylose production in the process of enzymatic saccharification.

One potential explanation for the poor xylan degradation efficiency and incomplete glucose yield is that the half-life of Ctec3, particularly at low enzyme concentrations, causes loss of activity as time progresses. While inhibitors could play a key role in the reduced yield of glucose at 5% enzyme dose, alternatively, the half-life of Ctec3 could become rate limiting more rapidly at low enzyme doses. While at an equivalent enzyme dose the impact of inhibitors can be measured, Ctec3 half-life could potentially obscure results when comparing enzyme doses.

A



B

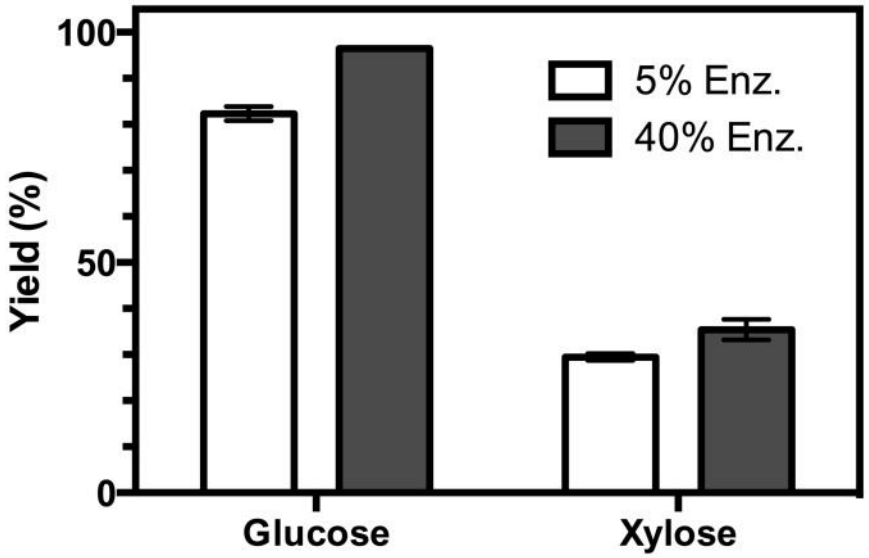


Figure 5- The effect of increasing enzyme dose on the yield of glucose and xylose from cellulose and xylan breakdown respectively.

(A) The effect of increasing enzyme concentration on the yield of glucose during the initial 96 hours. The condition of max digestibility (40% Enz) results in a gain in the region of 12% glucose yield with respect to normal conditions (5% Enz). (B) Concentrations of glucose and

xylose at 96 hours. There is a greater final yield of both glucose and xylose under the conditions of max digestibility (40% enzyme) compared to 5% enzyme. However the xylose yield in both cases are comparatively small (32% compared to 35%). Points represent mean of triplicates, error bars represent standard error.

3.1.2. Improvement in saccharification yield when clarified by Strata NH2

The improvements in glucose yield observed when increasing the dose of Ctec3 from 5% to 40% could be explained by the presence of inhibitors. As this was not the only explanation, studies into potential inhibitors were required. Such inhibitors can originate from multiple sources. Sources of inhibition include the side-products of lignocellulose decomposition, products of biological contaminants, or residual chemicals produced by fermentation of yeast over the duration of primary ensiling (Li *et al.* 2014) (Iyer and Lee, 1999). Solid Phase Extraction (SPE) was used to assist validate the hypothesis that inhibitors were affecting saccharification performance. Solid Phase Extraction (SPE) is a method by which biomass can be clarified, removing molecules which could act as inhibitors. The resultant changes in glucose yield between clarified media could be measured to determine the physical and chemical features of molecules most likely to be inhibitory.

With regards to SPE, the term “column” refers to plastic tubes which contain different sorbent material. Sorbents are material which adsorbs soluble molecules from a solution, removing different compounds from the DLB, and would enable discrimination of the most potent molecules. These columns are attached to a glass solid phase extraction apparatus, which itself is attached to a vacuum. Different sorbents are capable of interacting with different types of molecules, in this experiment 3 columns were used: Strata™ XL-C polymeric strong cation, Strata™ XL Polymeric reverse phase; Strata™ NH_2. Strata™ XL was a reverse phase column binding non-polar to weakly polar compounds, Strata™ XL-C was also reverse phase, but with an additional attraction to cationic compounds (bases). Strata™ NH2 was a normal phase column which interacted with strongly polar to weakly polar compounds. However, the sorbent side chains were also primed to bind anionic (acidic) molecules. Each of these columns were used to clarify pressate, with the resultant solution added back to biomass to alter performance of saccharification.

Three other conditions were added as controls. In the first, pressate that had been simply pressed out of the solid sorghum biomass by a wine press was added back to washed sorghum biomass. In order to display the effect of clarification of pretreated media, it was important to remove all soluble compounds from the solid biomass by washing, otherwise inhibitors still present on the solid could still impact enzyme activity. This DLB control is referred to as unfiltered pressate. In the second control, pressate that had not undergone solid phase extraction, however had been centrifuged and filtered to remove all solid particles was added to washed DLB, and the DLB sample was designated as filtered pressate. These two controls were selected in order to compare to clarified DLB to DLB that had not been clarified. Comparison between DLB containing filtered or unfiltered pressate would determine if any improvements in yield seen were from the preparation steps prior to SPE. A final control was prepared in which the washed DLB was brought to UDS=10% by addition of deionized water in place of pressate. The intent of using a DLB containing only water was to show the glucose yields that were attainable when no inhibitors were present in the sorghum biomass, and it was designated “deionized water.”

In order to validate the experiment and show that clarification could improve yields; an initial enzymatic saccharification was completed in duplicates of 10 g of clarified biomass as a proof of concept. Improvements in glucose yield at 96 hours were seen, with the most notable being between the sorghum biomass clarified by strata XL-C compared to “unfiltered pressate” sorghum biomass, although these improvements were undercut by a significant reduction in yield overall (Fig. 6).

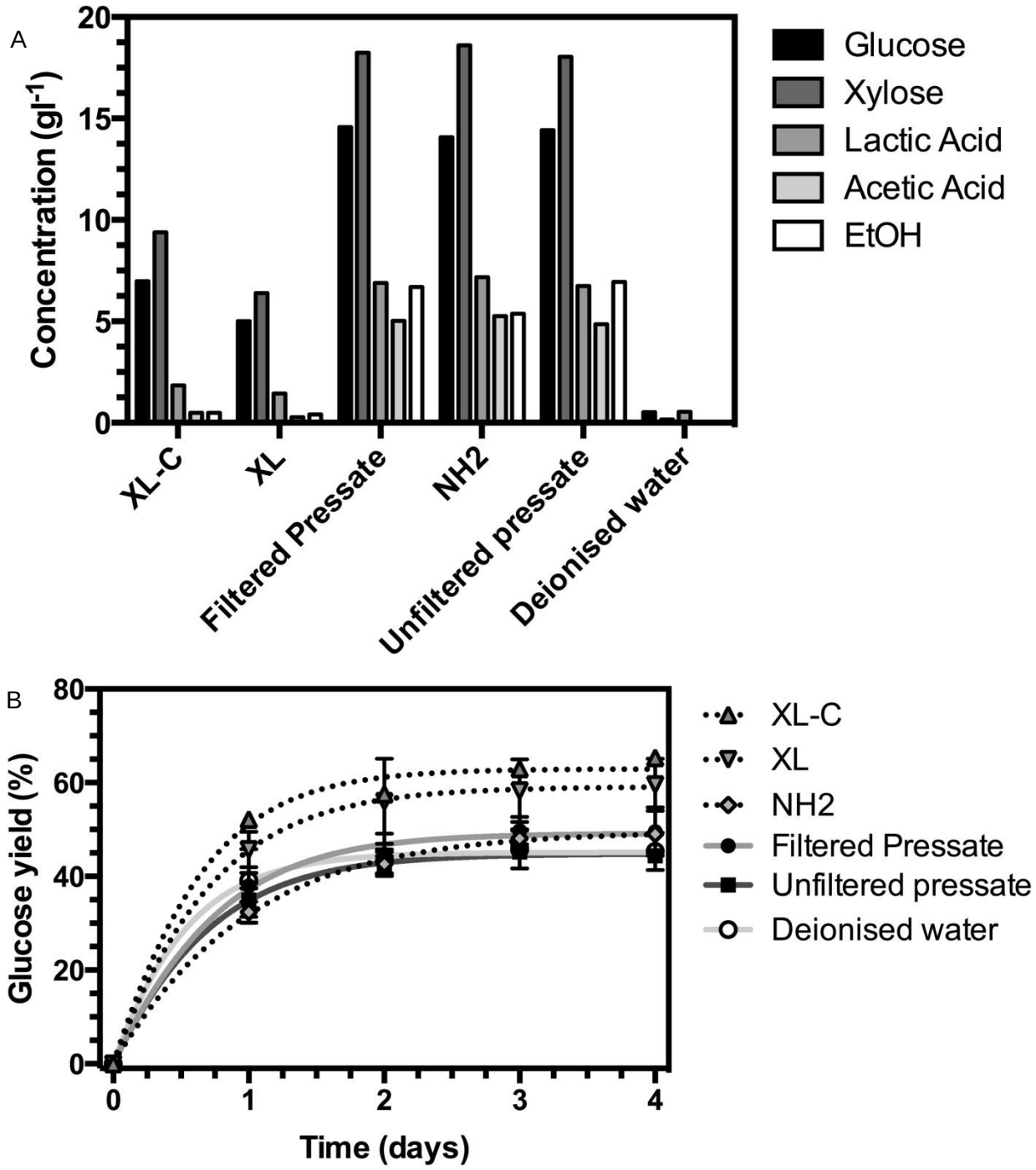
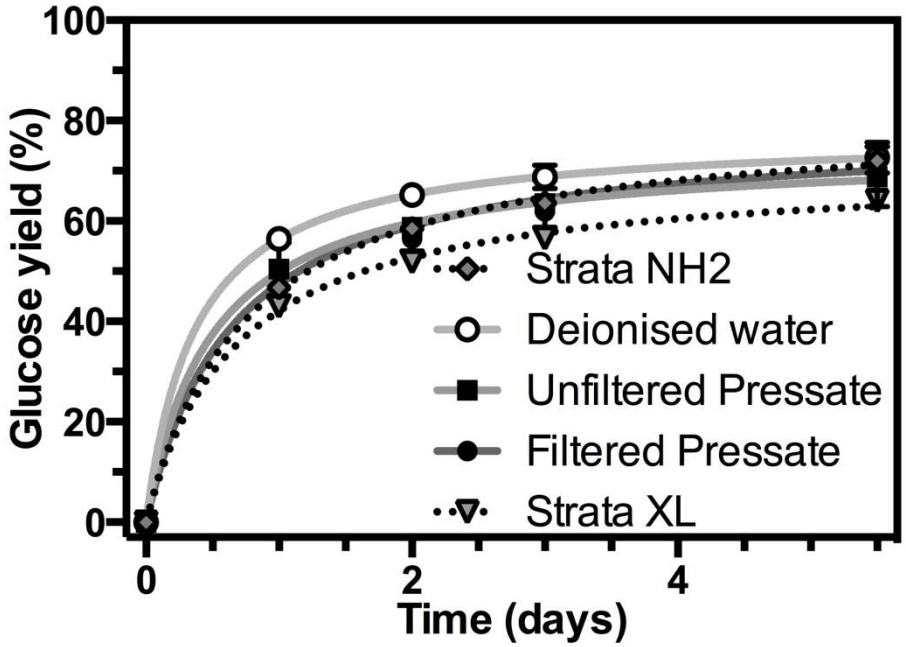


Figure 6 The effect of clarification by solid phase extraction on Ctec3 saccharification performance at small scale

(A) The concentration of the compounds glucose, xylose, acetic acid, lactic acid and ethanol. Concentration determined by HPLC. Reverse phase Solid Phase Extraction columns XL-C and

XL significantly reduce the concentration of all measured components. Normal phase solid phase extraction columns reduce only ethanol by a small amount with respect to unfiltered pressate. The available columns facilitated only one sample of filtrate to be prepared for each condition, therefore no error bars are present (B) The glucose percentage yield over 96 hours when Ctec3 added to 10 g of pretreated, clarified biomass; points represent average of duplicates, error bars represent standard error.

A



B

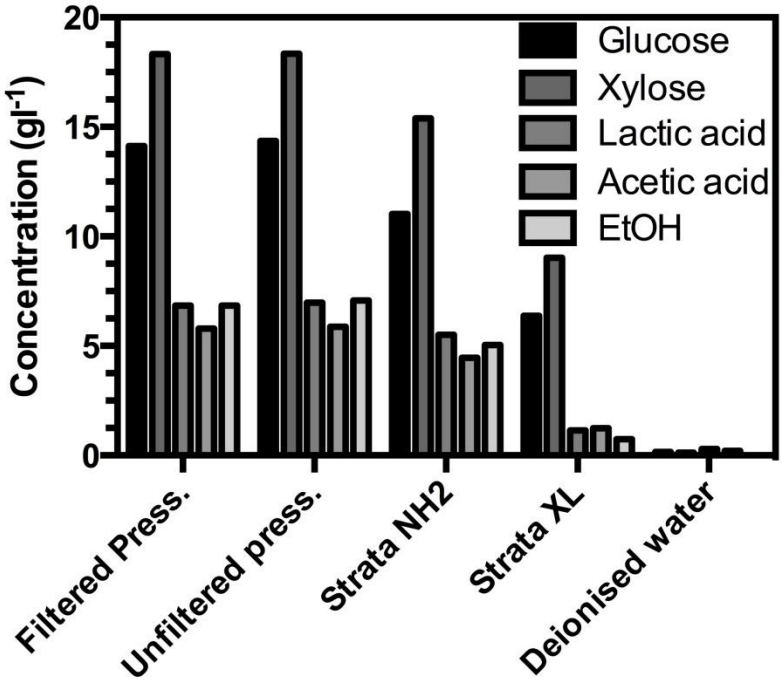


Figure 7: Solid phase extraction at standard scale with sorbents XL and NH2

(A) Saccharification of clarified biomass over 132 hours. Increasing the volume of solution which is clarified by SPE enables greater consistency between results. Points represent average of triplicates; error bars represent s.e. (B) the concentration of the compounds glucose, xylose,

acetic acid, lactic acid and ethanol. Concentration determined by HPLC.

To test the role of SPE in allowing identification of inhibitors, the effect of ethanol, lactic acid and acetic acid concentration was measured by observing how different SPE columns affected the concentration of these components and their effect on yield. Looking at small scale alone the conclusion could be reached that these three chemicals have an inhibitory effect on yield, as they are significantly reduced in concentration in Strata XL and Strata XL-C clarified biomass. XL-C was able to lower the concentration of ethanol (6.9 g l^{-1}), lactic acid (6.8 g l^{-1}) and acetic acid (4.9 g l^{-1}) in the pressate each to near 0 g l^{-1} (fig 6A). Compared to Strata NH2, Strata XL-C achieved improved absorption of ethanol, lactic acid and acetic acid to the sorbent to achieve glucose yields of 65.3% ± 0.2% compared to 44.5% ± 0.5% for “unfiltered pressate” (fig 6B).

In order to improve upon the imprecision of the results at small scale, the experiment was repeated with Strata XL and Strata NH2 at the standard scale, using triplicates of 75 g sorghum DLB. In order to clarify approximately 300 ml of sorghum pressate, 20 cartridges were used for each of the two columns tested. Strata NH2 heavily adsorbed the solvent, water, as well as adsorbing the polar components of the pressate. This meant the total volume of clarified Strata NH2 pressate obtained only 200 ml and the positive effects of the sorbent would be undercut by increasing the concentration of compounds that had not been attracted to the sorbent material.

Repeating this experiment at standard scale had the desired results of increasing consistency between the triplicate values. It was observed under these conditions that Strata NH2-clarified DLB improved yields of glucose production with respect to the “unfiltered pressate” control, in spite of a loss of 100 ml of water. The final yield of the strata NH2 clarified DLB was 72.0% ± 0.2% compared to the 67.4% ± 3.4% glucose yield of unfiltered pressate (Fig. 7A). The error for unfiltered pressate may lessen this conclusion however, with the two-tailed p value for this comparison, 0.45, being much greater than 0.05, meaning that the results are not statistically significant and may have been obtained through chance. While the kinetics of Strata NH2

clarified DLB show a reduction in initial rate of saccharification with respect to filtered and unfiltered pressate, before reaching a greater final yield, the high p value calls into question any conclusions that can be reached with this data.

A possible explanation for the poor rate of glucose production is that the concentration of some inhibitors may have increased as the solvent, water, was removed from the matrix. Small reductions in the concentration of other components of the biomass, such as ethanol, 1.49 g l^{-1} or acetic acid, 1.33 g l^{-1}, resulted in a small improvement in yield (Fig. 7B).

On the other hand, the performance of Strata XL clarified DLB was shown to be significantly reduced. This raised uncertainty about the results of the small scale experiment and whether ethanol, lactic acid and acetic acid were significant inhibitors. The final yield of 64.1% ± 1.8% was lower than that seen by unfiltered pressate suggesting that either the components removed were significant allosteric activators of cellulases, or that residual hexane remained from the conditioning step and acted as a new inhibitor of saccharification. Following hexane conditioning the cartridges were left under vacuum for at least 30 minutes to assist evaporation of all hexane molecules. However, if hexane molecules had remained bound to the column side chains, they would have been eluted during clarification of the pressate to lower the yield and kinetics of Ctec3. Observing the HPLC peaks compared to the filtered and unfiltered pressate samples, a new peak was found when analyzing the Strata XL clarified DLB, representing contamination of the DLB.

From these two experiments it was reasoned that polar compounds were likely to act as inhibitors of Ctec3 performance, in particular acetic acid, lactic acid and ethanol. However, due to uncertainty from the performance of strata XL and lack of statistical significance for Strata NH2 at standard scale, alongside the reduced saccharification following Strata NH2 clarification at small scale, further testing would be needed. Acetic acid, lactic acid and ethanol are stage 1

inhibitors produced during initial storage and ensiling, therefore greater insight into these compounds would allow validation of the hypothesis that stage 1 compounds cause inhibition of saccharification.

The control, deionized water showed superior kinetics at large scale (Fig. 6A). However, the final yield of 72.6% ± 3.0% was just 0.6% greater than Strata NH2 clarified biomass saccharification, and within error. Conversely, at small scale deionized water showed reduced kinetics compared to Strata XL and Strata XL-C (Fig. 5B).

3.1.3. Ethanol acts as an inhibitor of lignocellulose hydrolysis by Ctec3

Experimental design and statistical approaches can be used to understand the role of individual and synergistic interactions of inhibitors on biological systems with relatively fewer samples; this procedure is termed central composite design (CCD). Ethanol, lactic acid and acetic acid are components contained within the DLB, produced as a result of storage and ensiling of the sorghum biomass. The results of SPE supported the hypothesis that primary ensiling impacted saccharification, as improved glucose yields were seen when ethanol, acetic acid and lactic acid were reduced in concentration. It was therefore important to test for a correlation between high ethanol, lactic acid or acetic acid concentration and reductions in glucose yield, testing the hypothesis that primary ensiling produced inhibitors of the cellulose saccharification. As individual studies of multiple concentrations of ethanol, lactic acid or acetic acid would be time consuming and would not give insight into any synergistic effects, a CCD experiment was implemented.

Fifteen sorghum DLB samples were prepared for this experiment. Of the 15 DLB samples, each had a different combination of three concentrations of ethanol (8, 18.5 or 29 g l^{-1}), acetic acid (10, 15 or 20 g l^{-1}) and lactic acid (8, 12 or 16 g l^{-1}). The highest and lowest concentrations designated for ethanol, lactic acid or acetic acid were chosen in accordance to historical data of

minimum or maximum initial ethanol, lactic acid or acetic acid concentrations following RAPT pretreatment. As each component had three designated values, a complete study of all combinations would require 27 samples. Instead, the fifteen selected combinations were chosen in accordance with suggestions by DOE software, StatsGraphics, ranging for a combination of all low concentrations of the three components to an all high combination. Each of the 15 conditions condition were prepared in duplicates and hydrolyzed with 5% Ctec3 for 96 hours.

From studying saccharification of the 15 representative DLB samples, the effect of ethanol, acetic acid and lactic acid on glucose concentration was determined. The results concluded that ethanol was the only compound to inhibit glucose production with an absolute effect (Fig. 8A) and statistical significance, $p<0.05$ (Table 1). Increasing ethanol concentration from 8 g l^{-1} to 29 g l^{-1} resulted in a steep decrease in glucose production, with the concentration of glucose at 72 hours falling from 47.2 g l^{-1} to 45.6 g l^{-1} (Fig. 8B). This decrease was estimated to be of a linear nature.

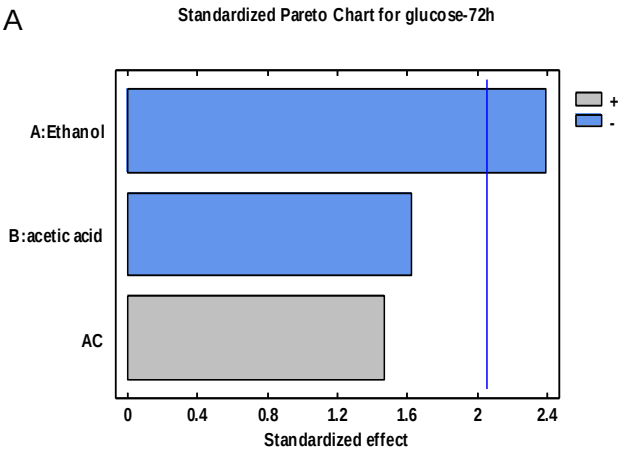
While acetic acid and lactic acid were not shown to have an effect on Ctec3 kinetics in isolation at the concentrations tested, it was of interest to determine whether these compounds could have a synergistic effect to either enhance or restrict the inhibition of ctec3 by ethanol. The data however showed no examples of synergistic effects, displayed clearly in the standardized Pareto chart of lactic acid and ethanol, which fails to reach the reference line (Fig. 8A), suggesting low importance of effect, This did not meet the test for statistical significance ($P>0.1$) (Table 1) and therefore does not act as an example of synergistic effects. The lack of interaction results in a flat estimated response surface, such as seen in Fig. 7C.

StatsGraphics ultimately suggested both an optimal condition and case of maximum inhibition for the parameters tested. Maximum inhibition, where component concentration of 29 g l^{-1} ethanol, 20 g l^{-1} acetic acid and 16 g l^{-1} lactic acid are implemented, obtained a yield of 66.6% ± 3.1% glucose at 72 hours. The second, a case of lowest inhibition, showed that 8 g l^{-1} ethanol,

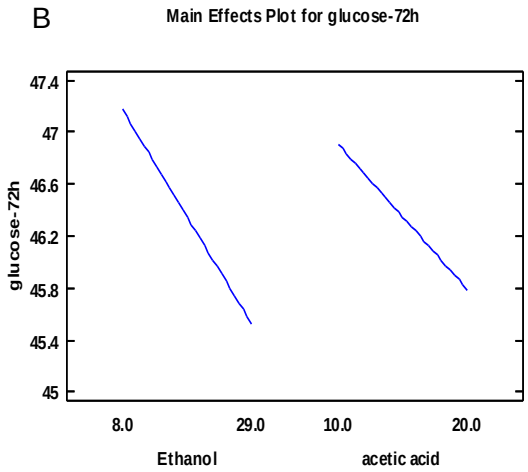
10 g l^{-1} acetic acid and 6 g l^{-1} lactic acid and achieved the yield of 81.0% ± 0.9% at 72 hours (Fig. 8D). A problem with this suggestion is that the data had suggested only Ethanol is an inhibitor of statistical significance, therefore increasing acetic acid and lactic acid should not be important.

In this experiment the CCD was completed by looking at glucose concentration in order to measure the effect of inhibitors on saccharification. Glucose concentration was selected as opposed to the glucose percentage yield used in other saccharification experiments. This was because of challenges associated with measuring the UDS of the samples. Unlike other experiments, the UDS for each sample was highly variable, leading to errors with regards to yield calculations which could mask results. Because of the errors in UDS measurement, the conclusion made by the CCD study needed to be verified by further testing.

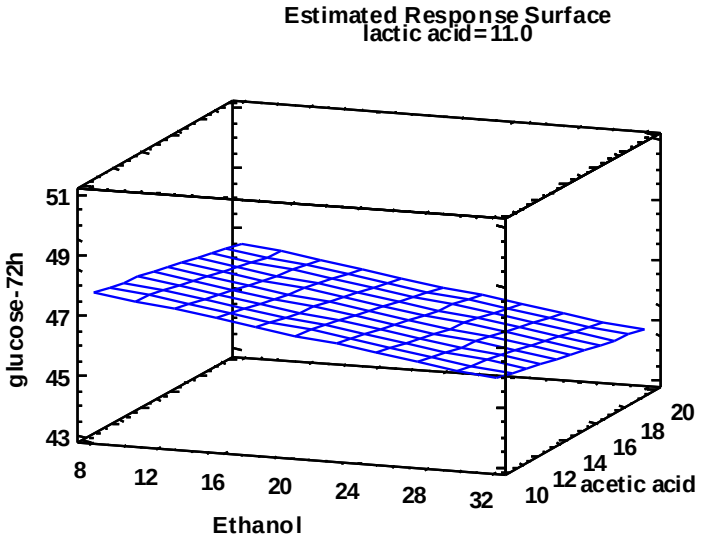
A


Standardized Pareto Chart for glucose-72h
A:Ethanol
B:acetic acid
AC
+
-
0
0.4
0.8
1.2
1.6
2
2.4
Standardized effect

B


Main Effects Plot for glucose-72h
glucose-72h
47.4
47
46.6
46.2
45.8
45.4
45
8.0
29.0
10.0
20.0
Ethanol
acetic acid

C


Estimated Response Surface
lactic acid=11.0
glucose-72h
51
49
47
45
43
8
12
16
20
24
28
32
Ethanol
10
12
14
16
18
20
acetic acid

D

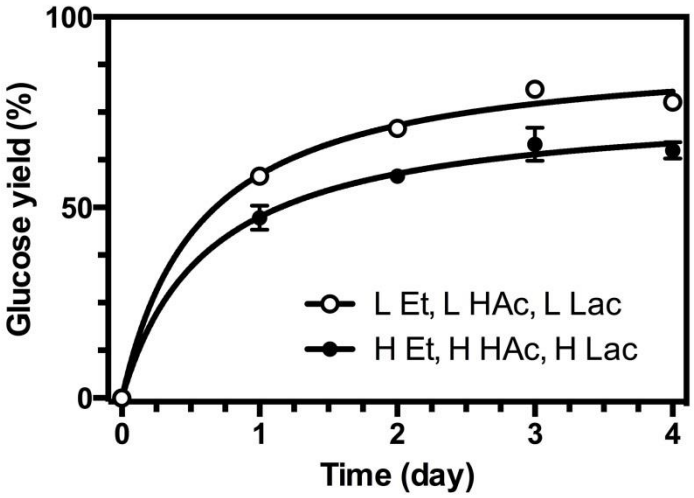

Glucose yield (%)
100
50
0
0
1
2
3
4
Time (day)
L Et, L HAc, L Lac
H Et, H HAc, H Lac

Figure 8: The inhibitory effect of ethanol on saccharification, irrespective of acetic acid and lactic acid concentration, calculated by Central Composite Design

(A) The standardized Pareto chart for glucose concentration at 72 hours. Values left of the reference line are representative of the condition being tested showing an effect that has potential importance, and is unlikely to be the result of error. Here increased ethanol concentration has a negative standardized effect. (B) The concentration of glucose in the presence of increasing ethanol or acetic acid concentration, displaying a linear inhibition – this is independent of standardized effect and could be the result of error (C) Estimated response surface representing the interaction of ethanol and acetic acid, the two-dimensional nature of the line suggests no synergy between the two conditions. Lactic acid concentration is maintained at 11 g l^{-1} in this model. (D) Glucose yield when all components (ethanol, lactic acid and acetic acid) are low in concentration (optimal) or high in concentration (sub-optimal). Samples taken from mean of duplicates, error bars indicate standard error.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Ethanol	13.6026	1	13.6026	5.15	0.0334
B:acetic acid	6.27648	1	6.27648	2.38	0.1375
C:lactic acid	0.430565	1	0.430565	0.16	0.6903
AA	0.965714	1	0.965714	0.37	0.5516
AC	5.11043	1	5.11043	1.93	0.1782
BB	0.503572	1	0.503572	0.19	0.6667
CC	1.91863	1	1.91863	0.73	0.4033
Total error	58.124	22	2.642		
Total (corr.)	86.8203	29			

Table 1: The ANOVA table of saccharification CCD.

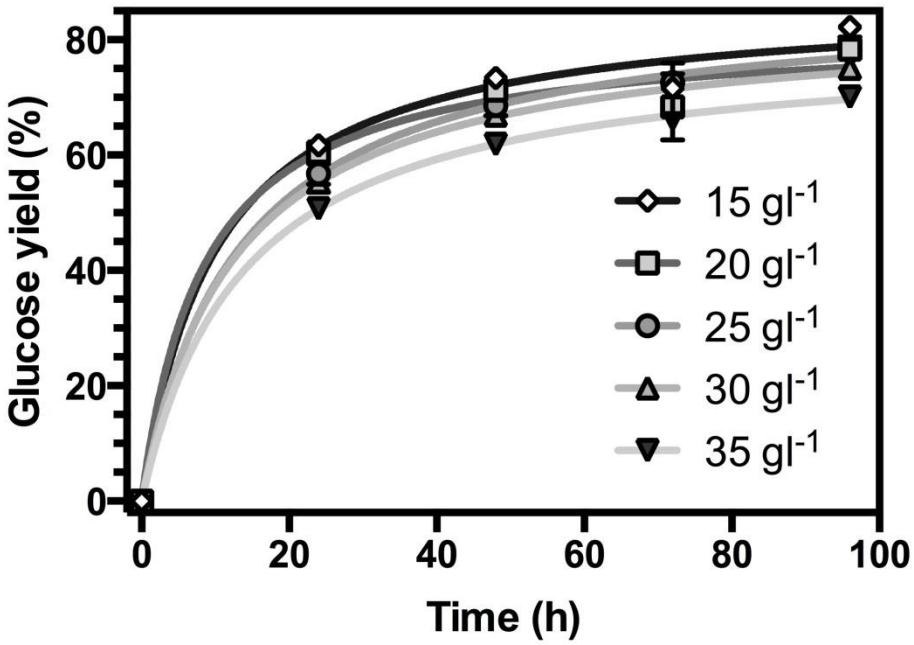
The ANOVA table, showing the probability of the compounds measured having an effect. A represents ethanol, B represents acetic acid and C represents lactic acid. Combinations of two letters represents potential synergistic effect. The p-value tests the null hypothesis that data from all groups are drawn from populations with identical means. A smaller p value means that results are not due to random sampling and thus the means are not identical between samples. The F ratio represents whether the results seen are the result of chance or a measured response, with a larger F value showing changes in values amongst groups is greater than the changes within a group, therefore the effect is less likely to be the result of error.

3.1.4. Effect of different ethanol concentrations on hydrolysis

In order to validate the results of CCD, it was important to look at how a greater range of ethanol concentrations effect the yield of glucose from DLB, to better characterize the nature of inhibition. Five samples of sorghum DLB were prepared for this experiment in which ethanol was present as one of five concentrations in each sample: 15, 20, 25, 30 or 35 g l^{-1}. The DLB was subsequently hydrolyzed by 5% ctec3 and comparisons drawn between the samples.

The results supported the linear inhibition suggested by CCD. Increasing the initial ethanol concentration above 15 g l^{-1} in DLB resulted in a gradual decrease in glucose production at 96 hours, reducing glucose yield from 82.2% ±1.7% to 70.1% ± 0.7% at an ethanol concentration of 35 g l^{-1} (Fig. 9B), two tailed p-value = 0.007. It should be noted that at a concentration of 25 g l^{-1} ethanol there was little change in glucose yield obtained compared to the yield when ethanol concentration was 20 g l^{-1}, however this result falls within error, with the kinetics of glucose production falling when ethanol concentration is 20 g l^{-1} following the initial 24 hours (fig 9A). The reduced kinetics seen for the 20 g l^{-1} ethanol sample may be the result enzyme denaturation during pH adjustment. The most significant depression in this experiment was between 30 g l^{-1} ethanol (75.1% ± 1.6% glucose yield) and 35 g l^{-1} (70.1% yield) which could suggest the pattern begins to change to an exponential decrease after this point, however greater initial ethanol concentrations have not been seen in practice. The two tailed p-value for this drop is 0.066, suggesting that it was statistically significant.

A



B

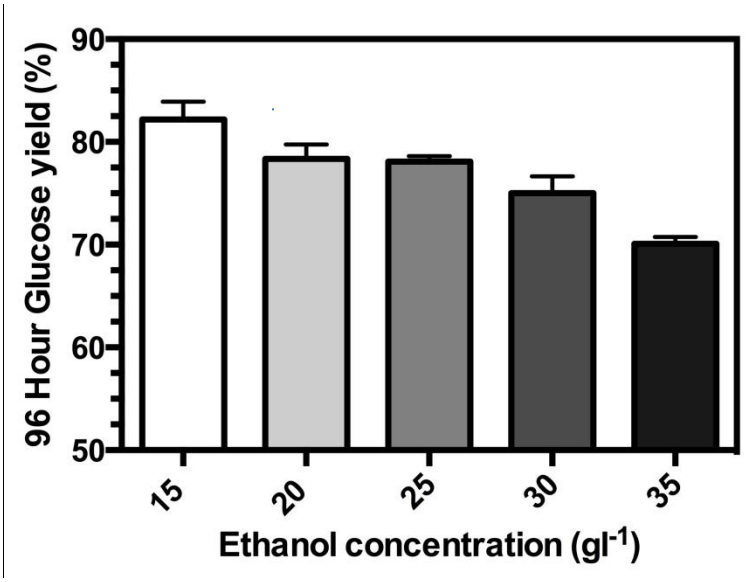


Figure 9: Characterization of the action of inhibition by ethanol on ctec3 performance (A) The kinetics of glucose yield over 96 hours when subjected to an ethanol concentration between 15 g l^{-1} and 35 g l^{-1}. Time points are measured every 24 hours, showing a reduced glucose yield with increased ethanol concentration. (B) Final glucose yield at 96 hours for the five ethanol concentrations tested. Results are the mean of triplicates, error bars indicate s.e.

3.1.5. Solid phase extraction shows improvements in yield when acetic acid, lactic acid and ethanol are at the same concentration.

Following studies on the effect of ethanol, lactic acid and acetic acid on saccharification, it was of interest to return to SPE in order to determine if any significant improvements could be seen when ethanol, acetic acid and lactic acid were brought to the standardized levels. By reducing the impact of primary ensiling, this study therefore measures the role of RAPT-derived compounds on saccharification. Furthermore, different sorbents could possibly allow determination of the inhibitor's nature.

It was found that both Strata NH2 clarified and Strata XL clarified DLB outperformed the standard control, achieving a final yield of 65.0% ± 1.3% and 64.7% ± 0.6% respectively. This is around 6% greater than the yield seen for the filtered pressate control, 59.0% ± 0.3% (Fig. 10A). The p-value for Strata NH2 improvement was found to be 0.012, and the p value for Strata XL improvement, 0.005. Both p values were therefore smaller than 0.05 and of statistical significance. The column Strata XL-C performed significantly worse in terms of glucose production than the pressate control, 55.1% ± 0.8%, however this could be the effect of residual hexane from conditioning steps being present in the media.

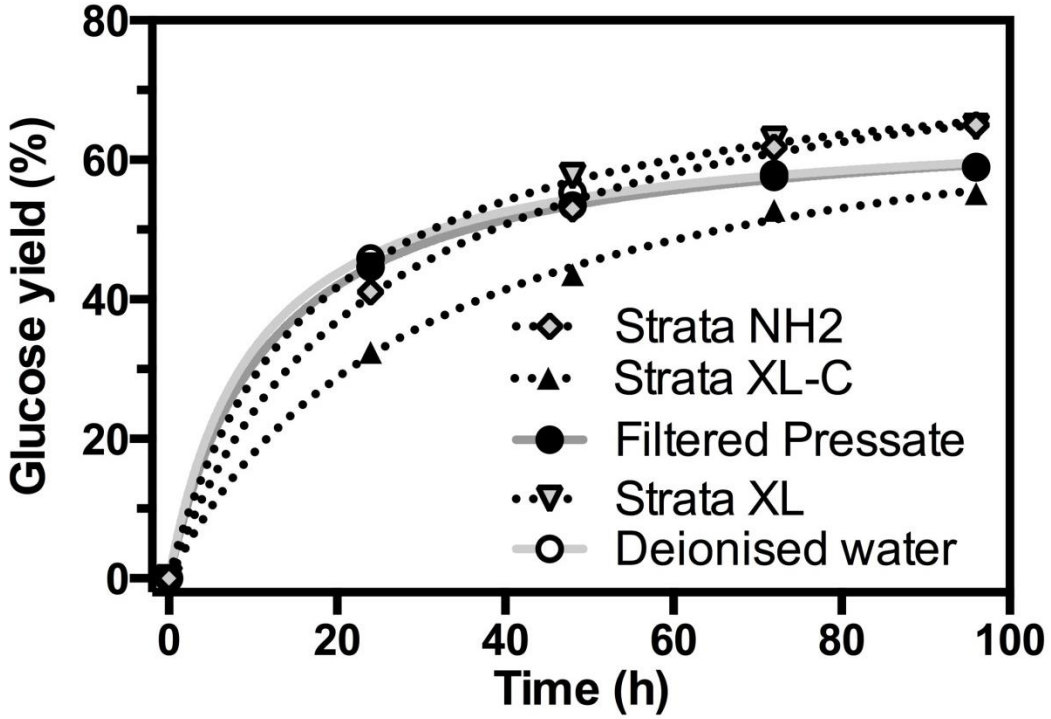
The kinetics of Ctec3 saccharification on clarified biomass differed depending on whether clarification was carried out using Strata XL or Strata NH2. When Strata XL is used, the kinetics in the initial 24 hours are nearly identical to filtered pressate, 44.7% ± 1.5% compared to 44.4% ± 1.5%. On the other hand, when Strata NH2 is used there are reduced kinetics during the initial 24 hours, 41.1% ± 0.9%, ($p=0.045$, smaller than 0.05) carrying through to 48 hours and only showing glucose yields greater than filtered pressate at 72 hours (Fig 10A).

Strata XL and Strata NH2 clarified biomass are able to achieve improvements over the filtered pressate control but vary in their kinetics. The difference in kinetics is a result of the substrate

specificity of the columns. Observing the change in concentration of acetic acid in pressate following clarification shows an imperfection in column selectivity. This work demonstrates that clarification of pressate by Strata XL can lower the concentration of the polar acetic acid by a greater level than Strata NH2, to 0.50 g l^{-1} and 3.14 g l^{-1} respectively, even when water is added back to the clarified biomass to compensate for solvent lost (Fig. 10B). This is in spite of Strata NH2 being produced to adsorb compounds such as acetic acid.

The deionized water control shows initial cellulose saccharification kinetics greater than any clarification method. However, following the initial time point, the kinetics slow and ultimately the final yield is equivalent to that of filtered pressate (Fig. 10A).

A



B

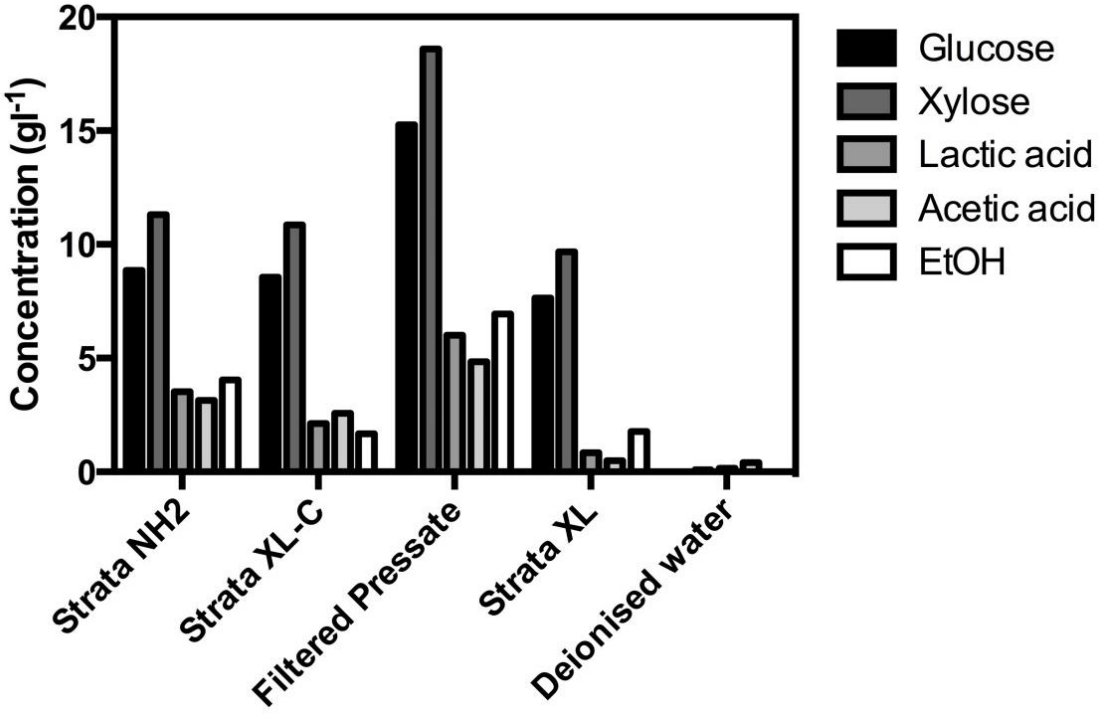


Figure 10: Use of solid phase extraction to determine the role of inhibitors excluding ethanol, lactic acid and acetic acid.

(A) Kinetics of glucose yield over 96 hours when different sorbent clarifications have been applied, followed by addition of acetic acid, lactic acid and ethanol. Clarification with Strata-XL and Strata-NH2 sorbents results in an increased final yield of ethanol. Concentration determined by HPLC. Each point represents an average of triplicates, error bars represent standard error. (B) Concentration of ethanol, lactic acid, acetic acid, glucose and xylose post clarification, measured by HPLC. Data shows a significant reduction in the concentration of all compounds post clarification, with Strata-XL showing the greatest overall reduction in lactic acid, acetic acid and ethanol concentration.

3.2. Fermentation

3.2.1. Ethanol, acetic acid and lactic acid act as inhibitors on fermentation rate of *Saccharomyces cerevisiae* strain c5fuel

A substantial body of evidence has demonstrated that ethanol, acetic acid and lactic acid act as inhibitors of wild type *S. cerevisiae* growth (Narendranath *et al.*, 2001) (Adeboye, 2014). It remains unknown whether these compounds would have the same inhibitory effect on growth and fermentation of transgenic yeast strains developed for fuel, notably the C5fuel strain of *S. cerevisiae*. C5fuel is characterized by the introduction of genes that confer new xylose catabolic pathways for ethanol production and enhanced resistance to metabolic inhibitors. It was important, therefore, to evaluate the role of ethanol, lactic acid and acetic acid on C5fuel fermentation. To best quantify the contributions of each compound to any deleterious effect on ethanol yield, and to investigate the potentially synergistic effects of the inhibitory compounds on C5fuel growth, a CCD was employed. Ethanol, acetic acid and lactic acid were introduced into the CCD as continuous variables and a number of experiments performed according to the models.

15 conditions of hydrolysate were selected, in accordance with historical data of maximum and minimum concentrations of ethanol, lactic acid and acetic acid, alongside a median value.

All three components tested were shown to have inhibitory activity that was of statistical significance on production of ethanol by C5fuel (Fig. 11A), $p<0.01$ (Table 2). An effects plot (Fig. 11B) predicted that as initial ethanol concentration was increased the yield of ethanol from fermentation was reduced dramatically, with a 10 g l^{-1} initial ethanol achieving a yield of 76% ethanol at 24 hours, compared to 30 g l^{-1} ethanol achieving a reduced yield of 56% at 24 hours. A similar reduction in ethanol yield was seen when increasing the concentration of acetic acid, with ethanol yield reduced once more from 76% to 56%. Importantly, unlike initial ethanol, this inhibitory effect was achieved by acetic acid over a relatively smaller increase in concentration,

from 5 g l^{-1} to 15 g l^{-1}. The effect was shown to be of a similar statistical significance to ethanol, $p<0.01$.

Of the inhibitors studied, lactic acid was shown to have the lowest standardized effect (Fig. 11A), although still of statistical significance, $p<0.01$ (table 2). Increasing the concentration of lactic acid from 6 g l^{-1} to 18 g l^{-1} was predicted to result in a reduction in ethanol yield of approximately 8% (Fig. 11B).

An interaction between ethanol and acetic acid that is of statistical significance, $p<0.01$, was observed during this experiment. These two components interact to cause a positive synergistic inhibition that works to significantly reduce the glucose yield. The Estimated Response Surface of the two inhibitors is shown to curve significantly downwards at the point where both acetic acid and ethanol begin to increase in concentration (Fig. 11C).

StatAdvisor also produced two conditions from the variables which would achieve either maximum inhibition or minimum inhibition. The first, in which maximum inhibition takes place had the concentrations of 30 g l^{-1} ethanol, 16 g l^{-1} acetic acid and 18 g l^{-1} lactic acid resulting in a final yield of 66.3% ± 0.6% ethanol. The second were the optimal conditions tested, 8 g l^{-1} ethanol, 5 g l^{-1} acetic acid and 6 g l^{-1} lactic acid, and resulted in a final yield of 77.6% ± 1.2%. Interestingly, under the optimal conditions a yield at 24 hours was measured as 80.7% ± 0.3%, before dropping towards the final yield. While this could be error, the calculated standard error does not suggest such an event, alternatively it may suggest that ethanol metabolism has taken place following complete fermentation. To reduce the impact of product consumption on conclusions drawn, ethanol yields will largely be focused on the initial 24 hours, or optimal yield, as opposed to the final yield.

Prism, the modelling program used, represented this data as a third order polynomial curve (Fig. 11D) however a more suitable model may have been a sigmoidal curve plateauing at 24 hours.

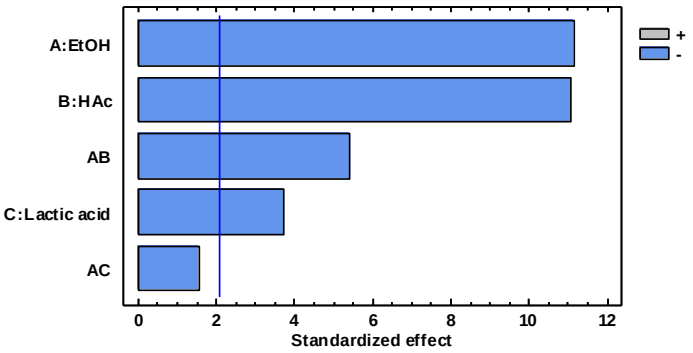
Alternatively, due to the lack of time points, a linear, point to point graph may have been a better choice.

Ultimately, analysis of the data was used to produce the model equation below, reflecting the inhibitory effect of initial ethanol, lactic acid and acetic acid on the glucose yield at 24 hours:

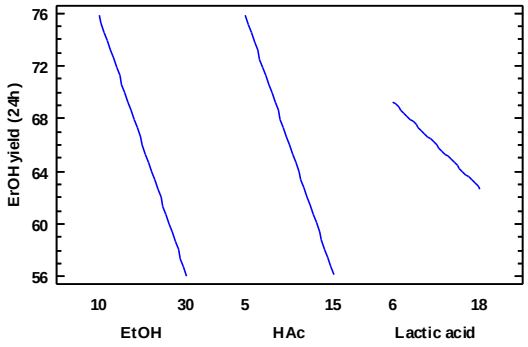
$$EtOH\ yield\ (24h) = 84.4433 + 0.39 * EtOH + 0.181 * HAc - 0.0391667 * Lactic\ acid - 0.10725 * EtOH * HAc - 0.025625 * EtOH * Lactic\ acid$$

Where Ethanol yield at 24 hours is in %, Ethanol (EtOH), Acetic acid (HAc), and Lactic acid are in g l^{-1}. R^2=92.4%

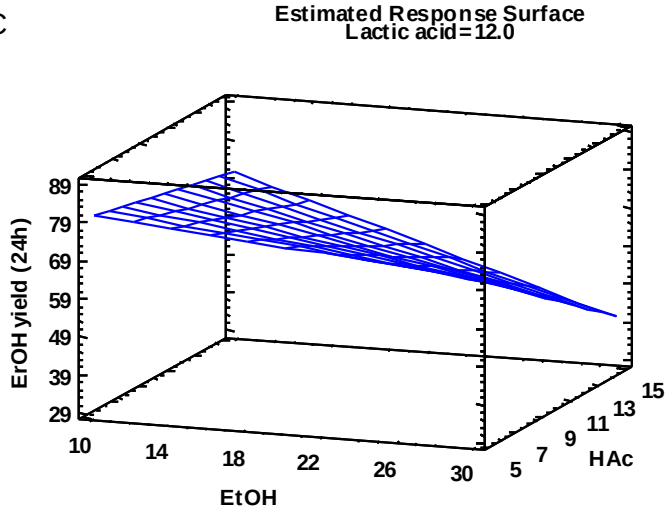
A


Standardized Pareto Chart for ErOH yield (24h)
A:EtOH
B:HAc
AB
C:Lactic acid
AC
+
-
0
2
4
6
8
10
12
Standardized effect

B


Main Effects Plot for ErOH yield (24h)
ErOH yield (24h)
76
72
68
64
60
56
10
30
5
15
6
18
EtOH
HAc
Lactic acid

C


Estimated Response Surface
Lactic acid=12.0
ErOH yield (24h)
89
79
69
59
49
39
29
10
14
18
22
26
30
EtOH
5
7
9
11
13
15
HAc

D

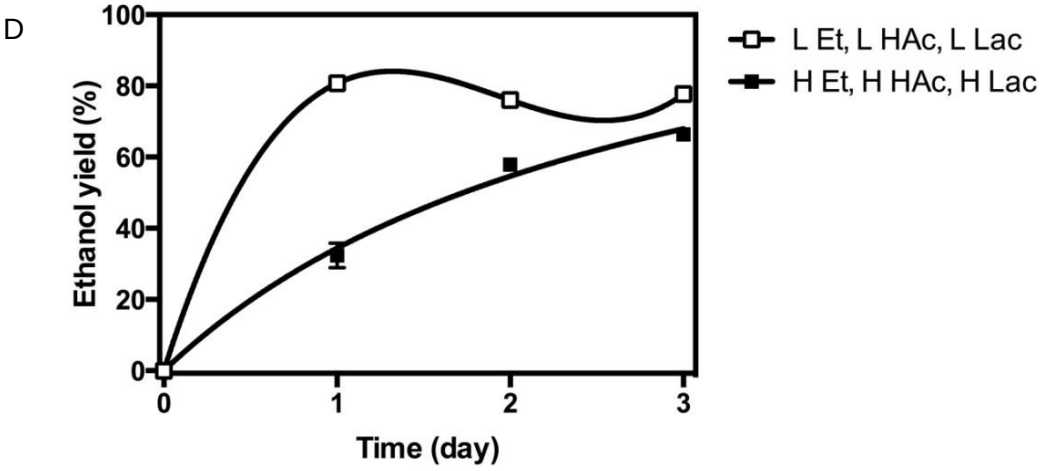

Ethanol yield (%)
100
80
60
40
20
0
0
1
2
3
Time (day)
L Et, L HAc, L Lac
H Et, H HAc, H Lac

Figure 11: The inhibitory effect of acetic acid, lactic acid and ethanol on C5fuel fermentation, as shown by CCD

(a) The standardized pareto chart, bars that pass the line at ~2 in the standardized effect are considered “statistically significant.” (b) a plot showing the effect of ethanol, acetic acid and lactic acid on ethanol yield at 24 hours (c) the estimated response surface representative of the inhibitory effect of acetic acid and initial ethanol concentrations on ethanol yield. The curved plot represents synergy. In this model Lactic acid concentration is kept at 12 g l^{-1}. (d) The actual ethanol yield measured under conditions of maximum inhibition (H Et, H Hac, H Lac) vs optimal conditions (L Et, L Hac, L Lac). Curve was modeled by Prism. Point represent an average of duplicates, error bars are s.e.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:EtOH	1960.2	1	1960.2	124.11	0
B:HAc	1928.65	1	1928.65	122.11	0
C:Lactic acid	219.122	1	219.122	13.87	0.0011
AB	460.102	1	460.102	29.13	0
AC	37.8225	1	37.8225	2.39	0.1348
Total error	379.067	24	15.7944		
Total (corr.)	4984.96	29			

Table 2 The ANOVA table for the effect of fermentation CCD.

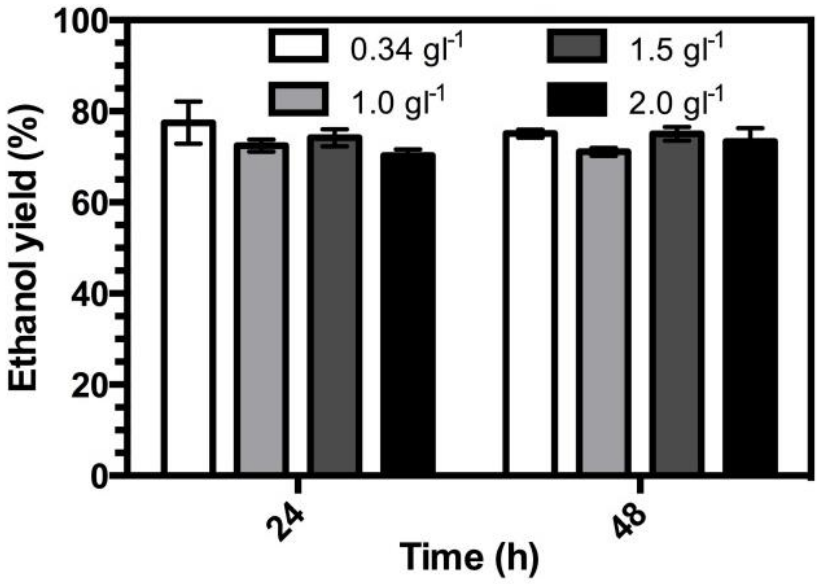
The ANOVA table, showing the probability of the compounds measured having an effect. A represents ethanol, B represents acetic acid and C represents lactic acid. The P-Value tests the accuracy of the data, a smaller p value means that results are not due to random sampling. The F ratio represents whether the results seen are due to chance or whether the results are a measured response, with a larger F value showing changes in values amongst groups is greater than the changes within a group, therefore the effect is less likely to be the result of error.

3.2.2. Furfural concentrations as high as 2 g l^{-1} do not inhibit C5fuel.

Furfural is a compound shown to act as an inhibitor of fermentative organisms; of interest, experiments have shown furfural has an inhibitory role on ethanol production in wild type *S. cerevisiae* (Sárvári Horváth *et al.*, 2003)(Field *et al.,* 2015). It was postulated that furfural may also function as an inhibitor to the genetically modified C5fuel strain. If so, this could be a significant concern for xylose catabolism, as it is a genetically modified process which may lack resistance genes to stabilise and so could be more susceptible to inhibition. To determine the role of furfural in C5fuel kinetics, the concentration was raised from 0.34 g l^{-1} to 2.0 g l^{-1} in an incremental manner, achieving four concentrations for the experiment (0.34, 1.0, 1.5 and 2.0 g l^{-1}). In the presence of up to 1.5 g l^{-1} furfural there was no significant reduction in the ethanol yield or kinetics of C5fuel (fig. 12A). However, when the concentration of furfural is 2.0 g l^{-1} there is a small reduction in ethanol yield, resulting in a lowered final yield of 77.7% ± 2.6% compared to the yield for 1.5 g l^{-1}, 79.6% ± 1.2%, although this falls within error. There is, nevertheless incomplete consumption of xylose at 48 hours, where 5.5% ± 0.7% of the sugars are retained compared to the 3.6% ± 0.1% at lower concentrations (fig 12B).

A question was raised over the course of this experiment about whether the measured furfural resistance is the effect of either the C5fuel propagation procedure, which enhances resistance to inhibitors, or genes have been encoded into C5fuel to confer resistance.

A



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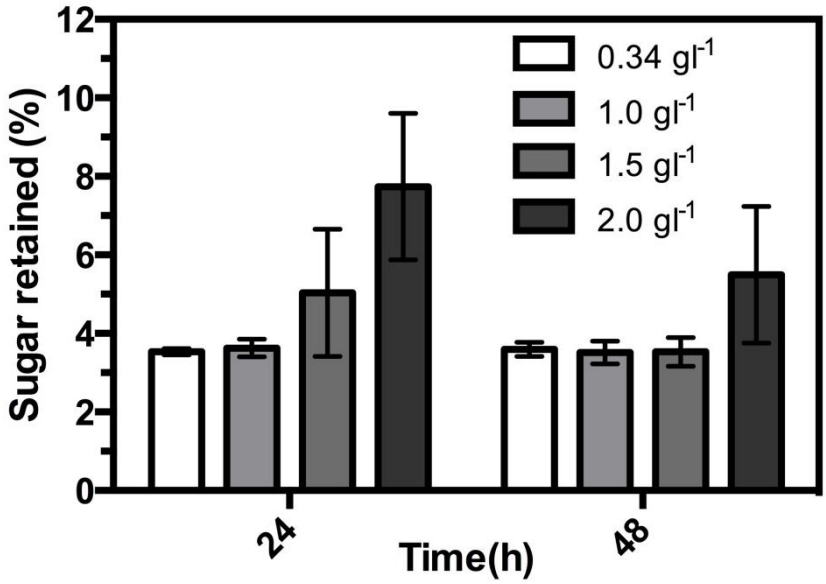


Figure 12: The effect of furfural concentration, up to 2gL^{-1}, on C5fuel fermentation performance

(A) Increasing the concentration of furfural at 24 or 48 hours causes only a small reduction in yield of ethanol at a concentration of 2.0 gl^{-1} (B) The percentage of sugar retained (glucose, xylose, acetic acid) increases with the increasing concentration of furfural. Error bars represent s.e.

3.2.3. Seed culture conditions an improved resistance to furfural

Propagation is a stage of rapid cell growth used to activate and increase the volume yeast cells prior to fermentation. In the RAPT procedure, propagation is a two-step process in which first the micro-organism was grown in media that enables it proliferate rapidly (pre-culture), followed by transfer to a culture that introduces stress and causes the cells to adapt to the conditions of the fermentation broth (seed culture). The role of the seed culture in RAPT was to condition the cell to potential inhibitors contained within the hydrolysate. This concept has been used in previous studies, such as adapting wild type *S. cerevisiae* to generate a strain capable of high temperatures and greater yields compared to wild type in presence of HMF (Wallace-Salinas *et al.*, 2013).

In the large scale application of the RAPT process there are a significant number of costs, including enzymes, water purification, heating of vessels and purchasing media. One of the potential methods through which money could be saved is through directly buying bulks of propagating *S. cerevisiae* strain C5fuel from Mascoma. Purchasing active cells would reduce the need for waste water that is used in the propagation strategy at large scale (replacing the YPD used at small scale), alongside removing the need to separate significant volumes of hydrolysate from fermentation needed to generate the seed culture media, reducing product. On the other hand if this decision affects yields, it could cause significant loses in final profits, negating any benefits of buying yeast cells in bulk. To determine the importance of propagating the yeast cells in a hydrolysate media, an experiment was attempted comparing the effect of a seed culture that adapts the cell to negative conditions, and a seed culture which simply allows cell growth.

A question was raised in the experiment of the effect of furfural on C5fuel performance, **3.2.2**, whether the conditioning of the C5fuel cells was responsible for the improved resistance to furfural or whether this was the result of encoded resistance genes. In order to determine the

role of seed culture in furfural resistance the experiment was repeated with furfural at two concentrations in hydrolysate samples, 0.34 g l^{-1} and 2.0 g l^{-1}. The cells used to ferment these hydrolysate samples had been cultured in one of two seed cultures: the first, a mixture of equal volumes of YPD (25 ml) to Hydrolysate (25 ml) or the second, a pure YPD solution (50 ml). The ethanol yields were compared at various time points.

It was found that using YPD as a seed culture results in a significant lag phase of ethanol production (Fig. 13A), 20.1% ± 0.4% compared to 80.1% ± 1.1% when the C5fuel was extracted from a YPD: Hydrolysate seed culture, causing reduced sugar consumption at 24 hours when the concentration of furfural is 2.0 g l^{-1} concentration (Fig. 13B). At 48 hours the yield was equal to all other compounds (Fig. 13A). The results of 2.0 gl^{-1} furfural on C5fuel cells propagated on a YPD seed culture suggests that furfural causes a reduced rate of sugar consumption in the C5fuel strain. Using the YPD:Hydrolysate seed culture to preadapt the cells to furfural enables C5fuel to immediately cope with increased furfural concentration up to 2.0 gl^{-1} sample and show near equivalent ethanol kinetics to a sample with furfural concentration at 0.34 gl^{-1}.

Prism displayed the ethanol yield data as a polynomial curve for most samples, with the exception of the C5fuel grown with 2.0 g l^{-1} furfural and YPD seed culture, for which a sigmoidal curve was produced (Fig. 13A). The sigmoidal curve was suggested due to the low yield of ethanol at 24 hours, followed by an ethanol yield equivalent to all other samples at 48 hours. A more suitable curve for the other three samples may have been a sigmoidal curve plateauing at 24 hours, and with a smaller initial growth phase compared to the 2.0 g l^{-1}, YPD sample, however without samples at 24 hours or earlier there is no way to confirm this model as correct.

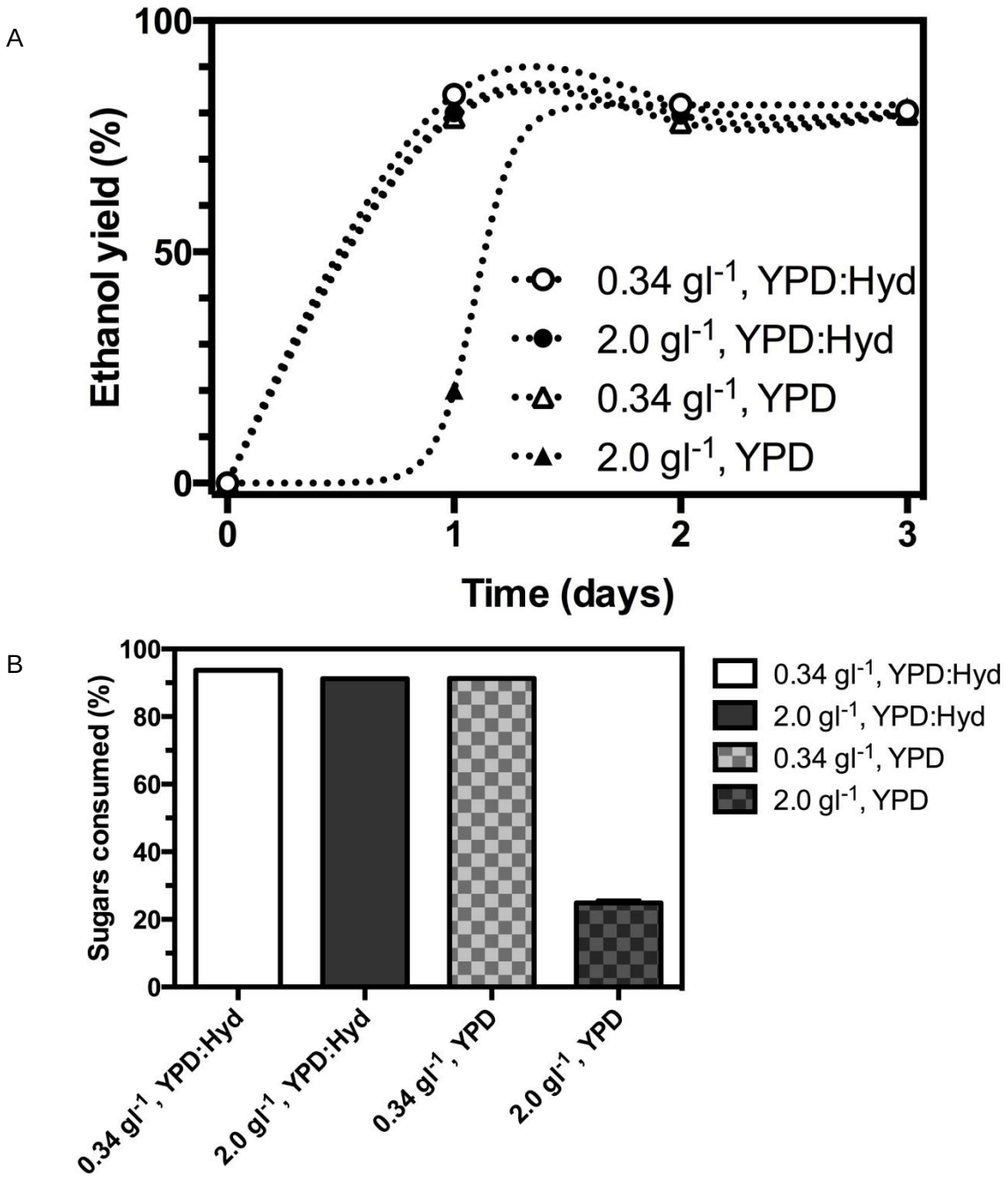


Figure 13: Fermentation of C5fuel in the presence of up to 2 gl^{-1} furfural, following growth on different seed culture.

(A) The kinetics of ethanol production over 72 hours for C5fuel in the presence of either 0.34 gl^{-1} furfural or 2.0 gl^{-1} furfural. C5fuel either proliferated on a seed culture of pure YPD or an equal mix of both YPD and filtered Hydrolysate. (B) Sugar consumption of C5fuel at 24 hours. Points

represent an average of triplicates, error bars represent standard error.

3.2.4. Solid phase extraction shows the ability to improve yield of ethanol to varying degrees depending upon column used

It was of interest to determine whether the SPE could be used to find the chemical and physical properties of inhibitors of fermentation. Compared to clarifying pretreated biomass, clarifying hydrolysate will not result in an identical clarification. The hydrolysate contains a different concentration of a variety of molecules including glucose, ethanol, and oligosaccharides compared to pretreated biomass. To ensure the concentration of inhibitors removed in fermentation was equivalent to those seen in the hydrolysis experiments, the product of the standard scale experiment completed in **3.1.2** was obtained and the concentration of glucose and xylose were raised to equal concentrations across all samples. Strata XL was not included in this experiment due to concerns that the results seen were due to hexane present in solution.

It was found that the column Strata NH2 achieved small improvements in the yield of ethanol, 81.6% ± 0.4% at 24 hours, with respect to the filtered and unfiltered pressate samples (79.9% ± 0.5% and 79.3% ± 0.4% respectively), the p-value for these results were 0.035 and 0.023 respectively, therefore $p<0.05$ and of statistical significance. In addition, the deionized water sample achieved significant improvements in 24 hour ethanol yield compared to the other conditions tested, 86.1% ± 0.3% (Fig. 14A). Final ethanol yields decrease slightly from the 24 hour yields as exposure to oxygen triggers metabolism of ethanol.

As ethanol, lactic acid and acetic acid have been demonstrated to have the potential to cause inhibition it was of interest to evaluate the effect of other inhibitors on fermentation yields and kinetics. In this case the product of the SPE experiment completed in section **3.1.5** were reused in order to produce hydrolysate with equal concentrations of glucose, xylose, ethanol, acetic acid and lactic acid between all samples, therefore differing in the concentration of pretreatment products. It was found that hydrolysate derived from biomass that had been clarified by either Strata XL sorbent or Strata NH2 sorbent resulted in the greatest yield improvements compared to untreated hydrolysate (Fig. 14B). Both samples were achieved a yield of 86.8% ± 0.4% and

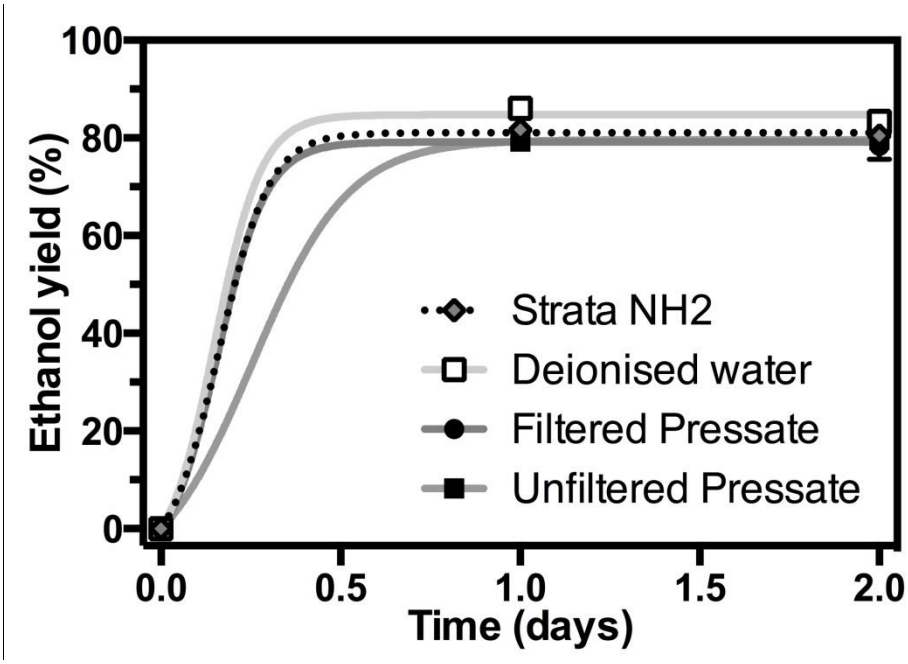
86.2% ±0.8% respectively at 24 hours, before dropping to ~82% at 48 hours. Conversely, deionized water was shown to have reduced kinetics, although achieving a final yield of 85% ± 1.3%. The hydrolysate sample derived from biomass with only added filtered pressate was shown to achieve a maximum yield of 80.6% ±1.2% ethanol production, although settled on a final yield of 78.0% ± 1.1% at 48 hours. For Strata NH2 clarified media, the improved yield with respect to filtered pressate control had a p value of 0.037, whereas Strata XL had a p value of 0.028. For both comparisons, $p<0.05$, meaning that the improved yields are of statistical significance.

Finally, the column strata XL-C resulted in a final yield of 79.1% ± 0.5%, which may suggest the presence of hexane was detrimental to fermentation or that the Strata XL-C column removed components of the hydrolysate that assisted ethanol production.

One problem with this experiment is that the concentration of glucose in the Strata XL sample was higher than desired due to a measuring error. This may have affected the results of fermentation, by providing a greater amount of substrate for C5fuel, meaning slightly more ethanol was produced, however the percentage yield can compensate for changes in substrate concentration.

Once more a polynomial graph was produced for the ethanol yield (Fig. 14B) representing Strata-NH2, Strata-XL and filtered pressate. A sigmoidal graph, similar to Strata-XL-C or deionized water may have been more suitable. However, the lack of data points could make an alternative argument for a simple linear, point to point graph.

A



B

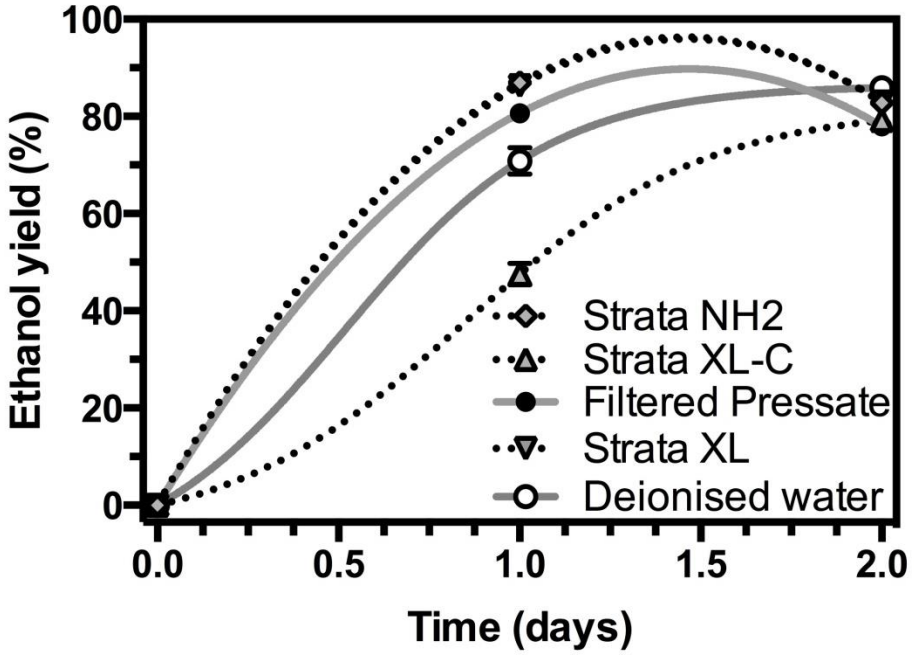


Figure 14: The effect of solid phase extraction on the activity of C5fuel fermentation (A) the fermentation of the hydrolysate produced in **3.1.2.**, (B) the fermentation of hydrolysate produced in **3.1.5.**, with glucose and xylose brought to equivalent levels, except in Strata XL where concentration is increased by 3 g l^{-1}. Data modeled by Prism. Points represent an average of triplicates, error bars represent standard error.

4. Discussion

4.1. Solid Phase Extraction reveals polar acids play a role in inhibition

Increasing the concentration of Ctec3 from 5% with respect to cellulose to 40% with respect to cellulose achieves a near maximum saccharification of glucose from cellulose in DLB, as was shown in figure 5A. If the final yields at 5% and 40% enzyme dose were equivalent, this would suggest that the RAPT pretreatment procedure was limiting the yield by preventing all cellulose from being accessed. Instead the increased activity when Ctec3 is dosed at 40% with respect to cellulose suggests that higher enzyme concentrations are able to resist components of the pretreated biomass. Therefore, at the operating Ctec3 dose of 5% with respect to cellulose, the cellulase enzymes are inhibited by components of the pretreated sorghum biomass below target yield of 87%. These results imply that inhibitors in the pretreated biomass are limiting saccharification.

Glucose percentage yield was measured by comparing the initial concentrations of glucose in media to the final concentration at 96 h. The measurements took place prior to enzyme addition, so to ensure that no initial hydrolysis was measured. Yield calculations were completed using an extensive excel macro document, which predicted the overall cellulose concentration from UDS, TS and cellulose percentages, however used an approximation for sugar added from the Ctec3 dose. While Novozyme's dosing guidelines indicates that doses between 1% and 6% would have little effect on yield calculations, the approximation would be inconsistent depending on the stabilizing solution used for each enzyme batch, implying that the true 0 hour measurement could be greater than 0%. This is a potential weakness of glucose yield calculations throughout this work.

There are limitations with the conclusion that increased enzyme dosage results in improved resistance to inhibitors. One key element to take into account is that the Ctec3 stabilizing solution, a mixture of sucrose and glucose which maintains enzyme activity in storage. As

mentioned above, Shell previously produced a formula for glucose yield analysis, approximating the glucose and sucrose added at low enzyme doses. However, at the heightened, 40% enzyme dose the sugars added would be much greater and may not be correctly predicted by glucose yield calculations. In order to aid with such calculations, a blank containing only enzyme should have been used for comparisons. Another explanation for reduced activity at the low enzyme dose is the biological half-life of Ctec3, which could affect the glucose yield differently when dosage varies between samples. A combination of both the lowered enzyme activity and the inhibitors may have acted synergistically to overstate the impact of inhibition on hydrolysis under 5% Ctec3 dosing conditions.

It is important to note that the RAPT procedure in combination with Ctec3 offers greater hydrolysis yields than many examples in literature. Saccharification methods such as hydrolysis of ozone pretreated cereal straw using NS50013 and NS50010 (García-Cubero *et al.* 2012) offered only a 40-50% glucose yield. Using the same enzymes with dilute acid pretreatment on wheat straw, on the other hand, resulted in an even smaller yield of 20% glucose, and the best pretreatment method, alkaline peroxide pretreatment, only achieved a yield of 64% following washing, and 49% without (Toquero and Bolado, 2014), a much lower yield than the 83% glucose seen for 5% w/w dosing with Ctec3 in this work. Use of Ctec3 in combination of a different pretreatment method, such as Ammonia Fibre Expansion pretreatment of corn stover, still results in reduced yields compared to RAPT studies, 68% (Bals *et al.* 2014).

While addition of 40% Ctec3 results in significant improvements that overcomes the limitations associated with the 5% Ctec3 dose with respect to cellulose, it also increases the amount of enzyme needed by a factor of 8. In Novozyme's recommendations, it suggests keeping doses between 1% and 6%, as higher doses would make costs impractical at large scale. An 8-fold increase in dosage is an inefficient tradeoff for a 12% gain in yield, therefore necessitating improvement of either the enzyme or the methodology of the procedure. Enzymes make up a

significant proportion of the operating costs of the RAPT procedure, therefore increasing the concentration of enzyme will result in significant loss in final profit. To achieve such improvements, it is important to understand the role of inhibitors on saccharification.

In order to determine the nature of inhibitors the process of solid phase extraction was applied. The results appeared to show that clarifying DLB by running the liquid phase (pressate) through a Strata NH2 sorbent results in small improvements in yield, from 67% to 72%. The improvement in glucose yield was seen in spite of water also being lost during clarification, increasing concentration of other inhibitors. Polar, acidic compounds that are likely to act as inhibitors include acetic acid and lactic acid, however this classification may also describe uronic, levulinic and formic acids (Palmqvist *et al.*, 2000). However, the results were not statistically significant, $p>0.05$, which meant further research was needed to confirm the hypothesis.

The degree by which desired compounds were adsorbed from the pressate during clarification was measured using HPLC, to determine the concentration of five compounds both before and after clarification. It was found that acetic acid, lactic acid, ethanol, glucose and xylose were removed to some extent during clarification, suggesting that the Strata NH2 column was not specific to acids alone. The lack of specificity would explain why water molecules were also removed from the sorghum biomass pressate, concentrating compounds remaining in the clarified solution. The removal of polar, non-acidic compounds from the pressate during clarification also raises questions as to whether the Strata NH2 sorbent had extracted non-polar acidic species such as phenolic acids.

It should be noted that that all clarification products from a single sorbent (i.e. Strata NH2) were combined before HPLC measurement, as a representation of the concentrations that were to be added to the dried biomass, and therefore there was standard error measurements were made. Measuring the combined clarified media may have caused flaws in clarification by individual

Strata NH2 columns to be missed, overstating the role in the sorbent to adsorb a particular compound. To reduce the risk of error, measuring the clarified products independently before and after combining would have offered increased accuracy.

As the study with Strata NH2 was not statistically significant, the hypothesis that improved saccharification would be measured following clarification of polar compounds from DLB had not been confirmed. To better understand the role of polar compounds, it was important to study potential inhibitors without changing the concentration of other chemicals. Of these species, it was likely that acetic acid, lactic acid and ethanol will be responsible for Ctec3 inhibition due to their previous activity in inhibiting cellulases and their effect at small scale on saccharification of the Strata XL and Strata XL-C clarified biomass.

The use of SPE to clarify DLB was not consistent in this experiment. Saccharification at standard scale demonstrated that Strata NH2 clarified sorghum biomass showed the greatest glucose yield compared to the pressate control samples, however the result was not statistically significant. This differs from the results at small scale, where Strata NH2 did not achieve any improvement. The inconsistency is likely the result of two points of error. The first potential point of error is the conditioning step, in which the columns are cleaned and primed for interacting with the target molecules by chemicals that inhibit Ctec3 performance through the column, such as methanol or hexane. It was found that a new peak in the HPLC profile of the Strata XL clarified biomass most likely representing hexane contamination, and therefore the risk of conditioning the column. The second potential reason is the loss of water when clarifying solution through the Strata NH2 column. Improper conditioning and removal of water reduces accuracy in predicting the effect of ethanol, lactic acid and acetic acid on Ctec3 activity from SPE alone. Both of these reasons could explain the large scale Strata NH2 data lacking statistical significance.

While the results of clarification suggest that ethanol, lactic acid and acetic acid would act as inhibitors of Ctec3, SPE cannot predict the role of just a small number of compounds. SPE

removes a large variety of compounds from a solution, any of which could be responsible for the effect, therefore testing the role of each compound is needed after SPE formulates the hypothesis.

4.2. Ethanol is a significant inhibitor of enzymatic saccharification

Taking into account the results of SPE, which demonstrated that polar compounds were associated with reduced glucose yield, this work demonstrated that ethanol was an inhibitor of saccharification, whereas organic acids acetic acid and lactic acids did not have an effect on Ctec3. Novozyme does not provide suggestions for what the inhibitors of Ctec3 may be and how to reduce their impact in the associated guidelines, necessitating research into the impact of ethanol on sorghum DLB saccharification.

Ethanol inhibits the cellulases of Ctec3, resulting in a reduction of 12% glucose yield from sugar T DLB at 96 hours when the concentration of ethanol is raised from 15 gl^{-1} to 35 gl^{-1} (Fig. 9). The inhibitory effect of ethanol was displayed first by CCD, where the effect of ethanol, lactic acid and acetic acid were studied both in isolation and combination via 15 representative conditions. The results of the CCD showed that only ethanol caused an inhibition of saccharification which could not otherwise be explained by error, suggesting that ethanol was the only chemical which effects Ctec3 kinetics of the three studied.

The CCD was used as a predictive measure on the effect of ethanol, lactic acid and acetic acid on yield (Fig. 8), but was not in its own right definitive, as the UDS measurements of the samples was error prone. As UDS is used to calculate available cellulose, and therefore the theoretical yield, this created a significant problem, preventing accurate glucose yields. Because of the inaccuracy, glucose concentration was instead used. CCD functioned to suggest the role of the three compounds, but was not definitive in its own right.

Due to the CCD experiment suggesting that ethanol was the only inhibitor of the three chemicals tested, an experiment that looked in greater detail about the effect of ethanol on Ctec3 activity

was completed. This experiment functioned to both validate the results of the CCD, and to determine the extent to which ethanol inhibited Ctec3 performance. The concentration of ethanol was increased in increments of 5 g l^{-1} in order to characterize the inhibition of ethanol, which was found to be linear, as predicted by CCD (Fig. 9B). The results suggested that a reduced ethanol concentration would likely result in an increased yield at 96 hours.

Ethanol is a known inhibitor of cellulase activity, however its inhibitory activity has previously been discussed with respect to simultaneous saccharification and fermentation, in which product ethanol is generated as saccharification progresses. Such studies have either used low ethanol concentrations (Qi *et al.* 2014), or different cellulases to Ctec3 (Ooshima *et al.* 1985). However, the Silenus project creates a new pathway by which ethanol can be introduced into the reaction conditions and inhibit Ctec3 activity.

The fact that the initial ethanol concentration acts as an inhibitor of Ctec3 performance is important due to the ethanol produced as a result of primary ensiling. Primary ensiling looks to maximize total ethanol production from sorghum by adding fermenting microbes during ensiling to complete fermentation of free sugars during the storage of biomass. The produced 1G ethanol can then be stored for a greater duration of time without significant consumption by microbes than is possible with glucose, and can be transported without significant reductions in yield. The improved storage capacity is because the metabolism of ethanol in an anaerobic environment is significantly more challenging than sugar metabolism, limiting contaminant growth. Alongside reducing sugars, contaminants also introduce compounds that may inhibit the fermenting microbe. Therefore primary ensiling not only produces a greater level of total ethanol, but also reduces contamination, meaning that less acetic acid and lactic acid will be produced.

The evidence of the inhibitory role for ethanol on Ctec3 saccharification of DLB, shows that while there are improvements made in total ethanol yields by producing 1G ethanol from sorghum grain immediately, there is also a risk of the 1G ethanol acting to inhibit DLB hydrolysis. At the

current stage of planning for the complete Silenus procedure, following ensiling the 1G ethanol, and other components of fermentation, are separated from the biomass through the use of a leachate stream. The leachate stream is channeled to a lagoon which is covered to maintain an anaerobic environment. The use of a leachate stream acts to separate excess liquid from solid. However, some of the liquid will be retained in the biomass, meaning there will still be high concentrations of ethanol. Refinement of this strategy could aid in reducing inhibition of Ctec3 by ethanol.

Leachate separation is an alternative to using a Superheated Steam Dryer (SSD) to remove ethanol. The SSD could be used to capture ethanol from solid biomass (Morey *et al*. 2014). However, use of the SSD was disfavored due to the associated costs. SSD introduces additional expense due to a high energy demand required for both heating and ethanol capture, which may lead to reduced profit at industrial scale. In addition, an argument is present that the retained ethanol would instead be volatized during the HESA recovery stage of RAPT, perhaps making the entire SSD procedure redundant. Nevertheless, the absence of SSD of ensiled biomass means that the biomass will retain more ethanol. Volatilization by RAPT takes the role of SSD. However, the two in combination would show increased efficiency. Therefore the initial ethanol concentration will be higher.

Acetic acid and lactic acid do not appear to have an effect on inhibition of Ctec3, which suggests that neither microbial contamination nor the byproduct of yeast fermentation will have significant effects on the ability of enzymes to hydrolyze pretreatment material. This is important as the CCD demonstrates that glucose production by enzymatic saccharification is dependent on reduced first generation ethanol production but not reduced acetic acid and lactic acid. Therefore, for saccharification alone, increased ethanol production will be a significant problem whereas contamination with acetic acid and lactic acid producing organisms is less likely to affect glucose yield.

4.3. Products of lignocellulose degradation inhibit activity of the enzymes

While the inhibitory effect of ethanol on Ctec3 is understood, ethanol alone does not explain the difference between yield at 40% Ctec3 dose and 5% Ctec3 dose. While the stabilizing solution or half-life of Ctec3 may be part of the reason for the difference in yield, unmeasured inhibitors present in the pretreated biomass pressate could also play a role. SPE was used to remove compounds of varying polarities and pK_a from DLB, followed by re-addition of ethanol, lactic acid, acetic acid, glucose and xylose to reduce the effect of these compounds on inhibition. By ensuring that the effect of ethanol, lactic acid and acetic acid was equivalent on Ctec3 for all samples, this work demonstrated that the products of RAPT act to cause significant inhibition of Ctec3.

Due to the different nature of each sorbent used, some conclusions could also be made about structural features of these inhibitors. It was found that that clarification of biomass by both Strata XL and Strata NH2 achieved improvements in glucose yield, with respect to DLB that had not been clarified. When the level of ethanol, lactic acid, acetic acid, glucose and xylose were equivalent between all samples the most effective column was found to be the Strata XL column, for which the sorbent selected for non-polar to weakly polar species. Strata NH2 on the other hand selected most strongly for polar, acidic compounds.

By looking at the glucose yield in the initial 24 hours it is possible to determine the kinetics of the clarification. Improving kinetics can reduce the timescale of saccharification. This saves operating costs for saccharification, particularly the costs to maintain the heat and rotation rate of pretreated biomass at levels optimal for Ctec3 performance. No improvements to kinetics were seen in the initial 24 hours of saccharification in either of the successful clarification methods. Strata XL clarified DLB displayed equivalent kinetics to the biomass control containing filtered pressate for the initial 24 hours, however Ctec3 ultimately achieved greater glucose yields at 48, 72 and 96 hours (Fig 9A).

Compared to the control, clarification with Strata NH2 showed reduced kinetics in the initial 24 hours. The reduced kinetics may suggest that some compounds present in the biomass activate the enzymes to accelerate yield, such as types of molecules or oligosaccharide molecules which could activate Ctec3 amylase activity. The presence of chloride ions, for example, has been demonstrated to cause k_{cat} allosteric activation of the amylase activity of cellulase EGX (Wang *et al*. 2003), and if present, would have been absorbed by Strata NH2, potentially explaining the reduced rate of glucose production. Alternatively, the Strata NH2 sorbent may have adsorbed components that act synergistically with Ctec3 inhibitors to reduce the inhibitor's effect.

It was of interest to test the theory by *Zhao et al.* that pheonolic acids would activate cellulases at moderate concentrations in a non-linear fashion (Zhao *et al.*, 2015). Unfortunately, the SPE columns that would remove non-polar acids were not available at the loading capacity as other columns and would therefore be less representative. Instead, the goal was to compare the Strata XL-C clarified biomass and Strata XL clarified biomass in order to elucidate the effect of non-polar acids. Strata XL-C adsorbs non-polar base molecules, whereas Strata XL adsorbs non-polar molecules. A major difference between the two would therefore be the amount of non-polar acid adsorbed allowing some determination of the effect of these molecules. The results seem to suggest that the target molecules of non-polar bases improve activity of the enzymes. However, by observing the HPLC spectra, a new peak is created following clarification suggesting that residual hexane from column conditioning could have been present, lowering the activity of Ctec3.

It should be noted that washed DLB was not able to replicate improvements in yield seen in previous clarification at standard scale, in spite of the greatest initial kinetics. The low yield is likely a result of pH inhibition, as deionized water lacks the buffering capacity of the biomass matrix. Furthermore, the effects may be exaggerated by the presence of ethanol.

Determining the nature of the chemicals responsible for inhibition is an important stage. Pretreatment, however, produces a large number of components in the DLB that are difficult to identify (Almeida *et al.* 2007). Due to the immense number of compounds detected, many of which show overlapping peaks, identification of which compounds are present and which of these compounds act as inhibitors will be a time consuming activity. Instead, the goal of SPE was to determine the chemical features of the most significant inhibitors by comparing the effect of different columns on Ctec3 activity. The conclusions that can be made from SPE studies is that non-polar species are likely to act as significant inhibitors of cellulases. These could include the phenolic aldehyde vanillin, which has inhibited cellulases at 5 g l^{-1}, but could also include many other lignin derived products (Li *et al.* 2014). Similarly polar, acidic compounds are likely to act as inhibitors, these include acetic acid, which has already been tested, but may also describe uronic acids, levulinic and formic acids (Almedia *et al.*, 2007).

The challenge however is to describe in detail which of these compounds would act as inhibitors clarified from biomass in SPE. There is evidence that polar compounds, such as ethanol and glucose, are also removed by Strata NH2 and Strata XL, which would suggest polar species that are not measured by HPLC are also removed, such as furfural and HMF. This reduces the accuracy in determining the nature of inhibitors.

The failure to remove acetic acid by Strata NH2 is an additional concern with regards to specificity (Fig. 9B). Strata XL removes greater volumes of acetic acid, a polar organic acid, than Strata NH2. This may be the result of water being more polar and therefore displacing the target molecules to some degree. However, it may also suggest that the column is interacting with stronger acids. A major concern is that the concentration of phenolic acids could be affected by Strata NH2. While Strata NH2 has the capacity to bind acids, it is uncertain under these conditions that the non-polar nature of the phenyl group would reduce affinity of phenolic acids

to the sorbent. If the sorbent can bind to non-polar acids, it is uncertain whether strong non-polar acids would be displace the polar organic acids such as acetic and uronic acids.

The results of the experiments on clarification of biomass display that RAPT, while still an improvement over the traditional dilute acid pretreatment, is nevertheless unable to completely overcome the production of inhibitors.

Future work would look at determining in greater detail the identity of the compounds removed, both to determine the efficiency of clarification and to identify notable compounds which could be responsible for inhibition. This could be completed by using washed material and adding compounds of interest in order to elucidate an effect. It will be of interest to add phenolic acids and bases known to be present in pretreated solution to view if they can act as activators of Ctec3 (Zhao *et al.* 2014). Washed material, however, is susceptible to significant changes in pH as fermentation proceeds, which can denature enzymes. In addition the saccharification performance of ctec3 is consistently lower in biomass brought to a UDS of 10% with deionized water than those seen when pressate is used instead. This could be explained by poor buffering capacity increasing susceptibility to pH changes, but may also reflect that components of the pressate are required to activate enzymes.

Solid phase extraction therefore demonstrates the role of pretreatment derived molecules in reducing the activity of ctec3. Identification of these compounds, which are predominantly phenolic in nature, will be a significant challenge.

Information on structural features that inhibit Ctec3 is useful for designing enzymes better able to resist inhibitors. An important element of this experiment, however, is that some compounds may act to increase activity of the enzyme. Studying this effect to determine whether it is the result of error, and if not, how to replicate and enhance will be useful in increasing enzyme activity.

4.4. Ethanol, lactic acid and acetic acid all act to inhibit *S. cerevisiae* strain C5fuel activity

The fermentative products of either the yeast used in primary ensiling or microbes contaminating sorghum biomass can be a significant issue to the ethanol yield generated by C5fuel. A CCD on the effect of acetic acid, lactic acid and ethanol on C5fuel shows that these three compounds achieve significant inhibition at a magnitude that cannot be explained by error. All compounds are also recorded as inhibitors of wild type *S. cerevisiae* further supporting this conclusion (Adeboye, 2014) (Narendranath *et al.* 2001). In addition the experiment determines that there is positive synergy when ethanol and acetic acid are in hydrolysate together. The positive synergy means that together these two compounds act to reduce yields of ethanol further than would be seen otherwise. The synergistic effect is displayed by the curve in the estimated response surface (Fig. 11C). The effect of these three compounds on fermentation is different from what is recorded for saccharification, where only ethanol has an inhibitory role (Fig. 8).

It is also important to note the model equation produced. The equation was produced using 15 conditions, with duplicates to reduce error, however has an R^2 value of 92.4%, meaning that while the data supports the equation, it is not a perfect fit. Further testing of the equation is needed to confirm accuracy. If this equation is accurate, it will enable a quick calculation of yield once the ethanol, lactic acid and acetic acid values are calculated. This means that at large scale the yields could be calculated in order to estimate ethanol from each batch of material, and therefore profits to be made. Any deviation from these calculations would suggest the presence of other inhibitors or components that have altered performance. The model could therefore allow errors to be captured quickly to prevent poor material being used throughout the procedure. In addition, the equation allows the consequences when changing methodology to be quickly understood, such as if volatilization was lowered or ethanol rich leachate was added to the biomass to reduce the UDS.

4.5. Inhibition of C5fuel by furfural highlights the importance of seed culture

This work demonstrated that C5fuel could tolerate furfural concentrations up to 2 g l^{-1}, much higher than seen in the hydrolysate in practice, with little loss of ethanol production or sugar consumption, only showing a small reduction in xylose consumption at 24 hours. The ability of C5fuel to resist furfural inhibition is important as it reflects that the RAPT procedure, which is less harsh in the use of high temperature when compared to dilute acid pretreatment, can be modified. As furfural is less inhibitory to C5fuel than wild type *S. cerevisiae* (Sárvári Horváth *et al.*, 2003)(Field *et al.* 2015), subjecting the biomass to higher temperatures in RAPT to reduce the time course of the experiment may not lead result in a loss in yield. Alternatively, it may suggest the flashing period could be extended to strip higher levels of HESA, ethanol and other volatiles from the biomass.

A hypothesis was then posed that the improved resistance may be in part due to the conditioning of C5fuel, not the resistance genes alone. In RAPT, multiple stages of media samples increasing in volume are used to proliferate the cells in order to generate large amounts of C5fuel from the lower amounts of C5fuel purchased. At this time, purchasing the media for proliferation was cheaper than buying C5fuel at the volumes needed for biofuel production, and could result in increased C5fuel activity. On the other hand, if the cost of buying C5fuel was to be reduced, Shell could introduce C5fuel immediately to the hydrolysate for hydrolysis.

While formulating the proliferation steps, a seed culture containing hydrolysate became part of the method. The hydrolysate was introduced in order to generate a stage in which the C5fuel is adapted to the conditions of the cell, as well as reducing the use of YPD. Reducing the use of YPD media is important as YPD can be expensive and would require extensive water purification at large scale, making it unprofitable. Addition of hydrolysate, however, lowers rate of growth, with concentrations greater than 50% resulting in 24 hours being needed to achieve a suitable OD for inoculation of hydrolysate for fermentation.

Due to the difficulty in producing large concentrations of cells in growth media at a rapid rate, interest had returned to looking at purchasing the desired volume of cells to directly inoculate the hydrolysate for fermentation. Evaluating the role of the seed culture on C5fuel performance was therefore important in determining whether this decision would affect yields of fermentation. Because furfural was a known inhibitor of wild type *S. cerevisiae* (Sárvári Horváth *et al.*, 2003)(Field *et al.* 2015), while shown to have little effect on the GMO strain C5fuel when the current procedure is applied, a study was completed looking at how different seed cultures affected C5fuel performance.

It was found that the use of a hydrolysate and YPD mixture to condition proliferating C5fuel cells was essential to achieving the desired kinetics in C5fuel in the presence of inhibitory concentrations of furfural. When C5fuel cultured in a pure YPD mixture, representative of C5fuel in the condition it would be purchased, was exposed to 2 g l^{-1} furfural in standard hydrolysate the conditions resulted in the cell showing a significantly reduced rate of sugar consumption, with only 25% of sugars, of which xylose consumption was just 4% (Fig. 13B). Not only does a reduced rate of fermentation mean that it will take longer to obtain desired yields, but increasing costs will also be recorded as equipment must be run for longer and temperatures must be maintained during this time.

These results demonstrate the necessity of proliferating cells internally rather than buying C5fuel in bulk from Mascoma, due to the role of the seed culture in conditioning the yeast. Conditioning is a process by which a cell is introduced to an intermediate stress in order to allow it to tolerate an extreme stress (Wallace-Salinas *et al.* 2013). While the effect of furfural at 2 g l^{-1} highlights how the lack of conditioning can affect the kinetics of an experiment, when taking into account the role of ethanol, lactic acid and acetic acid in reducing yields alongside kinetics, improper conditioning, and thus reduced expression of resistance genes could severely reduce final

yields. This is particularly important as little evidence is present in literature about the impact of conditioning *S. cerevisiae* to improve ethanol yields.

4.6. The use of SPE to clarify polar acids and non-polar compounds from the pretreated biomass will result in improved fermentation yields compared to standard hydrolysate.

The use of SPE on DLB suggested that products of lignocellulose decomposition and contamination were responsible for significant loses in glucose yield. Whether this held true for fermentation was also of interest, as the use of the seed culture conditioning stage may result in up-regulation of resistance genes and therefore reduce the impact of any potential inhibitors, as seen for furfural.

It is important to note that hydrolysate generated from Ctec3 saccharification will differ from the DLB, due to variation in oligomer and sugar concentration, particularly glucose and xylose. In addition, proteins will be present in the hydrolysate as a result of Ctec3 saccharification, which may influence clarification. This is of concern as the SPE sorbent will interact with the hydrolysate matrix differently when compared to the pressate matrix, producing clarified solution that differs in concentration of target molecules compared to DLB. For this reason hydrolysate that had been produced from the saccharification of SPE clarified DLB was used in this experiment as opposed to SPE clarified hydrolysate.

It was found that fermentation of the hydrolysate produced from both Strata XL and Strata NH2 clarified biomass resulted in an equivalent improvement in ethanol yield. This contrasts to the effects seen in saccharification, where Strata XL clarified biomass showed superior kinetics to Strata NH2 clarified biomass. The difference between performance in saccharification and fermentation therefore suggests that components that have achieve similar inhibition on C5fuel will not necessarily have the same effect on Ctec3.

Overall the data suggest that components removed from pretreated biomass by Strata XL and Strata NH2 achieve equivalent inhibition on C5fuel performance. Based on column preference, these are likely to be polar acids and non-polar compounds.

4.7. Imperfect selection accuracy limits the use of solid phase extraction to determine behavior of phenolic compounds

The use of solid phase extraction in order to determine the nature of the most significant inhibitors of saccharification and fermentation was different to the traditional application of SPE. Experiments that focused on SPE, such as **3.1.2**, were not only important in finding the role of inhibitors of saccharification and fermentation, but also allowed evaluation of the technique itself.

Solid Phase Extraction is a sample preparation and purification technique often used to assist in the analysis of a chemical matrix (Lindsey *et al.*, 2001). To achieve this, a solution is run through a sorbent containing side chains with moieties conditioned to interact with other molecules depending on their physical and chemical nature. The chains will then interact with complementary compounds such as acids, adsorbing them and removing them from the matrix.

The products of lignocellulose pretreatment are a broad range of compounds, often phenolic in nature. Due to the complexities of the matrix generated following lignocellulosic degradation, it was of interest to determine whether SPE could be used to identify a group of molecules that can act to achieve a particular effect, such as inhibition or activation of Ctec3. Importantly, it was of interest if SPE could facilitate the production of an accurate methodology that would determine which chemical or physical properties were associated with the most significant inhibition of both fermentation and saccharification.

In recent years technology associated with SPE has enabled solid phase extraction cartridges to go from clarifying material in terms of 100 mg of material in a 3 ml tube to being able to purify up to 5 g of target molecules in a sample matrix up to 20 ml. These “gigatubes” enabled large scale solid phase extraction to be completed. The ability to complete large scale solid phase extraction

to clarify a range of different compounds meant that for the first time SPE could be used in order to clarify high volumes of the liquid matrix of sorghum DLB (Corley and Iacono, 2015).

In order to study the effect of clarification on saccharification and fermentation, 3 columns were needed, one to remove polar species from a biomass matrix, acting to determine the role of polar compounds such as furfural in achieving inhibition, and two to remove different types of non-polar compounds in order to best clarify the behavior of phenolic compounds. In practice a column that selected for polar, acidic species was selected due to the volume of organic acids present in the biomass.

For SPE to be feasible in determining the chemical and physical nature of molecules responsible for achieving significant inhibition of saccharification and fermentation there must be a significant level of selectivity. In practice poor selectivity is a major problem with SPE that has obstructed definitive conclusions from being reached. By comparing the concentration of lactic acid and acetic acid to filtered pressate, we see that the sorbent XL-C, which selects for non-polar bases, reduced the concentration of these polar organic acids (Fig. 9B). This means that the columns lack suitable selectivity for binding only to their defined structures. Therefore, while results may suggest that one SPE column, and therefore one group of molecules such as polar acids, are responsible for inhibition, in practice we may be seeing the results of an overlapping group of chemicals, such as nonpolar acids. This limits interpretation of the results.

In addition, in order to guarantee that the effect seen is exclusively from the clarified biomass, it is important to completely wash and dry the solid phase of sorghum biomass to 100% UDS. The method of completely drying the sorghum biomass, however, raises concern that some of the lignin could depolymerize or sugars degrade under the high heat and pressure. The resultant lignin and degraded sugars could act to inhibit the saccharification enzymes.

SPE is an attractive method to determine features of a large group of molecules, particularly due to the variety of structures produced from lignin degradation, which can be difficult to identify. As

phenolic compounds are known to inhibit cellulase (Li *et al.* 2014) and *S. cerevisiae* activity (Almeida *et al* 2007), it is important to determine what chemical structures are most responsible for losses in yield. An understanding of the exact role of phenols could aid with determining how the products of lignin degradation are affecting the final yields of glucose and ethanol. In particular, it would be important to measure whether lignin degradation products were causing the large losses in yield or if the results seen are representative of chemicals that could be overlooked, such as citric acid. The imprecision of the columns, however, means that determining whether there are any features to look into, such as the role of polar acids, is a significant challenge.

4.8. Changes and refinements

Over the course of inhibitor studies certain elements of the sorghum saccharification and fermentation procedure would vary slightly. While these would not affect the individual experiments, the changes may lead to some inconsistency between experiments.

Of these changes, one of the major variations was pretreatment of the sorghum biomass. While using the same techniques, concentrations of acid and originating from the same ensiled biomass, every 9 kg of material would vary on the date pretreated. This could result in small variations in crystallinity of the cellulose polymer, lignin and concentration of components. UDS may also be inconsistent between DLB samples.

As the experiments proceeded new batches of Ctec3 were delivered, and while identical to previous enzyme, small changes in yields and kinetics could be seen due to the differences in age of the enzyme and exposure to room temperature. C5fuel samples were also delivered regularly and could face similar small changes.

One element of the procedure that was refined over the course of the experiment was the technique used to measure the UDS of pretreated sorghum biomass. Following challenges with the error prone UDS measurements for the CCD experiment on saccharification, a review was

made of how DLB was sampled. It was found that using residual biomass for UDS measurements, following the allocation of DLB for the experiment introduced significant error. This remaining DLB for UDS measurement would often differ in UDS when compared to the DLB samples taken from the whole sample. In addition it was important to mix the DLB samples in a container with a wide surface area, as opposed to using a falcon tube or flask, before taking 4 g aliquots for UDS measurement, so to best maintain consistency within material. Following these steps, UDS measurements were found to be consistent between samples.

4.9. Limitations in data points

In this work, 24 hour data points were collected for both fermentation and saccharification studies. While saccharification took place throughout the 96 hours measured, for fermentation ethanol yield would plateau between 24 and 48 hours. The lack of time points for fermentation created limitations in the modelling ethanol yields for figures 11D, 13A and 14, causing Prism to generate polynomial lines in place of a more suitable model. It could be reasoned that ethanol yield would be seen as a sigmoidal curve, starting with a lag phase, followed by exponential ethanol production and plateauing at 48 hours. This was seen for some conditions in figure 13A and 14, and a sigmoidal curve would take into account the lag phase of C5fuel growth in a new media. However without data points prior to 24 hours the exact shape of the line, length of the lag phase and degree of exponential growth cannot be concluded, meaning the shape could still be imprecise and the conclusions drawn are flawed.

This issue demonstrates a need for more frequent measurement in fermentation studies, at least during the initial 12-24 hours. A measurement every four hours would give greater insight into how C5fuel behaves and would facilitate improved models in Prism. As measuring all 24 hours could be challenging, a second set of flasks for each reaction condition could also be prepared, with fermentation started at the 12 hour point, producing samples staggered 12 hours behind the rest, suitable for measuring overnight time points.

One time point which could be limiting in this work is the 0 hour time point. Measured before addition of Ctec3 or C5fuel, this time point represents the base levels of glucose or ethanol used in yield calculations. Ctec3 is held in a stabilizing solution rich in glucose and sucrose. Addition of 5% enzyme would add small amounts of sugar to the reaction, potentially impacting yield calculations. An estimated volume of sugars added when adding Ctec3 is generated during yield calculations, based on previous controls used in Shell, however this measurement is an estimate, so while the yield is represented at 0%, in practice it may be slightly smaller or greater.

4.10. Future work

4.10.1. Evaluate CCD of fermentation

When determining the role of acetic acid, lactic acid and ethanol on saccharification and fermentation Stats Graphics generated a model equation for the effect of the three components on ethanol yield. It will be important to evaluate the model equation in order to determine whether it can accurately represent the effect of ethanol, lactic acid and acetic acid on fermentation. Incorrect models can give inconclusive results, whereas a fully functional model allows evaluations to be made of ethanol yield with respect to the initial concentration of ethanol, lactic acid and acetic acid without requiring experiments to validate decisions, therefore saving both time and costs.

In order to evaluate the model equation a series of experiments in which the results are compared to the predicted results from the model can be planned out. Incremental increases in ethanol, acetic acid and lactic acid concentration, attempts to replicate synergy and randomized fermentation samples could all be used to evaluate the accuracy of the model. One element to note is that the model is suitable for representing all sorghum hydrolysate samples, but would not be applicable for varieties of sorghum other than Sugar T. This means that the model can be used to justify decisions, but once new varieties of sorghum are used, the yields will differ from

predictions made by the equation due to differences in degree of hemicellulose degradation in acid pretreatment, the concentration of inhibitors and the extent of lignin depolymerization.

4.10.2. Methodology for reducing the concentration of ethanol, lactic acid and acetic acid

The results of saccharification and fermentation studies on the effect of ethanol, lactic acid and acetic acid reveal the need to reduce the influence of these three compounds on lignocellulosic ethanol production.

At the current research stage, enzymatic saccharification and fermentation within flasks are targeted to a yield of 87% for glucose and ethanol respectively. Therefore, it is important to evaluate techniques that may overcome inhibition of Ctec3 and C5fuel. A shared inhibitor of these two systems is ethanol, therefore one method to improve yields is to reduce the initial ethanol concentration in pretreated Sorghum biomass.

To reduce the initial ethanol concentration it will be important to introduce a control step in order to ensure that ethanol is not above a defined level of 20 g l^{-1}. Perhaps an optional SSD step could be introduced if the biomass fails this check (Morey *et al*. 2014), or an extended flashing period following RAPT, which may also strip higher concentrations of HESA (Weider and Blackbourn 2016). A study may be required to determine the effect of using SSD, or a process that can act as a suitable model, before pretreatment, looking at the energy demand against the amount of ethanol produced. If SSD is able to improve both glucose yield in saccharification and ethanol yield in fermentation, it could offset the price of an SSD stage. Alternatively, varying an experiment could look into the effect of altering the volatilization period following pretreatment. A possible problem with this concept, however, is that SSD and volatilization introduces high temperatures to the lignocellulose, which could lead to a lignin reaching its glass transition state and subsequently reduce accessible cellulose for saccharification (Tan *et al.* 2009).

Reducing the concentration of other components of the biomass may be more manageable than ethanol reduction. Acetic acid can be the produced from contaminating bacteria (Hofvendahl *et*

al. 2000), and *S. cerevisiae* may also produce acetic acid (Rantsiou *et al.*, 2012). It is possible to limit acetic acid and lactic acid production through the use of the antibiotic lactrol, in which virginiamycin is an active component (Hynes *et al.*, 1997). While this will reduce the concentration of organic acids produced by fermentation, use of lactrol can raise costs to levels that the improved yield may not compensate for. Alternatively, it has been shown that co-fermentation of *S. cerevisiae* with *Candida zemplinina* has resulted in a reduced acetic acid production, which could be an option for a safer grain ethanol production (Rantsiou *et al.*, 2012).Studies will need to ensure that the second microbe does not interfere with ethanol production and will not significantly affect production costs. Genetic modification is not permissible during these stages due to the increased risk that GMOs could be released to the environment.

It is important to note that acetic acid is a volatile and could be removed by SSD. However, due to acetylation of xylose in hemicellulose chains, SSD will not be effective in stripping all acetic acid from the biomass, and therefore would simply add costs for little improvement. Indeed, acetylation of xylan could be a major problem in reducing yields of 2G ethanol; therefore it will be important to measure the acetic acid concentration of biomass before and after pretreatment to determine the on yield. Acetylation plays a role in enabling xylan to interact with cellulose in order to compartmentalize cellulose from lignin. Importantly, the degree of acetylation varies between species (Busse-Wicher *et al.*, 2014), and could therefore play a role in crop selection.

Changing crop selection could be a method to reduce the impact of ethanol on Ctec3 and C5fuel. This work suggests that energy crops with high levels of both free fermentable sugars and lignocellulose, such as strains of sugar cane or maize (Ra *et al.*, 2012), may not be favored in the long term due to the high levels of inhibition by the produced 1G ethanol. Instead, focus should turn to crops with moderate levels of free sugars but high levels of lignocellulose. It is important note that focus exclusively on agricultural residues in an effort to avoid high initial

ethanol yields is not an acceptable action either, as 1G ethanol yields are needed to assist total ethanol production so that the complete lignocellulosic ethanol process is profitable. Instead a balance will be required between crops that produce effective yields of 1G ethanol, but do not significantly reduce Ctec3 and C5fuel activity. Suitable crop selection can either be achieved by looking at for strains of sorghum with lower grain yields but higher stem or leave production, a region of the plant rich in lignocellulose (Saini *et al.*, 2015). Alternatively testing other crop species, such as switchgrass or elephant grass could be an option.

Alternatively, instead of altering the RAPT procedure prior to saccharification, it will be important to evaluate the cellulase enzymes and GMO strains used in order to alter resistance towards inhibitors. Evaluating Ctec3 cellulases will require an understanding of the cellulose structure to elucidate the bonding mechanism of ethanol and how it achieves inhibition. This could be achieved by crystallization and X-ray diffraction studies with the intent of discovering the binding site and to determine the possibility of using genetic modification to reduce inhibition. Such techniques have been applied to find the binding site of Aza-sugars on Glycosidase Cex of *Cellulomonas fimi* (Notenboom *et al.*, 2000). Substitution of the amino acids identified as interacting with ethanol could aid in reducing inhibition, with the effect on kinetics analyzed following editing of the enzyme.

Genetic modification of C5fuel will need to take into account the inhibitory actions of acetic acid and lactic acid, as well as ethanol. Due to the synergy between acetic acid and ethanol, the greatest focus should be on one of these two compounds. The standardized values of ethanol, lactic acid and acetic acid used throughout saccharification and fermentation studies are assumed to represent the conditions seen in practice. Acetic acid is recorded in practice to have an average of 8 g l^{-1}, whereas ethanol is instead measured to reach an average of 22.5 g l^{-1}, meaning significant concentrations of ethanol are present in the hydrolysate, which may make it the more desired target for C5fuel strain resistance. On the other hand, acetic acid can achieve

equivalent reduction in ethanol yield as initial ethanol concentration, but over a smaller concentration increase, 2% g^{-1} compared to 1% g^{-1} for ethanol (Fig. 11B). Ultimately, the lower concentration of acetic acid suggests that ethanol is the more significant inhibitor, and therefore the compound resistance should be encoded for in C5fuel. In addition, it is unlikely that acetic acid concentration will rise above these levels, as 1G ethanol production reduces *Acetobacter* feedstock.

There are a number of methods by which ethanol tolerance can be achieved in *S. cerevisiae*. Directed evolution, growing multiple generations of C5fuel strains on media that incrementally increases in ethanol concentration, is one option, with the intent of producing a generation with significant resistance. Methods to achieve this include random mutagenesis, which produces multiple mutants of which some may show improved yields (Packer *et al.*, 2015). Alternatively, increasing expression of resistance genes could enhance inhibitor tolerance. One such method that has been proposed to improve ethanol resistance is the up-regulation of proton and potassium pumps by hyperactivation of *PMA1* and *TRK1*. Up-regulation of these ion channels, which enable proton export and potassium import, improved resistance of lab *S. cerevisiae* above even industrial strains (Lam *et al.*, 2014).

Unfortunately, due to the commercial nature of C5fuel as a product of Mascoma and the enzyme package Ctec3 being produced by Novozyme, information about the structures of enzymes or genes used in the cell is confidential to Shell. Genetic modification will instead be completed by the external companies, with guidance from Shell using the results generated to justify research.

4.10.3. Enhancing Solid Phase Extraction to best determine the nature of inhibitors

This work both demonstrated the potential and flaws of SPE in understanding the role of components of the pretreated sorghum biomass matrix in inhibiting saccharification and fermentation. SPE has been shown to contain notable flaws, however, the challenge analysis of

inhibitors. Future work should look into refining the procedure to improve the understanding of clarification through trouble shooting.

SPE sorbent will adsorb chemicals within a matrix in a sliding scale of importance, as designated by the column. Strata NH2, for example, will bind polar strong acids, before interacting with polar weak acids. The result of SPE demonstrated that once all polar acids are bound it can be seen by the results of SPE it will begin to interact with polar components, such as ethanol. It is possible that this scale of adsorption can be overcome by increasing the amount of target molecules, strong and weak acids, running through the sorbent.

It is important to note that SPE columns that adsorb polar compounds will also remove polar water molecules from the pressate solution, therefore making polar columns unattractive going forwards. Columns which bind non-polar groups could be refined. One potential reason that columns lack specificity is due to the low concentration of non-polar molecules in the biomass matrix. Therefore to improve specificity, a greater volume of solution could be run through each column, therefore displacing molecules weakly absorbed by the sorbent. This trouble shooting step could be used to evaluate whether specificity of the columns can be improved with respect to clarifying the pressate. By studying the change in concentration of polar molecules, and comparing to a standard non-polar compound an accurate loading volume can be determined. The goal of troubleshooting would be to retain significant amounts of non-polar molecules in the stationary phase, yet show little change in the concentration of polar compounds.

While use of HPLC can enable easy measuring of the concentration of ethanol, lactic acid and acetic acid, it is challenging to measure how non-polar compounds produced from lignin degradation. These columns are detected together as a single, tall peak unrepresentative of individual molecules. Instead, a new technique should be used for determining the effect of SPE clarification on soluble components of the sorghum biomass.

One method to determine the features heavily removed from biomass during clarification is Nuclear Magnetic Resonance Spectroscopy, which has been used for studying pretreatment products following organosolvent pretreatment (Berlin *et al.* 2006). Comparing the NMR spectra of different clarified media could be used to compare which chemical groups, such as carboxylic acids, are most heavily removed in each column. SPE has previously been combined with NMR to reduce noise in urine analysis, and demonstrated that each washed and eluted fraction can then be measured with greater precision for chemical analysis (Jacobs *et al.* 2012). This can be used to predict the types of molecules clarified in order to find the structures that affect the final glucose yield.

4.10.4. Enhance cell proliferation in a more representative seed culture

The role of seed culture in conditioning the cell to the toxic conditions of the hydrolysate has been shown to be highly important in maintaining desired kinetics. Use of a 50% hydrolysate seed culture can increase ethanol yield under stress (Fig. 13). However, the current procedure tested is not applicable to large scale. At large scale, YPD cannot be used due to its expense. It will therefore be important to produce a system by which either C5fuel is grown in a mechanism representative of large scale plans. In practice pure hydrolysate will be used for the seed culture, with the pre-culture replaced by a 50% hydrolysate, 50% waste water media.

Because of the increased use of hydrolysate, cells will be subjected to highly toxic conditions, lowering growth rate. In order to overcome this lowered growth rate it may be of interest to use directed evolution, growing multiple generations of C5fuel on a gradually increasing concentration of hydrolysate in a seed culture to accelerate proliferation under stress. This could be used to generate a strain of C5fuel that proliferates in unfavorable media, and may show improved resistant to the components of hydrolysate. Throughout the directed evolution studies, it will be important to take small samples of the culture in order to test fermentation capacity of the C5fuel generation. Regular measurements must be taken to determine the fermentation

capacity of C5fuel following directed evolution to ensure cells are not sacrificing essential mechanisms in order to increase proliferation.

It will also be of importance to study the Crabtree effect of C5fuel when the cell is proliferating. The Crabtree effect can result in notable reductions in cell growth as metabolism is shifted towards fermentation, and requires feeding strategies in order to overcome (Gómez-Pastor *et al.* 2011). One feeding strategy that does not limit proliferation is the fed-batch method. Fed-batch refers to introducing new media for the cell to metabolize in a discontinuous fashion, based on adding sugars only when the cell's rate of growth begins to slow. The rate at which new media is supplied in fed-batch strategies is calculated on carbon dioxide production. An optimal feeding strategy enables a greater *S. cerevisiae* growth rate than would be seen otherwise, increasing the number of cells and enabling the proliferation steps to be achieved in a shorter duration of time.

5. Conclusion

The work presented within this thesis evaluates the current limitations of the Silenus procedure used by Shell for second generation ethanol production, demonstrating that both primary ensiling and RAPT can result in reduced yields of lignocellulosic ethanol. Both saccharification of DLB by the enzyme Ctec3, and fermentation of hydrolysate by the *S. cerevisiae* strain C5fuel are subjected to inhibition as a result of conditions introduced by the current methodology. Ethanol, the product of primary ensiling, is an inhibitor of both Ctec3 and C5fuel, whereas acetic acid and lactic acid, products of contamination of the sweet sorghum crop, have no impact on Ctec3, but achieve statistically significant inhibition of C5fuel.

The library of compounds present in the DLB, produced as a result of lignocellulosic decomposition during RAPT, were shown to cause significant inhibition of both Ctec3 and C5fuel. RAPT therefore does not completely overcome the inhibitory effect of acid pretreatment on saccharification and fermentation, despite causing some improvements. Furthermore clarification of the DLB by SPE demonstrated that while non-polar compounds cause inhibition of Ctec3, clarification of polar acids caused rates of glucose production to be lowered, suggesting allosteric activation may take place to accelerate saccharification during the initial 24 hours. The importance of this research is that an understanding of inhibitors allows the Silenus procedure to be refined in order to improve yields. Refinements could vary from altering resistance of Ctec3 and C5fuel to inhibitors, or using conditions of pretreatment which generate smaller amounts of inhibitors within the DLB.

6. Materials and Methods

6.1. Biomass moisture content

Sorghum bicolor (sweet sorghum), var. Sugar T stock was selected as the biomass crop. Following harvest at ~90 days of growth, the sweet sorghum was first fermented in an ensiling pile with yeast. The products of ensiling were collected in a lagoon, a large body of solution covered by a tarp to limit aerobic respiration, and the solid (unfermented lignocellulosic) biomass was stored in buckets. The lignocellulosic biomass was then partially-saccharified using a concentrated acid pretreatment procedure via the external company Andrietz, having been provided to the company following ensiling. Acid pretreatment was completed using 3.75% w/w hydroxyethylsulfonic acid (HESA) at 125^{o}C to release xylose from hemicellulose, after which the pressure was increased alongside temperature to volatize the acid and remove it from solution. The HESA was then removed from the solution alongside some ethanol.

The resultant delignified biomass (DLB) can be described as a wet biomass, composed of a solid phase of cellulose, hemicellulose and residual lignin, and a liquid phase of sugars, organic acids, degradation byproducts of pretreatment and ethanol dissolved within water. The DLB is therefore composed of moist cellulosic biomass which is no longer recalcitrant to enzyme saccharification, and pockets of excess pools of liquid containing sugars and other soluble organic molecules. Any residual lignin is no longer bound to the cellulose or hemicellulose, and therefore will not impact lignocellulose. Recalcitrance refers to the ability of solid biomass to resist saccharification, often due to the cross linking of polysaccharides and polymers. In cases where moisture content of the sorghum DLB was too high, the sorghum samples were pressed in order to remove excess liquid, termed pressate, which contains free sugars and soluble inhibitors. In this experiment a standardized range of Un-Dissolved Solids (UDS) of 9-10% and Total Solids (TS), 15%, were selected for replicability, avoiding undesired effects on yield.

Pressing was performed in a stainless steel Fiesta wine press. The TS and UDS were then calculated by comparing the mass of a representative sample (20 g) of the resultant lignocellulose biomass before and after drying in a vacuum oven.

6.2. Analysis of metabolites

The concentration of ethanol, glucose, xylose, acetic acid, lactic acid and glycerol in DLB or hydrolysate were measured throughout hydrolysis and fermentation experiments. Measuring of metabolites was performed by high performance liquid chromatography (HPLC). Samples of pretreated biomass or hydrolysate solution were centrifuged at 25155 x g at 15 ˚C for 8 minutes within a SpinX Eppendorf tube. After centrifugation the solid phase, held by a filter, was disposed and 200 µl of the liquid phase was then mixed with 800 µl 0.01 M sulfuric acid in 2.0 ml Agilent HPLC vials. The samples were resolved on an Aminex HPX 87H column by a laboratory technician, analyzing samples at 65^{o}C with a flow rate of 0.2 ml min^{-1}, using 0.025 M sulfuric acid as the mobile phase and a sulfonated divinyl benzene-styrene copolymer as the solid phase, with measured samples stored at -20 ˚C. In order to determine the concentration of these compounds, solutions containing 0, 1, 5, 10, 15 or 20 g l^{-1} concentrations of these chemicals were prepared as standards. Samples were diluted 5-fold to ensure they were within the range covered by the standards.

6.3. Sorghum DLB hydrolysis

Prior to enzymatic saccharification, the concentration of ethanol, glucose, xylose, arabinose, lactic acid, glycerol and acetic acid were measured by HPLC. UDS, TS and cellulose values were then measured to enable yield calculations. Cellulose values were determined by taking a 4 g aliquot of pretreated biomass and subjecting the pretreated biomass to concentrated acid hydrolysis. The acid hydrolysis step can degrade cellulose to its glucose monomers; therefore by measuring the concentration of glucose produced after hydrolysis the percentage of cellulose that makes up the undissolved solids can be calculated.

For hydrolysis to be consistent between experiments a set of standardized concentrations are needed for the metabolites within the pretreated sorghum biomass. Unless otherwise specified, the concentrations of metabolites within the DLB were 23.5 g l^{-1} ethanol, 6.5 g l^{-1} glucose, 28.5 g l^{-1} xylose, 3.8 g l^{-1} arabinose, 6 g l^{-1} lactic acid, 1.2 g l^{-1} glycerol and 7.9 g l^{-1} acetic acid. These concentrations were chosen as they represented commonly seen values in post ensiled, post RAPT pre-treated biomass and enabled consistency between results. Once the concentrations of these components were brought to desired levels, the pH was raised to 5.3 via addition of 5 N ammonium hydroxide solution, unless otherwise specified, and the sample was incubated for one hour at 53˚C.

Following incubation, the enzyme mixture Cellic Ctec3, provided by Novozyme, was, unless otherwise specified, introduced at 5% dosing concentration with respect to the mass of cellulose within the biomass, using the equation below:

$$Mass\ of\ enzyme(g) = Mass\ of\ sample(g) * UDS(\%) * cellulose(\%) * Desired\ enzyme\ dose\ (\%)$$

Saccharification was completed in a shaking incubator at 200 rpm, 53^{o}C, with sample mass of 75 g unless otherwise stated. Every 24 h, the pH was adjusted to ensure that a value of 5.3 was maintained. At 96 h the hydrolysis was stopped and product was collected and stored at 4^{o}C. Ctec3 activity was measured by taking samples at regular time points to be analyzed by HPLC in order to observe changes in glucose and xylose production. The UDS, TS and cellulose levels of the pre-treated biomass were used as the basis of yield calculations, with glucose and xylose concentrations prior to enzyme addition used to determine the time 0h concentration, represented as 0%.

6.4. Microorganisms and medium

Propagation in the RAPT procedure propagation is a two-step process, in which first the micro-organism is grown first in a YPD pre-culture followed by a hydrolysate containing seed culture.

Saccharomyces cerevisiae, strain C5fuel was obtained from Mascoma LLC. Cells were cultured in 75 ml of 50 g l^{-1} YPD (yeast extract (20 g l^{-1})-peptone (10 g l^{-1})–dextrose (20 g l^{-1})) media for 10 h at 53^{o}C within an orbital shaker at 160 rpm. The media was contained within a 250 ml vented, baffled flask, and inoculated to produce the pre-culture.

The seed culture was then produced, composed of 50% YPD (25 ml) and 50% filtered hydrolysate (25 ml), unless otherwise stated.

In order to inoculate the seed culture, a sample of pre-culture was added. This took place after *S. cerevisiae* had proliferated in the pre-culture for 10 hours, ensuring the cells at a high optical density (OD), representing significant proliferation. The volume of the pre-culture aliquot was dependent upon the equation displayed below, where the targeted optical density of the seed culture was 0.05.

$$Inoculation\ volume_{preculture}\ (ml) = \frac{0.05 * Volume_{Media}(ml)}{OD_{preculture}}$$

OD was measured using a Thermo Scientific Genesys 20 Spectrophotometer at A_{600nm}

Proliferation of *S. cerevisiae* in the seed culture then followed for 14-17 h, 53 ˚C, 160 rpm, once more in a 250 ml baffled flask. Hydrolysate was filtered in this stage so to allow continued measurement of OD.

6.5. Fermentation

For fermentation to be consistent between experiments a set of standardized concentrations were needed for metabolites in the hydrolysate. Hydrolysate was brought to 23.5 g l^{-1} ethanol, 57.6 g l^{-1} glucose, 25 g l^{-1} xylose, 3.8 g l^{-1} arabinose, 6 g l^{-1} lactic acid, 1.2 g l^{-1} glycerol and 7.9 g l^{-1} acetic acid, unless otherwise stated, so that it could be used as fermentation broth. *S. cerevisiae*, strain C5fuel was grown on 50 ml hydrolysate in a 125 ml baffled volumetric flask, vented to enable carbon dioxide release while preventing oxygen uptake. C5fuel cultures were centrifuged (49314 x g, 10 min, 4^{o}C), the supernatant decanted and the pellet resuspended in 50

ml hydrolysate. The volume of seed culture to be centrifuged was calculated using the equation below in order to yield an initial concentration of c5fuel cells of 0.5 g l^{-1}.

$$Volume\ of\ seed\ culture\ to\ incubate\ (ml) = \frac{Volume_{media}}{0.689\ *OD} * 0.5$$

C5fuel fermentation commenced at pH of 5.8, 32^{o}C, with orbital shaker at 180 rpm for 72 hours. pH was measured and adjusted periodically in fermentation by addition of 5 N potassium hydroxide. The concentration of ethanol produced was measured every 24 h by HPLC measurement. It should be noted that opening the flask to take samples and adjust pH will introduce oxygen and thus enable aerobic respiration, leading to reductions in the kinetics of ethanol production.

The theoretical yield of ethanol is calculated with respect to the initial concentration of fermentable sugars glucose and xylose, with the concentration of arabinose, a monosaccharide which C5fuel could ferment in the future, also taken into account.

6.6. Solid Phase extraction

6.6.1. Washed Biomass production

For Solid Phase Extraction (SPE) to clarify DLB, the pressate was to be separated from the DLB. To achieve this, sugar T DLB was pressed to separate the inhibitor-containing liquid-phase, the pressate, from the polysaccharide containing solid phase of DLB. The pressed solid DLB sample was then centrifuged (12236 x g, 15 min, 20 ˚C) and remaining liquid phase was disposed. The biomass was washed with distilled water (3 L) over a vacuum to dilute inhibitors. Once inhibitors were removed the sorghum, DLB was dried to a UDS of 100% in a vacuum oven at 65 ˚C for 2 days, meaning it was devoid of all liquid.

6.6.2. Pressate clarification

The liquid phase of DLB (pressate) contains a range of soluble inhibitors and was therefore the focus of this experiment. To initiate the experiment, pressate was collected from pressing of 9 kg

DLB, obtaining as much as 3 L. In order to remove any undissolved solid particulates a system of centrifugation (29588 x g, 10 min, 15^{o}C) and vacuum filtration was applied to the pressate. A solid phase extraction kit was then prepared. 3 types of columns were available, all provided by Phenomenex:

- Strata™ XL-C polymeric strong cation,
- Strata™ XL Polymeric reverse phase,
- Strata™ NH_2

The reverse phase columns, Strata™ XL-C and Strata™ XL, were conditioned via running 15 ml methanol followed by 15 ml deionized water. The column Strata™ NH_2 was conditioned with 2x15 ml aliquots of hexane.

The columns available were provided as 20 gigatube cartridges which can be attached to the main SPE apparatus. Each gigatube cartridge had a loading capacity of 5 g / 20 ml, meaning they could load 20 mL of solution at a time and remove 5 g of target molecules from that solution. To prevent overloading these columns the decision was made to add only 15 ml aliquot of filtered pressate to each tube. Solid phase extraction was performed under vacuum at a pressure of 10-15 Hg until collection of clarified supernatant ceased. Aliquots of clarified supernatant that were processed by the same types of columns were then collected and combined to obtain suitable volumes of solution for UDS adjustment of dried biomass.

The clarified supernatant was added to washed, dried DLB in order to yield a series of samples with the UDS of 10%. Seven samples were prepared by mixing dried solid with either Strata™ XL-C product, Strata™ XL product, Strata™ NH_2 product, filtered pressate, unfiltered pressate or deionized water. No change was made to the concentrations of ethanol, lactic acid, acetic acid, glucose or xylose following solid phase extraction unless otherwise specified.

Following the first 24 hours, sample pH was adjusted using 5 M sodium hydroxide solution, or 1 M sodium hydroxide solution if the 5 M concentration led to changes to the pH of deionized

water that were undesirable in magnitude, even at low volumes. Due to the poor buffering capacity of the solution, the pH of the washed biomass brought to a UDS 10% with deionized water was balanced twice within each 24 h period.

Hydrolysis of the clarified sorghum biomass was completed at standard scale using triplicates of 75 g, as detailed in section 6.3, unless stated otherwise.

6.6.3. Addition of Ethanol, lactic acid, acetic acid

After testing of the effect of SPE clarification, the method displayed above was repeated, however five chemicals in the DLB were brought to the desired level. These compounds were ethanol (23.5 g l^{-1}), acetic acid (7.9 g l^{-1}), lactic acid (6 g l^{-1}), glucose (6.5 g l^{-1}) and xylose (28.5 g l^{-1}), as they were products of primary ensiling or products of saccharification..

6.6.4. Fermentation of product

To define the effect of DLB clarification on the performance of *S. cerevisiae*, the product of hydrolysate was obtained from the experiments detailed in 6.6.2. and 6.6.3. The concentration of glucose and xylose were adjusted so that they were at equal levels between all samples and fermentation followed, using the method detailed in 6.4. and 6.5.

6.7. Central composite design on hydrolysis for variables of acetic acid, lactic acid and ethanol

Sugar T DLB was separated into 15 x 200 g aliquots. Different combinations of ethanol, lactic acid and acetic acid concentration were selected in a three tiered, face-centered Central Composite Design (CCD) system. Reciprocal combinations of a high, low or medium concentration of ethanol (8 g l^{-1}, 18.5 g l^{-1} and 29 g l^{-1}), acetic acid (10 g l^{-1}, 15 g l^{-1} and 20 g l^{-1}) and lactic acid (8 g l^{-1}, 12 g l^{-1} and 16 g l^{-1}) were selected for each 200 g aliquot of sorghum biomass during this experiment to produce fifteen conditions. The highest and lowest concentrations designated for ethanol, lactic acid or acetic acid were chosen in accordance to historical data, with the fifteen selected combinations suggested by StatsGraphics.

Hydrolysis then followed as detailed above. The data was then processed by the modelling program StatsGraphics to determine the optimal conditions and any synergistic interactions.

6.8. Central composite design on hydrolysis for variables of acetic acid, lactic acid and ethanol

Hydrolysate produced from large scale hydrolysis was collected and 15 x 105 g samples were prepared, in which reciprocal combinations of three concentrations of ethanol (10 g l^{-1}, 20 g l^{-1}, 30 g l^{-1}), lactic acid (6 g l^{-1}, 12 g l^{-1}, 18 g l^{-1}) and acetic acid (5 g l^{-1}, 10 g l^{-1} and 15 g l^{-1}) were selected in accordance with suggestions by DOE software to obtain fifteen different conditions. In practice the levels of components were less than desired, in particular the acetic acid concentrations recorded which were targeted for 10, 15 and 20 g l^{-1}, as in the CCD experiment on saccharification.

The fifteen conditions were then run under the fermentation conditions detailed above in sections 6.4 and 6.5 and the data obtained was processed by the program StatsGraphics.

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8. References

Adeboye PT, Bettiga M, Olsson, L (2014) The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates, *AMB Express*. 4(46), ePublication available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4052683/ [15th May 2016]

Al-Hasan M (2003) Effect of ethanol–unleaded gasoline blends on engine performance and exhaust emission, *Energy Convers. Manage.,* 44(9); pp. 1547–61

Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae, *J. Chem. Technol. Biotechnol*., 82(4); pp. 340–49

Ahmad AL, Mat Yasin NH, Derek CJC, Lim JK (2011) Microalgae as a sustainable energy source for biodiesel production: A review, *Renew. Sustainable Energy Rev.*, 15(1); pp. 584-93.

Antizar-Ladislao B, Turrion-Gomez JL (2008) Second-generation biofuels and local bioenergy systems, *Biofuels Bioprod. Bioref.*, 2(5); pp. 455-69

Aro E.-M (2016) From first generation biofuels to advanced solar biofuels, *Ambio*, 45(Suppl 1); pp. 24–31

Apergisa N, Payne JE (2012) Renewable and non-renewable energy consumption-growth nexus: Evidence from a panel error correction model, *Energ. Econ*., 34(3); pp. 733–8

Bayané A, Guiot SR (2011) Animal digestive strategies versus anaerobic digestion bioprocesses for biogas production from lignocellulosic biomass, *Rev Environ Sci Biotechnol*, 10: pp. 43–62

Balan V, Chiaramonti D, Kumar S (2013) Review of US and EU initiatives toward development, demonstration, and commercialization of lignocellulosic biofuels, *Biofuels, Bioprod. Biorefin.*, 7(6); pp. 732-59

Bals BD, Gunawan C, Moore J, Teymouri F, Dale BE (2014), Enzymatic hydrolysis of pelletized AFEX™-treated corn stover at high solid loadings, *Biotechnol Bioeng.* 111(2); pp. 264-71

Belton PS, Taylor JRN (2003) Sorghum and millets: protein sources for Africa, *Trends Food Sci Tech*, 15(2), pp. 94-98

Behera S, Singh R, Arora R, Sharma NK, Shukla M, Kumar S (2015) Scope of Algae as Third Generation Biofuels, *Front Bioeng. Biotechnol.,* 90(1); ePublication available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4324237/ [16th July 2016].

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J. (2006) Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations, *J Biotechnol*., 125(2): pp. 198-209

Boden TA, Marland G, Andres RJ (2010). Global, Regional, and National Fossil-Fuel CO2 Emissions. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tenn., U.S.A., ePublication available: http://cdiac.ornl.gov/trends/emis/glo_2010.html [2nd July 2016]

Boretti A (2012) Towards 40% efficiency with BMEP exceeding 30 bar in directly injected, turbocharged, spark ignition ethanol engines, *Energy Convers. Manage.,* 57, 154–166

Brat D, Boles E, Wiedemann B (2009) Functional Expression of a Bacterial Xylose Isomerase in *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.*, 75(8); pp. 2304-11

Brown SW, Oliver SG, Harrison DEF, Righelato RC (1981) Ethanol inhibition of yeast growth and fermentation: Differences in the magnitude and complexity of the effect, *European J. Appl. Microbiol. Biotechnol.*, 11; pp. 151-5

Busse-Wicher M, Gomes TCF, Tryfona T, Nikolovski N, Stott K, Grantham NJ, Bolam DN, Skaf MS, Dupree P (2014), The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of *Arabidopsis thaliana*. *Plant J.*, 79; pp. 492–506

Carriquiry MA, Dub X, Timilsina GR (2011) Second generation biofuels: Economics and policies, Energy Policy, 39, p 4222–4234

Casey E, Sedlak M, Ho NW, Mosier NS. (2010) Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of Saccharomyces cerevisiae, *FEMS Yeast Res.*, 10(4); pp. 385-93

César AdS, Almeida FdA, Souza RPd, Silva GC, Atabanİ AE (2015) The prospects of using *Acrocomia aculeata* (macaúba) a non-edible biodiesel feedstock in Brazil, *Renew. Sustainable Energy Rev.*, 49; pp.1213-20

Cherubini F, Bird ND, Cowie A, Jungmeier G, Schlamadinger B, Woess-Gallasch S (2009) Energy- and greenhouse gas-based LCA of biofuel and bioenergy systems: Key issues, ranges and recommendations, *Resour. Conserv. Recycl.*, 53(8); pp. 434–47

Cherubini F (2010) GHG balances of bioenergy systems – Overview of key steps in the production chain and methodological concerns, *Renew. Energy*, 35(7); pp. 1565–73

Chisti Y (2007) Biodiesel from microalgae, *Biotechnol. Adv.*, 25(3); pp. 294–306

Corley CA, Iacono ST (2015) A rapid approach to isolating nitro-explosives from imidazolium and pyrrolidinium ionic liquid solutions using solid phase extraction (SPE) *Anal. Methods*, 7; pp. 6911-15

Corma A (2014) Introduction: Chemicals from Coal, Alkynes, and Biofuels, *Chem. Rev.*, 114 (3); pp. 1545–6

Cragg SM, Beckham GT, Bruce NC, Bugg TD, Distel DL, Dupree P, Etxabe AG, Goodell BS, Jellison J, McGeehan JE, McQueen-Mason SJ, Schnorr K, Walton PH, Watts JE, Zimmer M (2015) Lignocellulose degradation mechanisms across the Tree of Life, *Curr Opin Chem Biol.*, 29: pp. 108-19

Cray JA, Stevenson A, Ball P, Bankar SB, Eleutherio EC, Ezeji TC, Singhal RS, Thevelein JM, Timson DJ, Hallsworth JE (2015) Chaotropicity: a key factor in product tolerance of biofuel-producing microorganisms, *Curr. Opin. Biotechnol.*, 33; pp. 228-59

Creutziga F, Baiocchi G, Bierkandt R, Pichler PP, and Seto KC (2015) Global typology of urban energy use and potentials for an urbanization mitigation wedge, *Proc. Natl. Acad. Sci. USA,* 112(20), 6283–8

Dias De Oliveira ME, Vaughan BE, Rykiel Jr. EJ (2005) Ethanol as Fuel: Energy, Carbon Dioxide Balances, and Ecological Footprint, *BioScience* 55 (7): pp. 593-602.

Dutta S, De S, Saha B, Alam Md I, (2012) Advances in conversion of hemicellulosic biomass to furfural and upgrading to biofuels, *Catal. Sci. Technol*., 2, pp. 2025-36

EASAC (2012) The current status of biofuels in the European Union, their environmental impacts and future prospects. *EASAC policy report* 19, ISBN: 978-3-8047-3118-9, ePublication available at: http://www.easac.eu/fileadmin/PDF_s/reports_statements/Easac_12_Biofuels_Complete.pdf [4th August 2016]

EIA (2016) Monthly energy review: April 2016, pp. 28-29, table 2.1, DOE/EIA-0035(2016/4) ePublication available: http://www.eia.gov/totalenergy/data/monthly/archive/00351604.pdf [10th June 2016]

EPA (2016) Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990-2014, EPA 430-R-16-002, ePublication available at: https://www.epa.gov/sites/production/files/2016-04/documents/us-ghg-inventory-2016-main-text.pdf [15th June 2016]

Espinoza L, Kelley J (2014) *Grain Sorghum Production Handbook*, Arkansas: Cooperative Extension service, University of Arkansas, pp. 3-5

Everingham YL, Muchow RC, Stone RC, Inman-Bamber NG, Singels A, Bezuidenhout CN (2002) Enhanced risk management and decision-making capability across the sugarcane industry value chain based on seasonal climate forecasts, *Agric. Syst*., 74(3), pp. 459-77

Farzin YH and Tahvonen O (1996) Global Carbon Cycle and the Optimal Time Path of a Carbon Tax, *Oxf. Econ. Pap.* 48(4); pp. 515-36

Field SJ, Ryden P, Wilson D, James SA, Roberts IN, Richardson DJ, Waldron KW, Clarke TA (2015) Identification of furfural resistant strains of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* from a collection of environmental and industrial isolates, *Biotechnol Biofuels*. 8(33) ePublication available at: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-015-0217-z [30th August 2016]

García-Cubero MT, Palacín LG, González-Benito G, Bolado S, Lucas S and Coca M (2012) An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. *Bioresour Technol,* 107; pp. 229-34

Gelfand I, Zenone T, Jasrotia P, Chen J, Hamilton SK, Robertson GP (2011) Carbon debt of Conservation Reserve Program (CRP) grasslands converted to bioenergy production, *Proc. Natl. Acad. Sci. U S A.,* 108(33); pp. 13864–69.

Gnansounou E, Dauriat A, Wyman CE (2005) Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China, Bioresource Technol, 96(9); pp. 985-1002.

Gómez-Pastor R, Pérez-Torrado R, Garre E, Matallana E (2011). Recent Advances in Yeast Biomass Production, Matovic D (Ed.), Biomass - Detection, Production and Usage, InTech, EPublication available at: http://www.intechopen.com/books/biomass-detection-production-and-usage/recent-advances-in-yeast-biomass-production [2nd August 2016]

Goldemberg J, Guardabassi P (2009) The potential for first generation ethanol production from sugarcane, *Biofuels, Bioprod. Biorefin.*, 4(1); pp. 17-24

Guo M, Song W, Buhain J (2015) Bioenergy and biofuels: History, status, and perspective, *Renew. Sustainable Energy Rev.*, 42; pp. 712-25

Ha SJ, Galazka JM, Kim SR, Choi JH, Yang X, Seo JH, Glass NL, Cate JH, Jin YS (2011) Engineered Saccharomyces cerevisiae capable of simultaneous cellobiose and xylose fermentation, *Proc. Natl. Acad. Sci. U S A*, 108(2); pp. 504-9

Hansen J, Ruedy R, Sato M, Lo K (2010), Global surface temperature change, *Rev. Geophys*., 48(4), RG4004

Havlík P, Schneider UA, Schmid E, Böttcher H, Fritz S, R Skalský , Aoki K, De Cara S, Kindermann G, Kraxner F, Leduc S, McCallum I, Mosnier A, Sauer T, Obersteiner M (2011) Global land-use implications of first and second generation biofuel targets, Energy Pol., 39(10); pp. 5690–702

Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels, *Proc. Natl. Acad. Sci. U.S.A*., 103(30): pp. 11206–10

Hill N (2016). "SULTAN modelling to explore the wider potential impacts of transport GHG reduction policies in 2030." Ricardo Energy & Environment for the European Climate Foundation. Ref. DG-1509-55582, ePublication available at: https://europeanclimate.org/wp-content/uploads/2016/02/ECF-Transport-GHG-reduction-for-2030_Final_Issue21.pdf [accessed on 16th August 2016]

Hoekman SK, Broch A, Robbins C, Ceniceros E, Natarajan M (2012) Review of biodiesel composition, properties, and specifications, *Renew. Sustainable Energy Rev*., 16(1); pp. 143-169

Hofvendahl K, Hahn-Hägerdal B (2000) Factors affecting the fermentative lactic acid production from renewable resources, *Enzyme Microb Technol*. 26(2-4): pp. 87-107

Howard TP, Middelhaufe S, Moore K, Edner C, Kolak DM, Taylor GN, Parker DA, Lee R, Smirnoff N, Aves SJ, Love J. (2013) Synthesis of customized petroleum-replica fuel molecules by targeted modification of free fatty acid pools in *Escherichia coli, Proc Natl Acad Sci U S A.*, 110(19); pp.7636-41

Hu F, Ragauskas A (2012) Pretreatment and Lignocellulosic Chemistry, *Bioenerg. Res.*, 5(4); pp. 1043–66.

Hu J, Chandra R, Arantes V, Gourlay K, van Dyk JS, Saddler JN (2015) The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings, *Bioresour. Technol*., 186; pp. 149-53

Hynes SH, Kjarsgaard DM, Thomas KC, Ingledew WM (1997) Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation, *J. Ind. Microbiol. Biotechnol.*, 18(4); pp 284–91

IEA (2015) Key World Energy Statistics 2015, ePublication available at: https://www.iea.org/publications/freepublications/publication/KeyWorld_Statistics_2015.pdf [10th June 2016]

Iiyama K, Lam TBT, Stone BA (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes*. Phytochemistry* 29(3); pp. 733–737

Irwin S. (2016) The Profitability of Ethanol Production in 2015, *farmdoc daily* (6):3, Department of Agricultural and Consumer Economics, University of Illinois at Urbana-Champaign,

ePublication available at: http://farmdocdaily.illinois.edu/2016/01/the-profitability-of-ethanol-production-in-2015.html [accessed 17th August]

Iyer PV, Lee YY (1999) Product inhibition in simultaneous saccharification and fermentation of cellulose into lactic acid, *Biotechnol. Lett*, 21: pp. 371–3

Jacobs DM, Spiesser L, Garnier M, de Roo N, van Dorsten F, Hollebrands B, van Velzen E, Draijer R, van Duynhoven J (2012) SPE-NMR metabolite sub-profiling of urine. *Anal Bioanal Chem.* 404(8); pp. 2349-61

Janssen R, Rutz DD (2011) Sustainability of biofuels in Latin America: Risks and opportunities, *Energy Pol.* 39(10); pp. 5717–25

Joe M-H, Kim J-Y, Lim S, Kim D-H, Bai S, Park H, Lee SG, Han SJ, Choi J (2015) Microalgal lipid production using the hydrolysates of rice straw pretreated with gamma irradiation and alkali solution, *Biotechnol. Biofuels.*, 8; 125

Johari A, Nyakuma BB, Nor SHM, Mat R, Hashim H, Ahmad A, Zakaria ZY, Abdullah TAT (2015) The challenges and prospects of palm oil based biodiesel in Malaysia, *Energy*, 81; pp. 255–61

Jönsson LJ, Martín C (2016) Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects, *Bioresource Technol*., 199, pp. 103-12

Ju X, Bowden M, Engelhard M, Zhang X (2014) Investigating commercial cellulase performances toward specific biomass recalcitrance factors using reference substrates, *Appl. Microbiol. Biotechnol.*, 98(10); pp. 4409-20

Kampman B, Verbeek R, van Grinsven A, van Mensch P, Croezen H, Patuleia A (2013). "Bringing biofuels on the market: Options to increase EU biofuels volumes beyond the current blending limits." Delft, CE Delft, Available at: https://ec.europa.eu/energy/sites/ener/files/documents/2013_11_bringing_biofuels_on_the_market.pdf [accessed 16th August 2016]

Kallio P, Pásztor A, Thiel K, Akhtar MK, Jones PR (2014) An engineered pathway for the biosynthesis of renewable propane, *Nat. Commun.,* 5; 4731

Koçar G, Civaş N (2013) An overview of biofuels from energy crops: Current status and future prospects, *Renewable and Sustainable Energy Reviews*, 28; pp. 900–16

Kuepper G (1992) *Sweet Sorghum: production and processing*, Oklahoma: Kerr centre for sustainable agriculture, pp. 13-18

Kumar P, Barrett DM, Delwiche MJ Stroeve P (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production, *Ind. Eng. Chem. Res.* 48; pp. 3713–29

Lacis AA, Schmidt GA, Rind D, Ruedy RA, (2010) Atmospheric CO2: Principal Control Knob Governing Earth's Temperature, *Science*, 330(6002): pp. 356-59

Lam FH, Ghaderi A, Fink GR, Stephanopoulos G (2014) Engineering alcohol tolerance in yeast, *Science*, 346(6205); pp. 71-5

Li MF, Fan YM, Xu F, Sun RC, Zhang XL (2010) Cold sodium hydroxide/urea based pretreatment of bamboo for bioethanol production: characterization of the cellulose rich fraction, *Ind Crops Prod*, 32, pp. 551–59

Li C, Knierim B, Manisseri C, Arora R, Scheller HV, Auer M, Vogel KP, Simmons BA, Singh S (2010) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification*, Bioresource Technol.*, 101(13); pp. 4900-6

Li Y, Qi B, Wan Y. (2014) Inhibitory effect of vanillin on cellulase activity in hydrolysis of cellulosic biomass. *Bioresour Technol.* 167: pp. 324-30)

Lindsey ME, Meyer M, Thurman† EM (2001) Analysis of Trace Levels of Sulfonamide and Tetracycline Antimicrobials in Groundwater and Surface Water Using Solid-Phase Extraction and Liquid Chromatography/Mass Spectrometry, *Anal. Chem.*, 73; pp. 4640-4646

Luo L, van der Voet E, Huppes G, Udo de Haes HA (2009) Allocation issues in LCA methodology: a case study of corn stover-based fuel ethanol, *Int. J. Life Cycle Assess.*, 14; pp. 529–39

Ma M, Liu ZL (2010) Mechanisms of ethanol tolerance in *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol*, 87(3); pp. 829–845

Madigan M, Martinko J, Stahl, D, Clark D. (2012) *Brock Biology of microorganisms*, 13th edition, San Francisco: Pearson education, Inc.

McIntosh S, Zhang Z, Palmer J, Wong HH, Doherty WOS, Vancov T (2016) Pilot-scale cellulosic ethanol production using eucalyptus biomass pre-treated by dilute acid and steam explosion, *Biofuels, Bioprod. Biorefin*., 10(4); pp. 346–358

McKendr P (2002) Energy production from biomass (part 1): overview of biomass, Bioresour. Technol, 88(1): pp. 37–46

Modig T, Lidén G, Taherzadeh MJ (2003) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase, *Biochem. J.*, 363(Pt 3); pp. 769-76

Morey RV, Zheng H, Kaliyan N, Pham MV (2014) Modelling of superheated steam drying for combined heat and power at a corn ethanol plant using Aspen Plus software, *Biosyst. Eng*., 119; pp. 80-8

Moshi AP, Crespo CF, Badshah M, Hosea KM, Mshandete AM, Mattiasson B (2014) High bioethanol titre from *Manihot glaziovii* through fed-batch simultaneous saccharification and fermentation in Automatic Gas Potential Test System, *Bioresour. Technol*., 56: pp. 348-56

Mukhopadhyay A (2015) Tolerance engineering in bacteria for the production of advanced biofuels and chemicals, *Trends Microbiol.*, 23(8); pp. 498–508

Mullet J, Morishige D, McCormick R, Truong S, Hilley J, McKinley B, Anderson R, Olson S, Rooney W (2014) Energy Sorghum—a genetic model for the design of C4 grass bioenergy crops, *J. Exp. Bot.*, 65; pp. 3479–3489

Naik SN, Goud VV, Rout PK, Dala AK (2010) Production of first and second generation biofuels: A comprehensive review, *Renew. Sustainable Energy Rev*., 14(2); pp. 578–597

Narendranath NV, Thomas KC, Ingledew WM (2001) Effects of acetic acid and lactic acid on the growth of Saccharomyces cerevisiae in a minimal medium. *J Ind Microbiol Biotechnol* 26(3): pp. 171-7

Nicoletti G, Arcuri N, Nicoletti G, Bruno R (2015) A technical and environmental comparison between hydrogen and some fossil fuels, Energy Convers. Manage., 89; pp. 205–13

Noparat P, Prasertsan P, O-Thong S, Pan X (2015) Dilute Acid Pretreatment of Oil Palm Trunk Biomass at High Temperature for Enzymatic Hydrolysis, *Energy Procedia*, 79; pp. 924 – 929

Notenboom V, Williams SJ, Hoos R, Withers SG, Rose DR (2000) Detailed Structural Analysis of Glycosidase/Inhibitor Interactions: Complexes of Cex from *Cellulomonas fimi* with Xylobiose-Derived Aza-Sugars, *Biochemistry*, 39(38); pp. 11553-63

Oates LG, Duncan DS, Gelfand I, Millar N, Robertson GP, Jackson RD (2016) Nitrous oxide emissions during establishment of eight alternative cellulosic bioenergy cropping systems in the North Central United States, *Glob. Change Biol. Bioenergy*, 8; pp. 539–49

Office of Energy Efficiency and Renewable Energy (2016) Fuel economy guide. DOE/EE-1249, US Department of Energy/US Environmental Protection Agency, Washington DC

Olsson L, Hahn-Hägerdal B (1996) Fermentation of lignocellulosic hydrolysates for ethanol production, *Enzyme Microb. Technol*., 18(5); pp. 312-31

Ooshima H, Ishitani Y, Harano Y (1985) Simultaneous saccharification and fermentation of cellulose: effect of ethanol on enzymatic saccharification of cellulose, *Biotechnol Bioeng.* 27(4); pp. 389-97

Packer MS, Liu DR (2015) Methods for the directed evolution of proteins, *Nature Rev. Genet.,* 16; pp. 379–94

Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, *Bioresource Technol.*, 74(1); pp. 25-33

Papini M, Nookaew I, Uhlén M, Nielsen J (2012) *Scheffersomyces stipitis*: a comparative systems biology study with the Crabtree positive yeast *Saccharomyces cerevisiae*, *Microb Cell Fact.*, 11:136

Paterson AH, et al. (2009) The Sorghum bicolor genome and the diversification of grasses, *Nature*, 457(39): pp. 551-6

Peterson CL, Hustrulid T (1998) Carbon Cycle For Rapeseed Oil Biodiesel Fuels, *Biomass Bioenerg.*, 14(2); pp. 91-101

Promkhambut A, Younger A, Polthanee A, Akkasaeng C (2010) Morphological and Physiological Responses of Sorghum (*Sorghum bicolor* L. Moench) to Waterlogging. *Asian Journal of Plant Sciences*, 9: pp. 183-93

Qi B, Chen X, Yi S, Wan Y (2014) Inhibition of cellulase, β-glucosidase, and xylanase activities and enzymatic hydrolysis of dilute acid pretreated wheat straw by acetone-butanol-ethanol fermentation products, *Environ Prog Sustain Energy*, 33(2); pp. 497-503

Ra K, Shiotsu F, Abe J, Morita S (2012) Biomass yield and nitrogen use efficiency of cellulosic energy crops for ethanol production, *Biomass Bioenergy*, 37: pp. 330–4

Radtke CW, Hamilton PG, Kreitman KM (2015) Process for producing volatile organic compounds from biomass material, US8946491 B2.

Rahikainen JL, Martin-Sampedro R, Heikkinen H, Rovio S, Marjamaa K, Tamminen T, Rojas OJ, Kruus K (2013) Inhibitory effect of lignin during cellulose bioconversion: The effect of lignin chemistry on non-productive enzyme adsorption, *Bioresour. Technol.*, 133; pp. 270-8

Rantsiou K, Dolci P, Giacosa S, Torchio F, Tofalo R, Torriani S, Suzzi G, Rolle L, Cocolin L (2012) Candida zemplinina Can Reduce Acetic Acid Produced by Saccharomyces cerevisiae in Sweet Wine Fermentations, *Appl. Environ. Microbiol.* 78(6); pp. 1987-94

Raupach MR, Marland G, Ciais P., Le Quéré C, Canadell JG, Klepper G, Field CB (2007) Global and regional drivers of accelerating CO2 emissions, *Proc. Natl. Acad. Sci. USA*, 104(24), 10288 – 93

Rawat I, Ranjith Kumar R, Mutanda T, Bux F (2013) Biodiesel from microalgae: A critical evaluation from laboratory to large scale production, *Appl. Energy*, 103; pp. 444-67

Rosas-Lemus M, Uribe-Alvarez C, Chiquete-Félix N, Uribe-Carvajal S (2014) In Saccharomyces cerevisiae fructose-1,6-bisphosphate contributes to the Crabtree effect through closure of the mitochondrial unspecific channel, *Arch. Biochem. Biophys,* 555–556; pp. 66–70.

Rosillo-Callem F, de Groot P, Hemstock S l, Woods J (2007) *The biomass assessment handbook*, Abingdon: Routledge

Rubin EM (2008) Genomics of cellulosic biofuels. *Nature* 454, pp. 841–5.

Rutz D, Janssen R (2007) *Biofuel technology handbook*, Munchen: WIP Renewable Energies pp.65

Saha BC, Nichols NN, Qureshi N, Kennedy GJ, Iten LB, Cotta MA (2015) Pilot scale conversion of wheat straw to ethanol via simultaneous saccharification and fermentation, *Bioresour. Technol*., 175; pp. 17-22

Saha BC (2003) Hemicellulose bioconversion *J Ind Microbiol Biotechnol.* 30(5): pp. 279-91

Saini JK, Saini R, Tewari L (2015) Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments, *3 Biotech*. 5(4): pp. 337–353

Sárvári Horváth I, Franzen CJ, Taherzadeh MJ, Niklasson C, Liden G (2003) Effects of Furfural on the Respiratory Metabolism of *Saccharomyces cerevisiae* in Glucose-Limited Chemostats, *Appl. Environ. Microbiol.,* 69(4) pp. 4076–86

Shafiee S, Topal E (2009) When will fossil fuel reserves be diminished? *Energy Pol*., 37(1); pp. 181–9

Sharwood RE, Sonawane BV, Ghannoum O, Whitney SM, (2016) Improved analysis of C4 and C3 photosynthesis via refined in vitro assays of their carbon fixation biochemistry, *J. Exp. Bot.*, 67(10); pp. 3137-48

Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, Hogsett DA, Lynd LR. (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield, *Proc. Natl. Acad. Sci. U S A.*, 105(37); pp. 13769-74

Shekiro III J, Kuhn EM, Nagle NJ, Tucker MP, Elander RT, Schell DJ (2014) Characterization of pilot-scale dilute acid pretreatment performance using deacetylated corn stover, *Biotechnol. Biofuels*, 7(1); 23, online article accessible at: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/1754-6834-7-23 [10th august 2016]

Shoemaker C, Bransby DI, (2010) *Sustainable alternative fuel feedstock opportunities, challenges and road maps for six US regions. Chapter 9: The role of sorghum as a bioenergy feedstock.* Ankeny, IA: Soil and Water Conservation Society: pp. 149–59

Singh J, Suhag M, Dhaka A (2015) Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review, *Carbohydr. Polym.*, 117; pp. 624–631

Smeets EMW, Bouwman AF, Stehfest E, van Vuuren DP, Posthuma A. (2009), Contribution of N_2O to the greenhouse gas balance of first-generation biofuels. *Glob. Chang. Biol.*, 15(1); pp. 1–23

Solomon S, Qin D, Manning M, Marquis M, Aberyt K, Tignor MMB, Miller HL, Chen Z (2007) *Climate change 2007: the physical science basis*, New York: Cambridge press

Stewart JJ, Akiyama T, Chapple C, Ralph J, Mansfield SD (2009) The Effects on Lignin Structure of Overexpression of Ferulate 5-Hydroxylase in Hybrid Poplar, *Plant Physiol*., 150(2): pp. 621-35

Tan SSY, MacFarlane DR, Upfal J, Edye LA, Doherty WOS, Patti AF, Pringle JM, Scott JL (2009) Extraction of lignin from lignocellulose at atmospheric pressure using alkylbenzenesulfonate ionic liquid. *Green Chemistry*, 11 (3); pp. 339-45.

Tomás-Pejó E, Olsson L (2015) Influence of the propagation strategy for obtaining robust *Saccharomyces cerevisiae* cells that efficiently co-ferment xylose and glucose in lignocellulosic hydrolysates, *Microb. Biotechnol*., 8(6): pp. 999-1005

Toquero C, Bolado S. (2014) Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing, *Bioresour Technol.* 157; pp. 68-76

Tramice A, Melck D, Virno A, Randazzo A, Motta A, Trincone A (2009) Enzymatic synthesis and 3-D structure of anti-proliferative acidic (MeGlcUA) xylotetrasaccharide, *J. Mol. Catal. B: Enzym*., 61(3-4); pp. 129–35

Ulson de Souza AA, Ferreira FC, Guelli U Souza SM (2013) Influence of pretreatment of cotton yarns prior to biopolishing, *Carbohydr Polym*. 93(2); pp. 412-5

US Department of Agriculture (2014) Notice of Funds Availability (NOFA) for the Biomass Crop Assistance Program, *Fed. Reg.,* 79(112); pp. 33492-5

US Department of Agriculture (2015) Notice of Funds Availability (NOFA); Biofuel Infrastructure Partnership (BIP) Grants to States, *Fed. Reg.,* 80(115); pp. 34363-6

US Department of Agriculture (2016) Crop Production 2015 Summary, *National Agricultural Statistics Service*, ISSN: 1057-7823, pp. 57, ePublication available: http://www.usda.gov/nass/PUBS/TODAYRPT/cropan16.pdf [12th August 2016]

Van Hamme JD, Singh A, Ward OP (2003) Recent Advances in Petroleum Microbiology, *Microbiol. Mol. Biol. Rev.*, 67(4): pp. 503–49.

Vertès AA, Inui M, Yukawa H (2006) Implementing biofuels on a global scale. *Nat. Biotechnol*. 24(7); pp. 761-4.

Vieira ÉD, Andrietta Mda G, Andrietta SR (2013) Yeast biomass production: a new approach in glucose-limited feeding strategy, *Braz. J. Microbiol.*, 44(2); pp. 551-8

Wallace-Salinas V, Gorwa-Grauslund MF (2013) Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature *Biotechnol. Biofuels.,* 6(1):151

Wang B, Duan P, Xu Y, Wang F, Shi X, Fu J, Lu X (2016) Co-hydrotreating of algae and used engine oil for the direct production of gasoline and diesel fuels or blending components, *Energy*, ePublication available at: http://www.sciencedirect.com/science/article/pii/S0360544216303280 [20th July 2016] online article to be published

Wang J, Ding M, Li YH, Chen QX, Xu GJ, Zhao FK. (2003) A monovalent anion affected multi-functional cellulase EGX from the mollusca, *Ampullaria crossean*. *Protein Expr Purif.* 31(1); pp. 108-14

Wang WG, Lyons DW, Clark NN, Gautam M, Norton PM (2000) Emissions from Nine Heavy Trucks Fueled by Diesel and Biodiesel Blend without Engine Modification, *Environ. Sci. Technol.*, 34(6); pp. 933-9

Weider PR, Blackbourn RL (2016) Treating biomass to produce materials useful for biofuels, US20160122837A1

Yüksel F, Yüksel B (2004) The use of ethanol–gasoline blend as a fuel in an SI engine, Renewable Energy, 29; 1181–91

Zhang YH, Lynd LR (2004) Toward an Aggregated Understanding of Enzymatic Hydrolysis of Cellulose: Noncomplexed Cellulase Systems, *Biotechnol Bioeng.* 88(7): pp. 797-824

Zhao X, Tang J, Wang X, Yang R, Zhang X, Gu Y, Li X, Ma M (2015) YNL134C from Saccharomyces cerevisiae encodes a novel protein with aldehyde reductase activity for detoxification of furfural derived from lignocellulosic biomass, *Yeast*, 32(5); pp. 409-22.

Zheng Y, Pan Z, Zhang RH (2009) Overview of biomass pretreatment for cellulosic ethanol production, *Int. J. Agric. Biol. Eng.*, 2(3); pp. 51–68

Zheng Y, Lee C, Yu C, Cheng Y-S, Zhang R, Jenkins BM, VanderGheynst JS (2013) Dilute acid pretreatment and fermentation of sugar beet pulp to ethanol, *Appl. Energy*, 105; pp. 1-7

Zhou H, Cheng JS, Wang BL, Fink GR, Stephanopoulos G. (2012) Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng*. 14(6): pp. 611-22

Zhao J, Chen H (2014) Stimulation of Cellulases by Small Phenolic Compounds in Pretreated Stover, *J. Agric. Food Chem*, 62; pp. 3223−9

Zoglowek M, Lübeck PS, Ahring BK, Lübeck M. (2015) Heterologous expression of cellobiohydrolases in filamentous fungi -An update on the current challenges, achievements and perspectives. *Process Biochem*. 50; pp. 211–20