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DOCTORAL THESIS

Characterization of Agro-Industrial Residues and Development of Processing Strategies for Conversion to Bioethanol

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Abstract

Renewable sources of chemical energy, such as plant biomass, are needed for synthesizing future liquid transportation fuels. However, the structural complexity and heterogeneity of plant biomass can result in low rates of carbohydrate-to-fuel conversion and often requires costly pre-processing techniques. As a result, plant materials that are abundant, cheap to produce, are socially responsible and have an easily amendable composition are required. Two agro-industrial biomasses derived from Agave and Vitis vinifera (grape) marc are studied here to determine their chemical compositions, their efficiency of conversion to fermentable sugars and to estimate subsequent ethanol yields.

Project Summary

The first step in examining a source of plant biomass as a potential raw material for bioethanol production is to characterize its composition. In paper I, the compositions of two *Agave* species (*A. americana* and *A. tequilana*) are described. Whole leaf tissue, juice (stem and leaf) and fibrous bagasse were characterised. Of the dry mass of whole *Agave* leaves, 85−95% consisted of soluble carbohydrates, insoluble carbohydrates, lignin, acetate, proteins and minerals. *Agave* leaf biomass was particularly attractive as a lignocellulosic raw material for ethanol production, because it had a significantly lower lignin content (< 13% w/w) relative to other common biofuel feedstocks at >17% w/w [1]. On a fresh weight basis the majority of the *Agave* leaf mass was attributed to moisture (85%) and at harvest the leaves may be crushed to separate juice from the fibrous bagasse. Juice from the leaves and stem was rich in fermentable sugars (fructose, glucose and sucrose) and soluble fructans. Different processing methods were trialled to hydrolyse the fructans, resulting in a final concentration of 41−48 g/L of hexose monosaccharides available in the leaf juice. The fiber fraction was cellulose-rich (up to 50% dry w/w) and could be further processed using pre-treatments to increase availability of the monosaccharides.

Characterization of wine industry waste (grape marc) is described in paper II. Marc derived from two varieties of grape, Cabernet Sauvignon and Sauvignon Blanc, were compared. On a dry weight basis the composition of the grape marc was predominantly carbohydrate (34−50%) and lignin (26−41%). A higher abundance of soluble carbohydrate (glucose and fructose) was detected in marc from Sauvignon Blanc than in Cabernet Sauvignon residues. The carbohydrates identified in Cabernet Sauvignon were predominantly present as insoluble structural polymers of cell wall origin. The distribution and structure of component polysaccharides and their derivatives were investigated using

transmission electron microscopy (TEM) coupled with immunocytochemistry, high performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF-MS).

The chemical composition of plant biomass influences the processing methods, such as physical or chemical pre-treatments and/or enzymatic saccharification, needed to prepare the biomass for conversion to ethanol. In paper I it was concluded that separation of *Agave* biomass into different fractions (whole leaf, stem, juice and/or bagasse) at the time of harvest is better suited to efficient processing outcomes but that expensive pre-treatments were not practical for this biomass as a whole. However, after the moisture had been removed from *Agave* leaves a cellulose-rich (32−45 % mol) fibrous fraction remained. The accessibility of this raw material to enzymatic hydrolysis was investigated using a crude cellulase preparation. The rate of saccharification and overall yield of glucose (38−40%) liberated in the hydrolysate after a 48 h treatment was similar for both *A. americana* and *A. tequilana* leaf tissue. The grape marc described in Paper II was rich in the polymer lignin, which is intertwined with cellulose and non-cellulosic polysaccharides in a biocomposite that is resistant to conversion and necessitates pre-treatment to allow enzyme penetration. A dilute acid pre-treatment resulted in an approximate 10% increase in the amount of liberated glucose after enzymatic saccharification, presumably due to the hydrolysis of non-cellulosic polysaccharides (NCPs). However, no significant change in glucose release was observed from thermally treated marc compared to non-treated samples.

The yield of ethanol produced from *Agave* juice is described in Paper III. This research determines the impact of processing methods, ranging from none to autoclaving, and the use of different fermenting microorganisms on ethanol yields. To date, available information is mostly related to the fermentation of juice extracted from cooked *Agave* stems, which is reflective of the processes used in the tequila industry [2-5]. The data from

the present study challenged standard practices used for the fermentation of *Agave* juice such as sterilizing the juice and/or spiking the juice with sugars and nutrients prior to fermentation to provide an optimal environment for selected fermenting organisms (paper III). In addition, the potential of using *Agave* leaves in no-input fermentations, such that no acid or enzymatic hydrolysis, supplementation of nutrients or standardization of sugar content occurred, was investigated. The experimental data indicated that leaf juice derived from *Agave* does not benefit from a sterilization step, because the ethanol yields achieved were not significantly different to those from raw juice fermentations. The productivity of the fermentations was more strongly influenced by the selection of the microorganism. However, ethanol yields were reduced if fermentation was reliant solely on endogenous microorganisms. It was found that *Agave* leaf juice could be converted to ethanol at an efficiency of 78% using non-*Saccharomyces* yeast strains, and this would equate to a yield of 1881 L/ha/yr ethanol. This research also demonstrated that sugar to ethanol conversion efficiency could be further increased when leaf and stem juice is blended and fermented using a yeast directly isolated from *Agave*, namely *Kluyveromyces marxianus*.

Overall the work presented in this thesis describes the processing of two agro-industrial residues from a raw material through to fermentation products (ethanol, organic acids and glycerol). The characterization of the biomass was instrumental in informing the types of downstream processing, fermentation methods and microorganisms that might be used. The amounts of extracted carbohydrate and conversion efficiencies achieved under different processing scenarios were extrapolated to predict ethanol yields obtained if they were to be produced on a large-scale. This enabled comparisons with other commonly studied biomass feedstocks. The methodology and data generated from this study may be informative when investigating the practicality of using agro-industrial residues such as *Agave* and grape marc for commercial biofuel and/or biochemical production.

The thesis is based on the following papers

I. "*Prospecting for energy-rich renewable raw materials: Agave leaf case study"*

Kendall R. Corbin, Caitlin S. Byrt, Stefan Bauer, Seth DeBolt, Don Chambers, Joseph A. M. Holtum, Ghazwan Karem, Marilyn Henderson, Jelle Lahnstein, Cherie T. Beahan, Antony Bacic, Geoffrey B. Fincher, Natalie S. Betts and Rachel A. Burton

Doi: 10.1016/j.biortech.2015.06.030

II. "*Grape marc as a source of carbohydrates for bioethanol: chemical composition, pre-treatment and saccharification"*

Kendall R. Corbin, Yves S.Y. Hsieh, Natalie S. Betts, Caitlin S. Byrt, Marilyn Henderson, Jozsef Stork, Seth DeBolt, Geoffrey B. Fincher and Rachel A. Burton

Doi: 10.1371/journal.pone.0135382

III. "*Low-input fermentations of Agave tequilana leaf juice generate high-returns on ethanol yields"*

Kendall R. Corbin, Natalie S. Betts, Nick van Holst, Don Chambers, Caitlin S. Byrt, Geoffrey B. Fincher, and Rachel A. Burton

Candidates' contribution to papers

Paper I: Kendall Corbin completed the biochemical characterization of leaves, juice and bagasse from two species of *Agave*, the enzymatic saccharification and fermentation studies, preparation of the biomass for transmission electron microscopy and linkage analysis, and the majority of the writing.

Paper II: Kendall Corbin completed the majority of the experimental work including; compositional analysis of grape marc, pre-treatment and saccharification studies, sample preparation for microscopy and characterization of polysaccharide structure. In addition she wrote the majority of the manuscript.

Paper III: Kendall Corbin performed the fermentation studies including the screening of microorganisms, set-up of fermentation trials, HPLC analysis and data/statistical interpretation and wrote the majority of the manuscript.

Statement of Authorship

I, Kendall Corbin certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Kendall R. Corbin

May 2015

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Abbreviations

A:X	Arabinose:Xylose ratio
AIR	Alcohol insoluble residue
Ara	Arabinose
ASE	Accelerated solvent extractor
C5	Pentose
C6	Hexose
CDTA	Cyclohexane- 1,2-diamine tetraacetate
CAM	Crassulacean acid metabolism
Fruc	Fructose
FPU	Filter paper units
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
Glc	Glucose
GlcA	Glucuronic acid
HILIC	Hydrophilic interaction chromatography
HMF	5-(hydroxymethyl)furfural
HPLC	High performance liquid chromatography
HTL	Hydrothermal liquefaction
LAP	Laboratory analytical procedure
LCA	Life cycle assessment
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of flight mass spectrometry
Man	Mannose
Mol %	Relative percent molarity
MS	Mass spectrometry
NCP	Non-cellulosic polysaccharides
NGS	Next-generation sequencing
NREL	National Renewable Energy Laboratory

RGI	Rhamnogalacturonan I
Rha	Rhamnose
PBS	Phosphate buffered saline
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TFA	Trifluoroacetic acid
TSS	Total soluble solids
V/V	Volume per volume
WSC	Water soluble carbohydrates
W/W	Weight per weight
Xyl	Xylose
YPD	Yeast extract-peptone-dextrose

1.0 Background

The demand for chemical energy is projected to increase by 1.4% annually for the next 20 years, which represents a 32% increase relative to current demands and is equivalent to a rise of liquid fuel consumption (i.e. oil and biofuels) of 111 million barrels per day (Mb/d) [6]. The search for renewable and sustainable forms of chemical energy that could meet this demand is a major challenge on a global scale. Energy stored in plant biomass is a potentially renewable material that could contribute substantially to transportation fuel needs at costs competitive with fossil fuel [7]. Plant biomass can be produced in abundance, cheaply and is not geospatially restricted to one biome. Globally, it is estimated that 100 billion tonnes of land biomass (organic dry matter) and 50 billion tonnes of aquatic biomass are produced per year [8]. Of the plant biomass, agricultural, industrial and forest derived lignocellulosic residues are regarded as the largest source of carbohydrate available for making chemical fuels [9]. The lignocellulosic residues are a complex mixture of carbohydrates, polyphenol polymers and proteins that are difficult to separate into discrete, usable components. Hence, to convert this recalcitrant biomass into ethanol, fermentable monosaccharides need to be liberated from the network. The processing methods employed to make the carbohydrates accessible, such as pre-treatments and enzyme saccharification, can drastically increase the cost of ethanol (per litre) production [10,11]. Thus, for ethanol production from lignocellulosic biomass to be cost competitive, the biomass must be sourced cheaply, produced abundantly and require minimal processing to drive down investment costs at all stages of production.

Two agro-industrial feedstocks that have been identified recently as potential sources of biomass are derived from *Agave spp*. [12-14] and *Vitis vinifera* (grape marc) [15]. Both *Agave* and *V. vinifera* are currently used in well-established industries yielding high value end

products. However, in both instances the agronomical practices and techniques used by industry do not consume the whole plant, which results in a waste containing residual carbohydrates that could be used for bioethanol production. For example, the standard practice for the conversion of *Agave* to alcohol is to use the stem or piña tissue and to discard the enclosing leaves. Similarly, in the wine industry the soluble carbohydrates (glucose and fructose) in grapes are fermented for alcohol production, leaving a more complex carbohydrate-rich residue (grape marc) as a waste by-product. Although these feedstocks are grown specifically for the alcoholic beverage industries (tequila and wine respectively), there is the potential to add value by capitalizing on the "spent" biomass. In both cases, the material has been aggregated for wine or tequila production and hence there is no additional cost associated with collecting and transporting the biomass sources. This may be contrasted with the use of wheat straw, which must be harvested and transported to the biofuel production factory.

One limitation that undermines the practicality and feasibility of using these residual biomass sources for bioethanol (or biochemical) industries is the lack of information available regarding their chemical composition. An understanding of the cell wall composition serves as a starting point from which to further investigate processing (pre-treatment and enzymatic saccharification) and conversion methods (fermentation) of these biomasses to liquid fuel. Identifying easily amenable biomass sources represents a major technical hurdle that must be overcome before the full potential of lignocellulosic biofuel can be realized [16].

2.0 Production of bioethanol

Lignocellulosic biomass is structurally complex, which means that direct conversion of the raw material to bioethanol is not always practical or cost-effective. To achieve competitive conversion yields, the raw material often requires extensive processing. Pre-treatments and enzymatic saccharification are used to liberate individual monosaccharides from the biomass. Once the monosaccharides are released they can be converted to ethanol by fermentation using a specific bacteria or yeast strain. Further processing in the form of distillation separates water from ethanol and other fractions. An alternative processing stream, less frequently used, is the direct extraction of soluble carbohydrates, monosaccharides and oligosaccharides that can be directly fermented to ethanol. This approach often requires less initial processing but can result in a substantial amount of the biomass, and therefore the fermentable carbohydrates, being discarded.

To reduce costs at all stages of production, it may be advantageous to separate lignocellulosic material based on composition at the time of harvest or collection, that is, to treat the recalcitrant (insoluble polysaccharides) and less recalcitrant fractions (predominantly soluble polysaccharides) independently [17]. The price of lignocellulosic biomass ranges from $55−140/MT, which is less than the market price of corn on a tonner per tonne basis [19,20]. However this value underestimates the real cost of utilizing lignocellulosic biomass for biofuel production as costly processing steps are required to make this complex material more amenable for conversion. Current projections estimate that the breakdown of lignocellulosic biomass into a form that can be converted to bioethanol costs twice as much as using starch-based corn for ethanol production, namely at $0.39/litre and $0.21/litre, respectively [18]. The majority of the expenses for lignocellulose conversion are attributable to costly processing

methods required to degrade the biomass to make carbohydrates accessible for fermentation [10,11]. For example, it is estimated that moderate enzyme loadings such as a cellulase dosage of 15 filter paper units, FPU/g cellulose at a commercial scale could correlate to about a 30 g enzyme loading per liter of ethanol produced [21], at a cost of $0.03−$0.11/litre [20,22,23]. However, when saccharification and fermentation data from corn stover are applied to techno-economic modeling these values seem to be understated because the real cost of enzymes is $0.19−0.40/litre ethanol [24].

As a result, the conversion of biomass to biofuel is both a complex and cost-intensive process. In-depth reviews of different methods (e.g. fast-pyrolysis, slow-pyrolysis, (trans)-esterification, hydrothermal liquefaction, fermentation) in both the production of biodiesel and bioethanol have been published, with a focus on the current limitations and application of each methodology [25-28]. Although the end product for all processes is chemical energy, the conversion processes actually require energy input too. Therefore, for such processes to be economically viable, the input energy costs must be drastically less than output energy values. In the present study, we focused on an alcoholic fermentation system for conversion (Figure 1), where input energy was derived directly from the metabolic processes of the fermenting organism, potentially reducing conversion costs. There are multiple stages during the production of ethanol that may be targeted for improvement, including:

1) Refining harvesting and/or processing techniques
2) Increasing monosaccharide availability
3) Identifying efficient low cost pre-treatments
4) Ensuring complete utilization of carbohydrates during fermentation
5) Optimizing recovery of distilled products
6) Increasing production of co-products and “adding value”

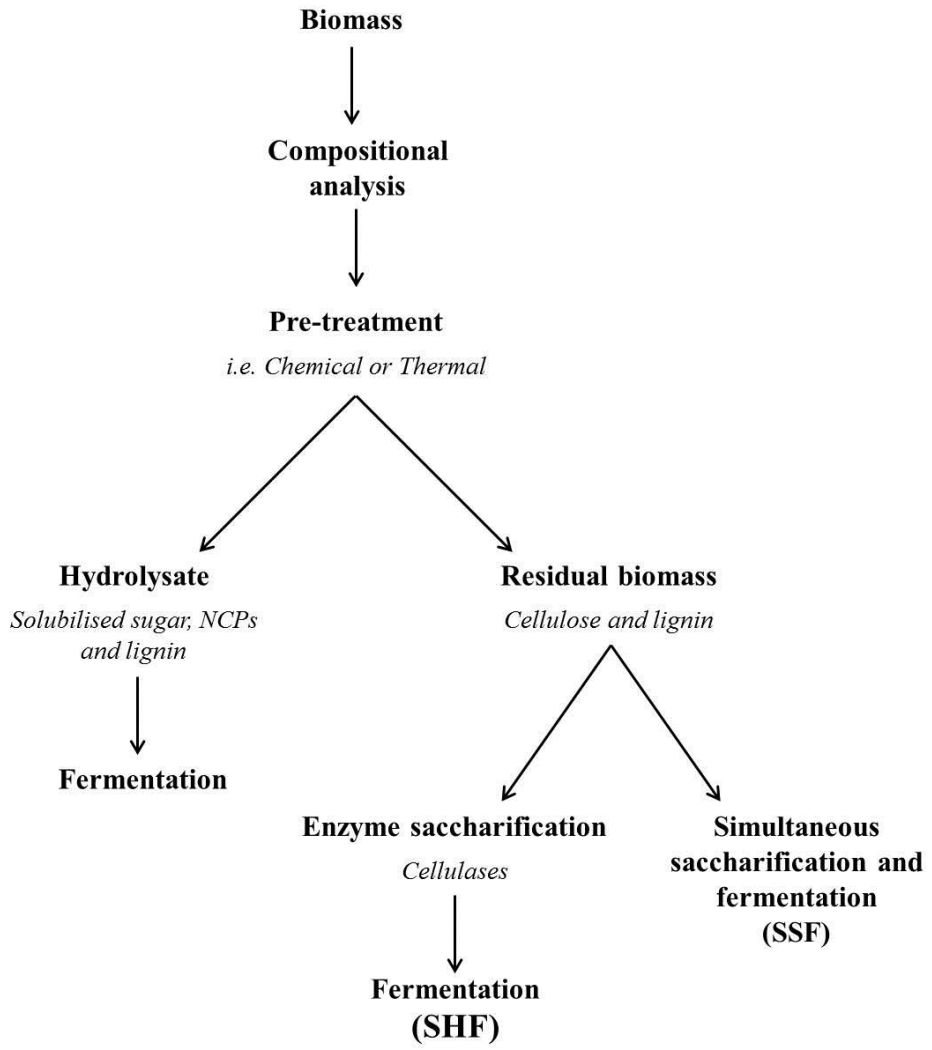

Figure 1 Simplified outline of a generalized processing scheme for the conversion of plant biomass to bioethanol

Plant lignocellulose is recalcitrant to conversion in its native form. Compositional data is informative when selecting appropriate downstream processing methods, including pre-treatments. Pre-treatments are used to break or loosen the cell wall matrix. For example, chemical and thermal pre-treatments separate the biomass

into two fractions; the hydrolysate and the insoluble residual biomass. The hydrolysate is rich in solubilised carbohydrates that are predominantly derived from non-cellulosic cell wall polysaccharides (NCPs) and may be fermented to ethanol. The residual biomass is predominantly cellulose and lignin, which can be further processed using enzymes and fermented (separate hydrolysis and fermentation; SHF). Alternatively the residual biomass may be converted to ethanol in one step via simultaneous saccharification and fermentation (SSF). It should be noted that this approach does not take into consideration the direct fermentation of soluble carbohydrates that may be extracted from the biomass.

3.0 Selecting lignocellulosic biomass

The first step in producing biofuel from plant biomass is to identify suitable feedstocks that are high yielding and can be produced cheaply. Although 90% of the dry weight of most plant materials is carbon, stored in the form of energy-rich cellulose and non-cellulosic polysaccharides, it is rarely in a form that can be utilized directly by fermenting microorganisms [9]. Cell wall polysaccharides form a network that is held together by covalent and non-covalent linkages between the wall polysaccharides themselves or to the non-carbohydrate polymer lignin. The presence of lignin reduces the access of enzymes to cellulose, which slows the rate and efficiency of hydrolysis [29,30]. In a biofuel context, lignin has a negative impact on the conversion of lignocellulosic biomass into bioethanol, and a high amount of lignin is not a favourable characteristic for a dedicated biofuel feedstock.

Conversely, carbohydrates stored in plant biomass that can be broken down with minimal processing are desirable. However, the polymer predominantly targeted for

bioethanol production, and which is not broken down particularly easily, is cellulose. The sheer mass of cellulose available outweighs its relative recalcitrance as it is the most abundant terrestrial natural biopolymer [31]. Cellulose is a linear polymer composed of β-(1,4)-linked glucosyl residues. As the cellulose chains are synthesized, 18−24 parallel glucan chains aggregate via H-bonding and van der Waal forces to form a para-crystalline microfibril [32]. When chains do not aggregate in an ordered fashion, amorphous regions are formed [16] and these non-crystalline regions are more accessible for enzymatic hydrolysis. Thus, the relative degree of crystallinity in cellulose influences the rate of biochemical conversion to the fermentable monosaccharide glucose [33].

The remaining carbohydrate component of lignocellulosic biomass consists of heterogeneous polymers known as the non-cellulosic polysaccharides. Non-cellulosic polysaccharides originate from cell walls and represent about 20–35% of lignocellulosic biomass and up to 50% of walls in some cereal grains [34,35]. These polysaccharides are generally synthesized in the Golgi and exported, partial or complete, to the cell wall via secretory vesicles, where they form a gel like matrix [36-38]. Once incorporated in the cell wall they become intertwined with the cellulose microfibrils, adding strength while providing flexibility and porosity to the wall [36]. Compared to cellulose, non-cellulosic polysaccharides have lower degrees of polymerisation and are less crystalline, resulting in polymers that are more easily hydrolysed with dilute acid or mild pre-treatment conditions [39]. Structurally, non-cellulosic cell wall polysaccharides include xyloglucans, heteroxylans, heteromannans, pectic polysaccharides and (1,3;1,4)-β-glucans, which are composed of pentose sugars (D-xylose and L-arabinose), hexose sugars (mainly D-galactose, D-glucose and D-mannose), and uronic acids [40].

When investigating the potential of a particular biomass source to produce cheap and plentiful ethanol, attention is usually focused on these three cell wall components (Table 1). However, additional cell wall components can affect breakdown, processing and conversion of biomass to bioethanol. Thus, a mass balance should be calculated that accounts for the structural complexity of both carbohydrate and non-carbohydrate components. For this reason in-depth compositional analyses are diagnostic when selecting the best methods for downstream processing of the biomass.

Table 1 Composition of lignocellulosic biomass (% w/w)

	Lignocellulosic biomass	Cellulose	NCP	Lignin	Citations
Grasses	*Miscanthus*	38−42	21−23	18−21	[41,42]
	Sorghum	15−34	12−18	6−16	[43,44]
	Sugarcane	20	10	6	[42]
	Switchgrass	33−45	25−35	6−18	[43,45]
	Coastal Bermuda grass	26−32	19−25	15−20	[46,47]
	Energy cane	33	23	16	[42]
Woody species	Beech	43	32	24	[48]
	Eucalypts	40−44	10−19	28	[49,50]
	Pine	33−43	20−21	27−35	[42]
	Poplar	27−37	14−25	21−25	[42,51]
	Spruce	40	31	28	[48]
	Willow	26	14	23	[42]
Waste	Barley straw	37−38	26−37	16−19	[52,53]
	Corn stover	36−38	28−29	17−21	[54,55]
	Municipal solid waste	33	9	17	[56]
	Newspaper	40−62	25−40	18−30	[56,57]
	Rice straw	39−42	20−32	13−14	[58,59]
	Rye straw	33	22	20	[46]
	Sunflower stalks	34−39	20−34	17−18	[60,61]
	Rapeseed straw	37	24	17	[62]
	Olive tree prunings	25	16	19	[63]
	Wheat straw	30−39	39−50	15−17	[57,64]

Lignocellulosic biomass is predominantly composed of cellulose, NCP (non-cellulosic polysaccharides) and lignin. Compositional information about plant biomass is essential when making informed decisions regarding the best processing and conversion methods to be used for biochemical and biofuel synthesis. The amount and complexity of polymers is directly correlated to the yield of ethanol that may be produced. Data are presented as percentage of dry weight (% w/w).

4.0 Potential feedstocks for bioethanol production in Australia

Much of the land that has fallen out of agricultural production worldwide is classified as semi-arid [65]. The environmental stresses associated with semi-arid regions include inconsistent rainfall, extreme temperatures, nutrient and mineral depleted soils and increased levels of salt. Such conditions are commonly observed throughout Australia, which has the largest proportion of semi-arid land of any continent (33% of the mainland) [12,66]. The feedstocks currently used in Australia for ethanol production are molasses, grain sorghum, wheat and waste wheat starch, which is a residue from flour production [67]. Fuel crops that are either suitable for these environmental conditions or could add value to existing agricultural industries in Australia include *Agave* and *Vitis vinifera*, respectively.

4.1 *Agave* (*A. tequilana and A. americana)*

Historically *Agave* has been utilized in the production of alcoholic beverages, fiber, and sugar additives. In Australia selected species (i.e. *A. americana, A. fourcroydes* and *A.*

sisalana) were grown commercially as dedicated sources of the fiber sisal as early as the 1890s [13]. However, economic instability, warfare and agricultural modernization resulted in the industry becoming too costly. With the collapse of the sisal industry, *Agave* plantations were deserted, leaving the *Agave* plants to acclimatize to the Australian environment. The presence of these robust, naturalized species can be found in a range of environmental niches in Australia across regions with rainfall as low as 107mm/yr and as high as 4140 mm/yr (Figure 2) [68]. In addition, these plants have historically been used as ornamentals for landscaping purposes.

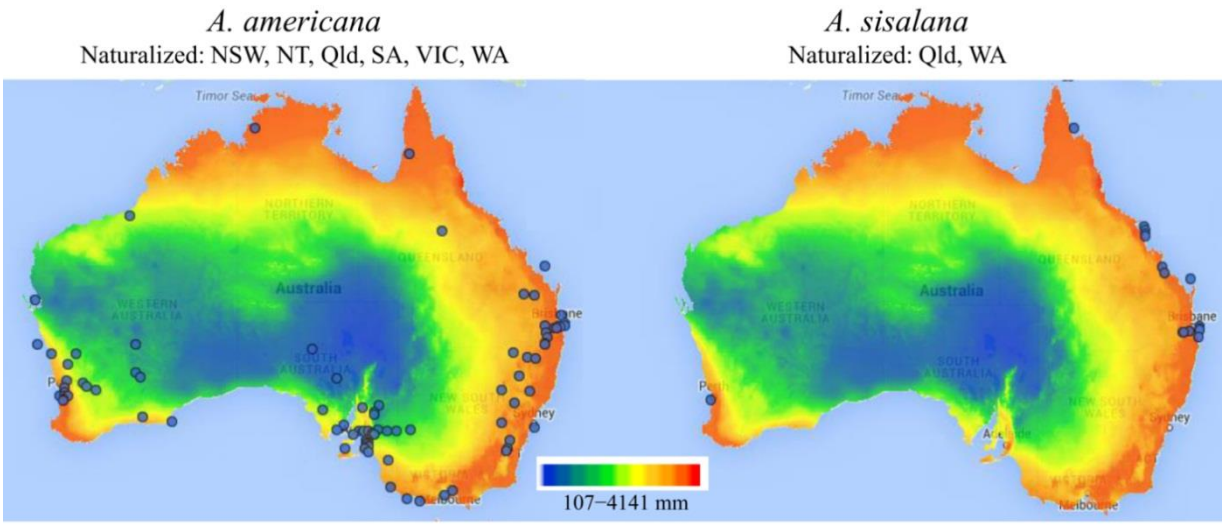

Figure 2 Regions of naturalized *A. americana* and *A. sisalana* in Australia

Naturalized *Agave* plants (blue dots) are tolerant to a range of environmental conditions, which has enabled their adaptation to regions of poor or inconsistent rainfall (blue; 107 mm/yr) and likewise in optimal growing regions (red; 4141 mm/yr rainfall) [68].

Recently *Agave* has been attracting attention as a potential dedicated biofuel feedstock. Factors that contribute to its appeal are its ability to thrive on semi-arid land, which is facilitated by its high water use efficiency that is derived from possession of the Crassulacean acid metabolism (CAM) photosynthetic pathway [12]. Based on a desktop study, the variety *A. tequilana* was identified as the prime species to be trialed in Australia for bioethanol production. It is high yielding for both carbohydrate and fiber content, it can thrive in soils and under climate conditions common to Australia and its cultivation and processing technologies have been optimized on a commercial scale in other regions of the world [13]. Also, because this feedstock is known to accumulate fructose-rich polymers, there is also the potential to tap into other secondary markets that use fructose-based compounds [67]. In June 2009, the first planting of *Agave* (*A. tequilana*) grown specifically for biofuel production took place in Australia. The plants in the trial were generated by tissue culture propagation of accessions that were imported from Mexico [67].

4.2 Grape marc derived from *Vitis vinifera*

Australia is one of the global leaders in wine production, for which 1.6 MT of grapes were harvested in 2012 (Figure 3a, [69]). A significant byproduct of the wine industry is the grape marc. Grape marc is the term used to describe the waste material left after grapes are crushed during wine production. Marc is composed of grape skin, pulp, seeds, stem and some residual juice. South Australia is the leading producer of grapes in Australia, although they are grown in all states (Figure 3b). From the grapes harvested in 2012 for wine production, a total of 316,000 tonnes of the waste material was produced (155,000 t from white grapes and 161,000 t from red grapes), which represents a substantial amount of untapped carbohydrates

that could be exploited for growing biofuel markets. If conversion of this agro-industrial waste could add value to wine making, it could stimulate economic growth for the viticulture industry and provide concomitant environmental benefits without creating a food versus fuel competition for land, given that no extra plantings above the current acreage under harvest would be necessary [70,71].

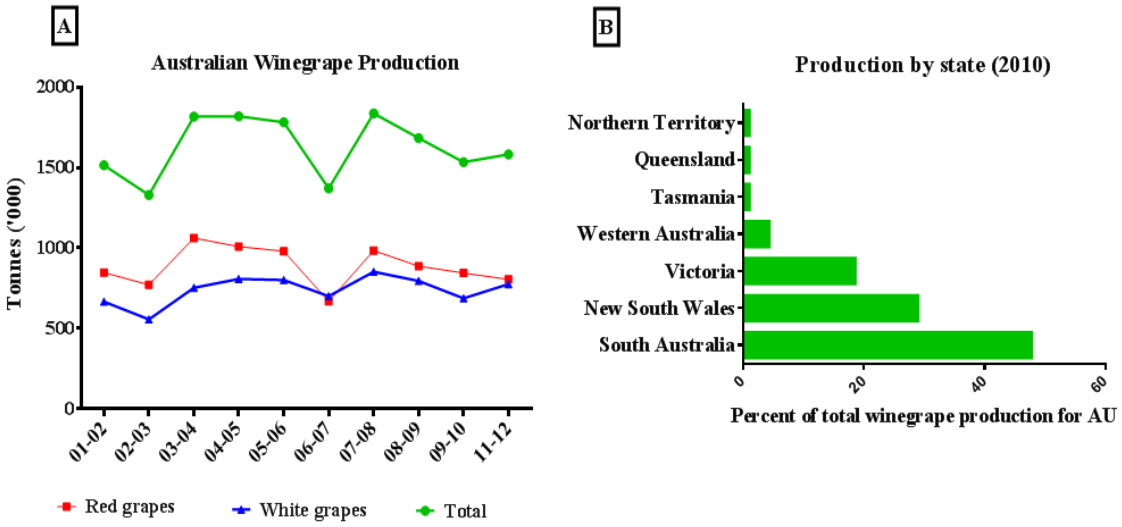

Figure 3 Grape production in Australia

There is a reservoir of the agro-industrial waste grape marc in Australia, which may be capitalised on for biofuel production. Over the last ten years the production of grapes in Australia has been steady (the dip in 2007 was weather related) (A) and grapes are the 12th most important commodity in terms of production [69,72]. In Australia, grapes are grown in all states, with the majority of grape production in South Australia (B) [73]. Figures adapted from [72] and [73], respectively.

5.0 Compositional analysis of selected biomass

To obtain a holistic assessment of a particular biomass, a set of standard procedures for biomass compositional analysis have been established by the National Renewable Energy Laboratory (NREL) in the USA. These methods measure all parameters that contribute to the mass of the raw biomass and create a summative mass closure. The established methods also measure process intermediates that will be generated during the conversion of the biomass to biofuel. The standardized methods for biomass analysis include sample preparation and the quantification of the mineral content (soluble and insoluble), protein, extractives (carbohydrates and minerals), structural carbohydrates, lignin (soluble and insoluble) and degradation by-products [74]. The reproducibility of these standardized methods when conducted on a small scale has been confirmed [41,42,51]. A mass balance was created for *Agave* (paper I) and grape marc (paper II) by quantifying both soluble and insoluble components of the biomass.

6.0 Pre-treatment solubilizes cell wall polymers

Biomass pre-treatment refers to initial steps that are taken to convert biomass from its native recalcitrant state into a form that can be more readily hydrolysed [11]. Methods used for the solubilisation of polysaccharides into oligosaccharides or monosaccharides include the use of physical pre-treatments that reduce the particle size or moisture content of the biomass, chemical treatments, thermal exposure or biological treatments (Figure 4) [75] in a feedstock-dependent manner. The composition of the biomass and the severity of the pre-treatment directly affect the degradation of polymers, the percentage of residual carbohydrates, cost and

the efficiency of the subsequent hydrolysis. For example, if it is necessary to remove lignin from the cell wall matrix, biological pre-treatments using white-rot fungi have been shown to be most effective [76]. Chemical pre-treatments are effective for hydrolysing non-cellulosic polysaccharides and disrupting microfibrils, whilst thermal pre-treatments partially depolymerise lignin, although recondensation may occur [76].

Pre-treatment may generate by-products, some of which can inhibit fermenting organisms in downstream steps [11] such as 5-(hydroxymethyl) furfural (HMF; from hexose carbohydrate degradation) and formic acid or levulinic acid (from the breakdown of HMF) [77]. The accumulation of inhibitory compounds negatively affects the fermenting organisms by causing an extension of the lag phase, slowing the growth rate and hence lowering cell density, and ultimately decreasing ethanol production [77-80].

The net gain incurred by different pre-treatment methods is difficult to determine because of factors such as processing, capital investment, chemical recycling and waste treatment system costs [81]. Different pre-treatments will liberate varying amounts and types of digestible substrates (Figure 4). When selecting a pre-treatment for a specific feedstock it is important to consider the composition and the downstream methods that will be employed. The selected pre-treatment should produce not only the greatest yield of hydrolysates (overall energy output), but also incur the lowest operational cost.

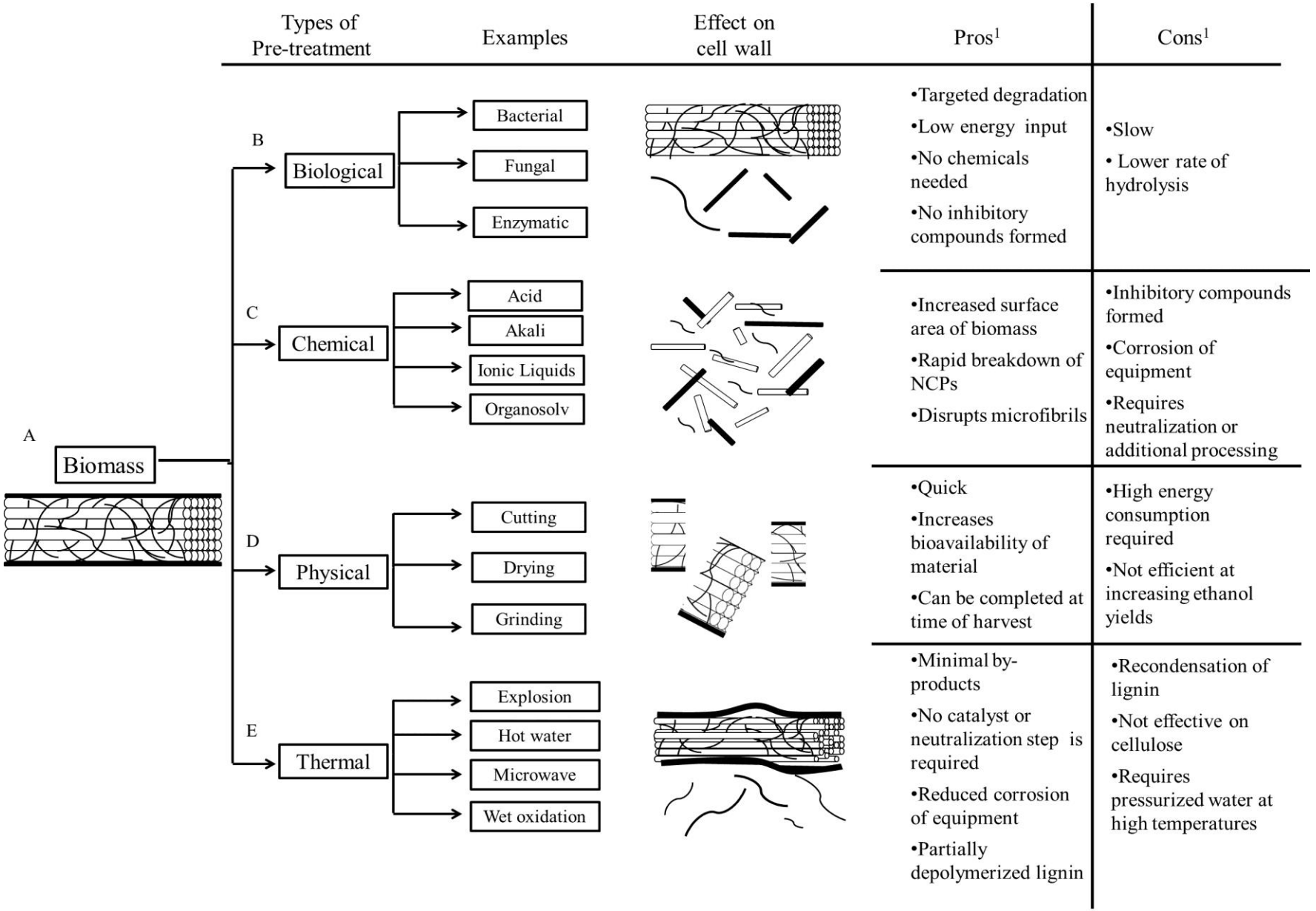
Types of Pre-treatment
Examples
Effect on cell wall
Pros[1]
Cons[1]
A
Biomass
B
Biological
Bacterial
Fungal
Enzymatic
•Targeted degradation
•Low energy input
•No chemicals needed
•No inhibitory compounds formed
•Slow
• Lower rate of hydrolysis
C
Chemical
Acid
Akali
Ionic Liquids
Organosolv
•Increased surface area of biomass
•Rapid breakdown of NCPs
•Disrupts microfibrils
•Inhibitory compounds formed
•Corrosion of equipment
•Requires neutralization or additional processing
D
Physical
Cutting
Drying
Grinding
•Quick
•Increases bioavailability of material
•Can be completed at time of harvest
•High energy consumption required
•Not efficient at increasing ethanol yields
E
Thermal
Explosion
Hot water
Microwave
Wet oxidation
•Minimal by-products
•No catalyst or neutralization step is required
•Reduced corrosion of equipment
•Partially depolymerized lignin
•Recondensation of lignin
•Not effective on cellulose
•Requires pressurized water at high temperatures

Figure 4 Selected pre-treatment methods differentially affect the breakdown and liberation of polymers from the cell wall

Lignocellulosic biomass is a complex network of carbohydrate and non-carbohydrate polymers (A). In its native form plant biomass is recalcitrant. Pre-treatment is the initial processing step used to convert raw biomass into a form that can be more readily hydrolyzed. Biological pre-treatments are efficient at removing lignin from the network, leaving a carbohydrate-enriched fraction (B). Chemical pre-treatments may result in complete breakdown and fragmentation of cell wall components (C). Physical pre-treatments are used to reduce the particle size of the biomass (D). The use of thermal pre-treatments may loosen bonds between and within polymers but lignin is not completely removed (E). [1][11,76,82]

7.0 Enzymatic saccharification increases the amount of fermentable monosaccharides

Pre-treatments are used to increase the solubilisation of carbohydrates in lignocellulosic biomass. However, it not uncommon for more recalcitrant carbohydrates, such as cellulose, and non-carbohydrate components, such as lignin, to remain in the residual biomass (non-solubilized component). Depending on the efficiency of the pre-treatment, enzymatic saccharification is usually required to further degrade polysaccharides into their respective constituents. The three types of cellulase enzymes used for biomass processing are endoglucanases, exoglucanases and β-glucosidases. Endoglucanases increase the number of

chain ends and significantly decrease the degree of polymerization by hydrolysing the interior glucosidic linkages of cellulose molecules [83]. Exoglucanases shorten the chains incrementally by acting on the reducing and non-reducing ends of cellulosic chains or released oligosaccharides [84]. The β-glucosidase enzymes act on the short oligomers such as cellobiose, a disaccharide produced from partial hydrolysis of cellulose. If biological or thermal pre-treatments are used, the biomass may need to be treated with enzymes specific to non-cellulosic polysaccharides to liberate both pentose and hexose monosaccharides. For xylose-based non-cellulosic polysaccharides, xylanases may be used to degrade linear -(1,4)-β-xylan chains. Other enzymes which may be used to hydrolyse non-cellulosic polysaccharides include mannanases, galactosidases, arabinanases and a range of pectin degrading enzymes [85,86].

The efficiency of enzymatic saccharification is driven by enzyme concentration and influenced by substrate properties such as degree of polymerization, relative crystallinity, accessible surface area, linkage types, and the presence of lignin. These properties differ between feedstocks [83]. Feedstocks that have less recalcitrant cell walls, due to lower lignin levels or to lower cellulose crystallinity, may have similar hydrolysis rates for treated and non-treated material. Although the use of enzymes may increase the amount of available sugar for fermenting organisms, the actual cost associated with enzymatic saccharification in large scale production schemes is still a topic of much debate. In some cases a dependency on enzymes to break down carbohydrates at a commercial scale would be a significant financial drain, but it may be that costs will become manageable with the advancement of technology and infrastructure [24].

8.0 Anaerobic fermentations may be used for bioethanol production

After hydrolysis, monosaccharides or low molecular weight oligomers can be fermented to ethanol. Anaerobic fermentation of hexoses and pentoses generally produces two major end-products, carbon dioxide and ethanol, as well as small amounts of heat. The rate of sugar consumption and 'completion' of the fermentation can be monitored by the release of carbon dioxide. In simplified terms, the activation of the fermentation pathway begins when sugar molecules are transported across the plasma membrane of yeast. The transporters have a higher affinity for certain sugars in a heterogeneous sugar solution, but the rate of uptake decreases with depletion of the preferred sugar(s) over time [87,88].

Alcoholic fermentations involve complex chemical and enzymatic reactions in either a step-wise or continuous manner until conversion is complete, slows or is inhibited. The physical breakdown of polysaccharides can result in a decreased rate of fermentation or conversion. For example, the liberation of acetate substituents from polysaccharides can be toxic to susceptible fermenting microorganisms such as *Pichia stipitis*. In addition, fermentations can cease due to environmental stresses of pH, osmolarity, temperature, and oxygen level, due to decreased sugar utilization, decreased cell growth, or to intolerance of reactants and/or end products [89,90]. Ethanol itself can also exert inhibitory effects on the fermenting organism through plasma membrane disruption, disrupting passive proton flux, damaging intracellular enzymes and causing cell death [91,92]. Inhibitory compounds such as furfural and its derivatives, produced as byproducts of some pre-treatments, can inhibit glycolytic enzymes and aldehyde dehydrogenase activity, resulting in an accumulation of

acetaldehyde that lengthens the lag phase for microorganisms such as *Saccharomyces cerevisiae* and *Escherichia coli* [93,94].

The productivity of the selected microorganism is dependent on the organism's ability to thrive within the environment containing the substrate and on possessing the biological mechanisms required to convert the carbohydrates present to ethanol. However, microorganisms cannot function effectively outside a narrow range of parameters and selecting robust microorganisms best suited to converting a particular biomass is therefore essential.

Currently, *Saccharomyces cerevisiae* is one of the most commonly used yeasts for alcoholic fermentations [95], although the efficiency of other fermenting organisms i.e. *Pichia stipitis*, *Clostridium cellullulolyticum* and *E. coli* have been investigated [96]. However, many yeast and bacteria strains cannot ferment pentose sugars (C5), rendering up to 45% of the carbohydrates in some raw feedstocks unusable for ethanol production [97]. In order to improve conversion yields, recombinant DNA approaches have been used to introduce genes from organisms capable of naturally fermenting pentose sugars such as fungi, insects, microorganisms from ruminant guts and *Pichia stipitis* into yeast [98]. Bacteria may likewise be an option to use in fermentations, although problems with catabolite repression have been reported [99].

A twostep process, that is separate hydrolysis and fermentation steps (SHF), is the conventional method used for bioethanol production, because both processes can be conducted under their optimal conditions (Figure 1). Alternatively, genetic manipulations targeting the production and regulation of hydrolytic enzymes in fermenting microorganisms has resulted in the breakdown and conversion of carbohydrates to ethanol in a one step process [100]. Some organisms have the genetic machinery to synthesise these enzymes naturally, which could be

further exploited. For example the organism *Kluyveromyces marxianus* secretes fructanase to hydrolyse fructans and the liberated fructose and glucose monomers are converted to ethanol concurrently [101]. The ability of a microorganism to synthesise cell wall polysaccharide-degrading enzymes facilitates a natural simultaneous saccharification and fermentation (SFF) process. This approach may be more economical at a large scale because costs associated with enzyme addition are removed and conversion is completed in a single reactor vessel.

9.0 Objectives of the thesis

The main objective of this project was to investigate both the potential and limitations of two widely different sources of agro-industrial residues for bioethanol production. The materials selected were the leaves of *Agave americana* and *Agave tequilana* plants, and grape marc (Cabernet Sauvignon and Sauvignon Blanc) waste from the wine industry. For the conversion of carbohydrates derived from these feedstocks to be economically viable and sustainable, each stage of production must be optimized. There are six main areas where the production system can be optimized (see 5.0 Production of bioethanol). This study was focussed on identifying the limitations and opportunities in the areas of: 1) minimizing costs associated with harvesting the feedstock, 2) increasing monosaccharide availability, 3) minimizing the need for and costs associated with pre-treatments and saccharification; and 4) ensuring complete utilization of carbohydrates during fermentation.

The present study assessed the composition of novel sources of biomass and created a toolset of methods and techniques for the quantification and processing of the biomass into bioethanol. Lessons obtained from this study will help to define the feasibility, practicality and sustainability of using these feedstocks, namely *Agave* leaves and grape marc, for biofuel

production. In addition, compositional information generated may be of value to growing areas of research and commercialization such as the conversion of plant biomass into value-added chemical compounds.

10.0 Materials and Methods

Materials and methods used in this thesis are described in the related papers I-III. Where additional methods were used to generate unpublished data, as presented in the appendices, details are provided below.

10.1 Comparison of water extraction methods

Four methods were used to extract water-soluble carbohydrates from *A. tequilana* leaves (n=6). Following extraction, glucose, fructose and sucrose were quantified by hydrophilic interaction chromatography (HILIC) as described in paper I. The extraction conditions were as follows:

Treatment #	**Solid/Liquid ratio (mg/mL)**	**Time**	**Temperature**
1	~50 mg/2.5 mL water	15 min	80°C; water bath
2	~50 mg/2.5 mL water	15 min- continuous shaking	RT
3	~50 mg/2.5 mL water	1 h	121°C; autoclave
4	~20 mg/1 mL water	1 h- shaken every 15 min	90°C; water bath

10.2 Fluorescent Immuno-microscopy

Embedded tissue (as described in paper I) was sectioned at 1 μm using a diamond knife on a Leica Ultracut R microtome. Sections were rehydrated with phosphate-buffered saline (PBS), treated with 0.05 M glycine, and blocked with 1% w/v bovine serum albumin in PBS. Sections of *Agave sisalana* leaf were incubated with LM19 (Plant Probes, UK) in a moisture chamber at room temperature, washed and incubated with the 588 anti-rat IgM secondary antibody

(diluted 1:50, Thermo Scientific, Australia) for 2 h in a light-excluding humidity chamber. Following incubation with secondary antibodies, slides were washed and counterstained with 0.1% (w/v) Calcofluor White. Sections were mounted in 90% (v/v) glycerol. Images were captured using a Zeiss M2 Axio Imager fitted with an MRm Rev. 3 AxioCam, and the filter sets 43 dsRed (Alexa 588) and 49 (Calcofluor).

10.3 Monolignol analysis: S/G ratio

The monolignols *p*-coumaryl alcohol (**H**), coniferyl alcohol (**G**) and sinapyl alcohol (**S**) in *A. tequilana* leaves, bagasse and stem bagasse (n=2) were quantified by thioacidolysis and gas chromatography/mass spectrometry following a published method [102].

10.4 Total phenolic and anthocyanin content

A modified Folin-Ciocalteu method was followed [103]. Dried, ball milled material (10 mg) was extracted with acidified methanol in a dark chamber for 2 h. An aliquot of the supernatant was incubated with 10-fold diluted Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, USA) for 5 min at room temperature. Following incubation sodium carbonate (60 g/L) was added and samples were left for 90 min (room temperature) before an absorbance reading using a spectrophotometer (Thermo Fischer, Waltham, MA, USA) set at a wavelength of 725 nm. A standard curve was generated using ferulic acid in the concentration range 0–6 µg/µL. For quantification of anthocyanins the same extraction solvent and conditions were used. Cabernet Sauvignon grape marc was extracted twice using acidified methanol. Anthocyanins extracted from grape marc were separated and quantified using HPLC, as previously described [104]. Briefly, an Agilent 1100 HPLC (Agilent Technologies, Germany) equipped with a Phenomenox Synergi Hydro-RP column (150 x 2 mm) and a photodiode array detector (520

nm) was used. Anthocyanin compounds were identified from their elution time with respect to malvidin-3-glucoside and quantified as malvidin-3-glucoside equivalents.

10.5 Immuno-electron microscopy: grape marc

Grape seed, skin and stem were separated and fixed in a solution of 0.25% v/v glutaraldehyde, 4% v/v paraformaldehyde and 4% v/v sucrose in phosphate-buffered saline (PBS), dehydrated in an ethanol series, infiltrated in LR White resin (ProSciTech Pty Ltd, Australia), and polymerized in a gelatin capsules at 58°C for 48 h [20]. Sections of 70−90 nm were collected on collodion-coated nickel grids and labeled [20] using the primary antibodies LM19 (diluted 1/20), an antibody for partially methyl-esterified homogalacturonan and LM20 (diluted 1/20), an antibody for methyl-esterified homogalacturonans. The secondary antibody goat-anti-rat IgM (diluted 1/30; Jackson ImmunoResearch Labs Inc., USA) was used. Sections were imaged using a Philips CM100 Transmission Electron Microscope at an accelerating voltage of 80 kV.

10.6 Screening of yeast: assimilation and biomass accumulation on glucose

Ten microorganisms were obtained from the ARS Culture (NRRL) Collection, National Center for Agricultural Utilization Research (Table D-1; Peoria, IL, USA) (paper III). Yeasts were streaked from glycerol stocks (20% v/v) on YPD (yeast extract: peptone: dextrose medium) plates and placed at 28°C. A single colony was picked and cultured in liquid YPD overnight at 28°C with shaking at 120 rpm. Cell suspensions were diluted to an OD_{600} (Varian Cary 50) of 0.5 for spot assays [105]. Serial dilutions were spotted on plates of induction medium (pH 4.5) [106] supplemented with glucose (20 g/L) and grown for 24 h at 28°C. Accumulation of biomass for five strains (paper III) when cultured in glucose was assessed. Briefly, a standardized cell count (5×10^6 cells/mL) was inoculated into 10 mL of YPD media

and cultivated anaerobically at 28°C with agitation at 160 rpm; n = 2. After 24 h one mL of culture was transferred to pre-weighed tubes and the cells pelleted by centrifugation at 10000 rpm for 10 min. The supernatant was discarded and the pelleted cells washed with an equal volume of sterilized water to remove any excess glucose. Pellets were dried overnight at 35°C before the final weight was recorded.

11.0 Results and Discussion

11.1 Paper I: Highlights

Agave leaves are fruit-like – rich in moisture, soluble sugars and pectin and low in lignin

Leaf fiber is composed of crystalline cellulose (47–50% w/w) and non-cellulosic polysaccharides (16–22% w/w)

Juice pressed from the *Agave* leaves accounted for 69% of the fresh weight and was rich in glucose and fructose.

Hydrolysis of the fructan oligosaccharides doubled the amount of fermentable fructose in *A. tequilana* leaf juice samples

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Signature		Date	13/05/15

Name of Co-Author	Seth DeBolt		
Contribution to the Paper	Helped evaluate and edit manuscript, aided in assessment of saccharification efficiency		
Signature		Date	

Name of Co-Author	Natalie Betts		
Contribution to the Paper	Supervised development of work Helped interpret data Edited and helped evaluate manuscript		
Signature		Date	8/5/15

Name of Co-Author	Geoff Fincher		
Contribution to the Paper	Supervision, developed concept, helped with drafts.		
Signature		Date	11/5/15.

Name of Co-Author	JELLE LAHNSTEIN		
Contribution to the Paper	ANALYTICAL METHODS		
Signature		Date	12/5/15

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Contribution to the Paper	Assisted in conceptualising, setting-up and maintaining field-plot. Assisted in sampling. Provided mentoring on the Agave system and CAM. Read manuscript.		
Signature	Prof Joseph A M HOLTUM	Date	11/05/2015

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Signature		Date	12/5/15.

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Contribution to the Paper	Development of project, project guidance, manuscript revision, light microscopy and assistance with method development.		
Signature		Date	12/5/15

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Signature		Date	7/5/15

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Signature		Date	10/5/2015

Name of Co-Author	Stefan Bauer		
Contribution to the Paper	Provided assistance with experimental techniques.		
Signature		Date	05/07/2015

Name of Co-Author	Don Chambers		
Contribution to the Paper	Provided agave plant materials for testing and analysis		
Signature		Date	09/05/2015

Prospecting for energy-rich renewable raw materials: Agave leaf case study

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Abstract

Plant biomass from different species is heterogeneous, and this diversity in composition can be mined to identify materials of value to fuel and chemical industries. *Agave* produces high yields of energy-rich biomass, and the sugar-rich stem tissue has traditionally been used to make alcoholic beverages. Here, the compositions of *Agave americana* and *Agave tequilana* leaves are determined, particularly in the context of bioethanol production. *Agave* leaf cell wall polysaccharide content was characterized by linkage analysis, non-cellulosic polysaccharides such as pectins were observed by immuno-microscopy, and leaf juice composition was determined by liquid chromatography. *Agave* leaves are fruit-like – rich in moisture, soluble sugars and pectin. The dry leaf fiber was composed of crystalline cellulose (47–50% w/w) and non-cellulosic polysaccharides (16–22% w/w), and whole leaves were low in lignin (9–13% w/w). Of the dry mass of whole *Agave* leaves, 85–95% consisted of soluble sugars, cellulose, non-cellulosic polysaccharides, lignin, acetate, protein and minerals. Juice pressed from the *Agave* leaves accounted for 69% of the fresh weight and was rich in glucose and fructose. Hydrolysis of the fructan oligosaccharides doubled the amount of fermentable fructose in *A. tequilana* leaf juice samples and the concentration of fermentable hexose sugars was 41–48 g/L. In agricultural production systems such as the tequila making, *Agave* leaves are discarded as waste. Theoretically, up to 4000 L/ha/yr of bioethanol could be produced from juice extracted from waste *Agave* leaves. Using standard *Saccharomyces cerevisiae* strains to ferment *Agave* juice, we observed ethanol yields that were 66% of the theoretical yields. These data indicate that *Agave* could rival currently used bioethanol feedstocks, particularly if the fermentation organisms and conditions were adapted to suit *Agave* leaf composition.

Introduction

Plant biomass is a source of chemical energy that can be converted to combustible transport fuels and biochemicals by fermentation or chemical conversion of plant-derived sugars [1]. Currently, plant materials from farming-intensive food production systems, such as corn, wheat grain or cane sugar, are being used to make bioethanol and biochemicals. In the future, alternative sources of energy-rich plant material from low-input systems that are independent from the food chain will be needed [2,3].

Plant biomass contains soluble and structural sugars: for example the vacuoles of storage cells in the stem of sugarcane contain high concentrations of sucrose, a soluble disaccharide and the cell walls in the trunks of willow trees contain a large amount of cellulose, a structural sugar composed of glucose [4]. The composition of historical agriculture plant species have been reported (Table P1-1; [5]); however, the relative importance of plant species is likely to change as agricultural industries adapt to new markets and climate change. Research into novel plants may reveal non-food sources of valuable raw materials. One example of a plant species that is likely to gain importance is *Agave*. Historically *Agave* has been used for production of alcoholic beverages, fibers, chemicals and sugar additives [6] and there is growing interest in using *Agave* for biofuel production.

Alcoholic beverages such as tequila and mescal are made from the stem tissue of *A. tequilana* plants that are 8–12 years old. Fructans in mature stem tissue are degraded by heat to release fermentable fructose [7] and the leaves, which account for up to 66% dry weight of the biomass, are discarded [8]. *Agave* is a productive water-use efficient plant that grows in regions with extreme environments [9-11] and recent literature has considered the potential for using *Agave* as a feedstock for bioethanol production [12-18]. However, the composition of *Agave* leaf tissues from plants at an earlier stage in

development has not been well characterized and may represent an energy-rich raw material that can be produced rapidly in a low-input system [19,20].

There are standard protocols for determining the composition of plant biomass, such as the analytical procedures published by the United States Government National Renewable Energy Laboratory (NREL) [21-26]. Biomass composition analyses may include determination of moisture content, total solids, acid-soluble and insoluble residues and the amount of water soluble carbohydrates (WSC), starch, mineral, lignin, protein, crystalline cellulose and non-cellulosic polysaccharides. In the context of using biomass to make biofuels and biochemicals, it is of interest to determine not only the amount of fermentable sugars that can be extracted from plant biomass, but also the amount of inhibitory compounds that are formed during processing which may interfere with conversion of the biomass to bioethanol [27]. For example, acetic acid is generated from the hydrolysis of acetyl groups associated with non-cellulosic polysaccharides. Weak acids like acetic can reduce yeast growth and ethanol yields by prohibiting monosaccharide metabolism and causing intracellular anion accumulation [27]. In addition, the compositions and proportions of sugar present in soluble forms and structural forms, and the recalcitrance of these structural sugars are important as they influence the processing methods and costs. These data are also used to estimate the bioethanol yields for a feedstock of interest.

Here, the composition of *Agave* leaves is determined, including a detailed analysis of the fermentable and non-fermentable compounds in *A. americana* and *A. tequilana*. The efficiency of enzymatic hydrolysis of *Agave* leaf cellulose and hydrolysis of fructans in juice samples is quantified. Compositional data is then extrapolated to calculate theoretical ethanol yields and *A. tequilana* leaf juice is fermented using two *Saccharomyces cerevisiae* strains. These compositional and fermentation data can be used to inform the development of biotechnology to exploit this energy-rich raw material.

Material and Methods

Plant material

A. tequilana and *A. americana* plants were approximately 2–3 y old at the time of harvest and had begun to reproduce asexually. The heights of the plants from the base to the tip of the tallest leaf were at least 2 m. Six plants of *A. tequilana* were harvested from Ayr (Queensland, Australia) and six plants of *A. americana* were harvested from the Adelaide Hills (South Australia, Australia). From each individual plant stem tissue and at least three leaves were collected. Permission for the described field studies were granted by either the crop manager or land owner.

The stem and leaves were separated at the time of harvest and fresh weights recorded. Juice from the stem tissue of each *A. tequilana* plant was collected after shredding (Cutter-Grinder CG03, Jeffco) and three leaves per plant (*A. americana* and *A. tequilana*) were collected for compositional analysis. A subset of the remaining leaves was pooled and two experimental shredders were used to extract juice (Cutter-Grinder CG03, Jeffco and Food processor, Abode). Wet bagasse was dried at a 60°C to constant moisture content. Juice and whole leaves were transported to the University of Adelaide on dry ice and stored at –80°C. Prior to analysis, samples were cut into 200–400 mm^2 pieces, weighed, lyophilized (Labconco-Freezone, Missouri, United States) and moisture loss was calculated. Dried leaf material was ground in a 25 mL stainless steel grinding jar with one 7 mm steel ball. The grinding jars were shaken at 30 Hz for 3 min (Retsch mill MM400, Retsch GmbH; Haan, Germany). A flowchart of methods employed for compositional analysis is included in Figure P1-1.

Fiber extraction

Whole leaves were frozen at –80°C and subsequently thawed at room temperature. Fibers were pulled from three plants of each species and separated from the vegetative tissue manually. The fibers were further cleaned using forceps to remove any attached pith tissue. Fibers (1–2 mm) were dried overnight at 60°C. Dried fibers were hydrolyzed using 1M sulfuric acid (H_2SO_4) for 3 h at 100°C [28], cooled and centrifuged at 28 000 g for 5 min. The monosaccharides in the supernatant were analyzed using high-performance liquid chromatography (HPLC). Derivatisation and quantification of monosaccharides was completed according to [29] with modifications to the gradient conditions. Elution was performed with 10% acetonitrile, 40mM ammonium acetate (A) and 70% acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient for solvent B is as follows: 0–9.5 min, 8% B; 9.5–10 min, 17 % B; 10–11.5 min, 100% B; 11.5–14.5 min, 8% B.

Total soluble solids (TSS) in *Agave* juice

Aluminum pans (Fisher Scientific, Australia) were dried at 60°C and their initial weight recorded. Juice samples were centrifuged at 10 000 g for 10 min and 2 mL aliquots of supernatant were added to the pans and heated at 60°C for 48 h, leaving a solid residue in the pan. The final weight of the pan and solid residue was subtracted from the initial weight to calculate the total soluble solids (TSS).

Crystalline cellulose

Crystalline cellulose in leaf tissue and fiber-enriched samples was determined using a modified Updegraff method according to [30].

Elemental analysis and protein and mineral (ash) quantification

Samples for the elemental analysis included 300 mg of dry, ball milled, whole leaf tissue or 1 mL of juice. Elements (Al, Ca, Fe, Mg, P, K, Na, S and Zn) were measured

using a closed tube nitric acid/hydrogen peroxide digest and radial view inductively couple plasma-optical emission spectrometry [31].

The total nitrogen content was measured by the Waite Analytical Services, University of Adelaide using complete combustion gas chromatography (Carlo Erba Instrument) and 100 mg of biomass or 1 mL of juice. The nitrogen value was converted to an estimate of the protein content using the nitrogen factor (NF) 6.25 [21]. Mineral content of extracted and non-extracted material was calculated by heating samples to 500°C for 3 h [22].

Water- and ethanol-soluble carbohydrates in *Agave* leaves

Leaf samples were dried at 60°C and extracted sequentially in water, 95% v/v ethanol and 70% v/v ethanol at 80°C for 15 min using a 1:5 ratio of biomass to extraction liquid. The residual biomass was dried at 60°C.

The total fructan and (1,3;1,4)-β-glucan content in water extracts was measured using commercial assay kits (Fructan HK-Megazyme: AOAC Method 999.03 and AACC Method 32.32 and AACC Method 76.13, Mixed-Linkage Beta-Glucan-Megazyme: AACC Method 32-23, AOAC Method 995.16, EBC Methods 3.11.1, 4.16.1, 8.11.1 and ICC Standard Method No. 166; International Ireland Ltd., Wicklow, Ireland), respectively.

Glucose, fructose and sucrose in water extracts were measured by hydrophilic interaction chromatography (HILIC), using a Prevail Carbohydrate ES column (150 × 4.6 mm) (Alltech; Illinois, United States) on an Agilent 1200 series liquid chromatography instrument equipped with an evaporative light scattering detector (Alltech ELSD 800). The mobile phase consisted of water (A) and 90% acetonitrile (B) at a flow rate of 1.0 mL/min at 20°C. The gradient for solvent B is as follows: 0–18 min, 94.5% B; 18–19 min, 64.5% B; 19–20 min, 0% B; 20–30 min; 94.5% B.

The pectin-enriched polysaccharide content in water extracts was determined using an ethanol precipitation method according to [32].

Solvent was removed from water and ethanol extracts separately by centrifugal evaporation (Savant SC110 Speed Vac, Thermofisher; Massachusetts, United States). The concentrated material was hydrolyzed using 1M sulfuric acid (H_2SO_4) for monosaccharide analysis using HPLC, as previously described [29].

Measurement of structural carbohydrates, lignin and acetyl content

For compositional analysis, samples were extracted using an Automated Extraction System (ASE) following [23]. *Agave* leaves (cut to 2−4 mm in size); aluminum pans and Whatman GF/C 55 mm glass microfiber filters (Sigma-Aldrich, United States) were dried at 105°C. Extraction cells (11 mL) were fitted with pre-weighed filter paper and 1 g of dried material added. Material was extracted with three water cycles followed by three 190 proof ethanol cycles at 100°C (ASE300, Dionex). Extraction settings were modified to 60 s nitrogen purges following extraction, 5 min static time and 120% rinse volume. Following extraction the remaining alcohol insoluble residue (AIR) and filter paper were placed in pre-weighed aluminum pans and dried at 105°C. Dried, extracted biomass was ground using a Retsch mill MM400, as previously described. The percentage of extractables was calculated based on the difference between the initial weight (before water and ethanol extraction) and final weight (after extraction).

Following extraction the alcohol insoluble residue was analyzed following [24]. Briefly, a 30 mg sample of dried ground material was treated with 13.5M sulfuric acid at room temperature for 1 h. The samples were diluted to 0.75M acid and autoclaved at 121°C for 15 min and centrifuged for 10 min at 10 000 g. The supernatant was collected for monosaccharide, acid-soluble lignin and acetate analyses. A sugar recovery standard for monosaccharides was carried through the acid hydrolysis as outlined in [25].

Monosaccharides were measured following derivatisation as previously described using HPLC. The acid-soluble lignin content was measured using a spectrophotometer (Thermo Fischer, Waltham, MA, USA) set at a wavelength of 205nm and calculated following LAP-004 using the extinction coefficient value 110 L/g-cm [26]. The acetyl content in the supernatant was analyzed at 60°C using an Aminex HPX-87H column (300 x 7.8 mm) (Bio Rad; California, United States) on a 1100 series liquid chromatography instrument. Elution was performed isocratically with 2.5mM H_2SO_4 at a rate of 0.5 mL/min [33]. Starch was measured in extracted samples following a commercial assay (Total Starch-Megazyme: AOAC Method 996.11; International Ireland Ltd., Wicklow, Ireland).

The residual biomass was washed to a neutral pH and filtered through pre-dried and pre-weighed Whatman GF/C 55 mm glass microfiber filters (Sigma-Aldrich, United States). The filter paper and collected sample residue was heated to 105°C overnight and weighed (*M1*). The material was ash corrected by heating at 500°C for 3 h and weighed (*M2*). The lignin content was calculated based on the difference between *M2 – M1* divided by the initial weight.

Linkage analysis of cell wall residue in whole leaf

Lyophilized leaf material was ground in a 25 mL stainless steel grinding jar with one 7 mm steel ball. The grinding jars were shaken at 30 Hz for 3 min (Retsch mill MM400, Retsch GmbH; Haan, Germany) until all cells were ruptured. Samples were extracted sequentially with 80% v/v ethanol on ice, and acetone and methanol at room temperature. Samples were digested with α-amylase (*B. licheniformis*; EC 3.2.1.1) to remove starch. Linkage analysis and carboxyl reduction of the material followed [34].

Enzymatic saccharification

For saccharification, Celluclast 1.5 L (cellulase preparation from *Trichoderma reesei*) and Novozyme 188 (cellobiase preparation from *Aspergillus niger*) (Sigma-

Aldrich; St Louis, MO, USA) were mixed in equal volumes. Enzymatic activity of the cellulase cocktail was measured according to the National Renewable Energy Laboratory (NREL) analytical procedure, Measurement of Cellulase Activities (LAP 006) [35]. The saccharifications used an enzyme concentration of 60 filter paper units (FPU). Alcohol insoluble cell walls were prepared according to [36]. Modifications to the micro scale saccharification were made using equivalent amounts of 0.02 g cellulose for all samples (NREL; LAP 009) and the total reaction volume reduced to 1.5 mL [37,38]. The glucose concentration was measured using a Yellow Springs Instrument (YSI) glucose analyzer (Yellow Springs, OH, USA) over 48 h, n=3.

Analysis of hydrolyzed juice fraction

Samples of diluted, centrifuged, juice were treated with trifluoroacetic acid (TFA) to a final concentration of 0.2M TFA or fructanase (Fructan HK-Megazyme: AOAC Method 999.03; International Ireland Ltd., Wicklow, Ireland). For the TFA hydrolysis, juice and acid were mixed in equal proportions and samples were heated at 80°C for 1 h. For enzymatic hydrolysis, juice and enzyme mix were combined in equal proportions and samples incubated at room temperature for 30 min, then heated to 100°C for 15 min to deactivate the enzyme. Carbohydrates in the raw and treated juice samples were measured by HILIC, using a Prevail Carbohydrate ES column (150 × 4.6 mm) as previously described.

Microscopy

Fresh tissue was fixed in a solution of 0.25% glutaraldehyde, 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 24 h at 20°C. Samples were washed twice with PBS, dehydrated in an ethanol series, infiltrated in LR White resin (ProSciTech Pty Ltd, Australia), and polymerized in a gelatin capsules at 58°C for 48 h [39,40].

Light microscopy

Embedded *Agave* leaf tissue was sectioned at 1 μm using a diamond knife on a Leica Ultracut R microtome. Sections were collected and dried onto poly-L-Lysine-coated microscope slides and stained with either toluidine blue (Sigma-Aldrich, United States) or methylene blue/basic fuchsin (ProSciTech Pty Ltd, Australia). Sections were viewed using a Leica light microscope (Version 4.3) and images captured with a Zeiss M2 Axio Imager fitted with an MRm Rev. 3 AxioCam.

Immuno-electron microscopy

Ultrathin sections of 70-90 nm were collected on collodion-coated nickel grids and labeled following Aurion Immunogold Specific Localisation Methods [41] using the primary antibodies LM19 (diluted 1/20), LM11 (diluted 1/500), LM20 (diluted 1/20) (Plant Probes, UK), or (1→4)-β-Mannan (diluted 1/50; Biosupplies, AU) [42-44]. Diluted (1/30) secondary antibodies goat-anti-rat IgM (LM19, LM11 and LM20; Jackson ImmunoResearch Labs Inc., USA) and goat-anti-mouse IgG (Mannan; ProSciTech, Australia) conjugated to 25 nm gold particles were used (GAM IgG/M, ProSciTech, Australia). Labeled sections were examined and imaged using a Philips CM100 Transmission Electron Microscope.

Preparation of inoculums, fermentation conditions and analysis

Two *Saccharomyces cerevisiae* strains (Y-139 and Y-636) were kindly provided by the ARS Culture (NRRL) Collection, National Center for Agricultural Utilization Research (Peoria, IL, USA). Strains were streaked on 1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar (YPD) plates. Plates were grown overnight at 28°C and a single colony picked. The single colony was grown in YPD liquid broth (28°C) in a shaker incubator (120 rpm). The YPD cultures were used to inoculate autoclaved *Agave* leaf juice (100 mL) at a cell density of 5 x 10^6 cells/mL. Juice samples were autoclaved (121ºC, 15

min) and centrifuged at 5000 rpm for 10 min to remove excess leaf tissue, prior to inoculation. The fermentations were completed in Erlenmeyer flasks (250 mL) with side arm sampling ports and sealed with water-filled airlocks. The fermentation flasks were placed in a shaker (150 rpm) set at 28°C for 96 h. The cells were removed from the fermentation broth by centrifugation (1m / 10 000 g) and the supernatant stored at -20°C until analysis. Ethanol concentration was determined using an Aminex HPX-87H column (300 x 7.8 mm) (Bio Rad; California, United States) as described above, following [33].

Results and Discussion

Processing of *Agave* biomass: leaf and stem fractions

One feature of *Agave* plants that differs from traditional biofuel feedstocks is its high moisture content and inversely, its low water requirements. The seasonal water requirement of *Agave* (300–800 mm/yr) is minor compared with other biomass sources such as sugarcane (*Saccharum* spp., 1500–2500 mm/yr) [18]. The lower water requirement for *Agave* is attributed to its ability to store large volumes of water in its leaves (>83% w/w) (Figure P1-2). This water storage is common for crassulacean acid metabolism (CAM) plant assimilatory organs and aids in buffering the plant against periods of extended drought [45]. Such physiological characteristics make *Agave* a favorable biofuel feedstock for dry, marginal regions. However, moisture content directly contributes to biomass weight, which affects transport and processing costs. Separating *Agave* juice from the biomass at the time of harvest may result in higher yields and lower input costs such as transportation.

The above-ground portion of *Agave* plants can be separated into leaves and stems (Figure P1-3a). For 3 year old *Agave* plants, the ratio of leaf: stem dry weight is 4:1, but becomes more variable with age [8]. Whole leaf and stem tissue may be dried and ground

to remove excess moisture and to reduce particle size (Figure P1-3b). Alternatively, crushing the leaves by mechanical force releases 69% of the wet weight (Figure P1-2a) as a sugar-rich juice (Figure P1-3c). The biomass that remains after crushing is a fibrous bagasse, which may be further dried to remove excess moisture (Figure P1-3d).

Analysis of the whole leaf fraction

Pectic polysaccharides occur in crystal sheaths

The morphology of *Agave* cells and the spatial localization of polysaccharides in the leaf tissue was investigated. Transverse sections of *A. tequiliana* leaf were stained with toluidine blue to observe the morphology of the tissues (Figure P1-4a). Toluidine blue recognizes carboxyl groups on polysaccharides and proteins, and shows the distribution, but not amount or structure, of polysaccharides. Staining was observed in and around the parenchyma cells, with sclerenchymatous fiber cap cells staining very brightly. Further examination revealed that the sclerenchymatous fiber caps around the vascular bundles in *A. tequilana* (Figure P1-4b) had thicker cell walls than in *A. americana* (Figure P1-4c). These fiber caps surrounding the xylem and phloem cells are the main structural support for the leaves [46], and the thicker cell walls explain the more erect leaf morphology of *A. tequilana* plants.

Crystal clusters were identified at the junction between cells in *Agave* leaf tissue (Figure P1-5a). Crystals have been identified in a range of photosynthetic organisms but the abundance, distribution and crystal structure varies between organisms and within tissue types [47]. The accumulation of crystals is correlated with oxalic acid production in plant tissue during normal development and in fungal-plant symbiosis [48]. A pectin-specific antibody that detects methyl-esterified homogalacturonan (LM20) [44] revealed the presence of pectic polysaccharides in the sheath surrounding the crystals (Figure P1-5b). There is conflicting information about the sheath surrounding the crystals in *Agave*

plants; our results support a finding that polysaccharides are present [49], but this is not consistent with another report indicating that no polysaccharides are present in this sheath [50].

Labeling of partially (LM19; [44]) and fully (LM20; [44]) methyl-esterified homogalacturonan was also observed in xylem parenchyma cell walls in both species (Figure P1-6). Both linkage analysis and results from the water soluble fraction confirm that high levels of pectins are present in *Agave* leaves. However, the amount of pectin-enriched polysaccharides in water extracts of *A. tequilana* was five times higher than in *A. americana* (Table P1-2); whereas linkage analysis indicated that homogalacturonan levels were considerably higher in *A. americana* (17.6 mol%) than in *A. tequilana* (6.5 mol%; Table P1-3). These data indicate that pectins in *A. tequilana* leaves may be more soluble than those in *A. americana*.

The distribution of other cell wall polysaccharides was investigated using antibodies specific to xylan (LM11) [42] and (1→4)-β-mannan [43]. Xylan labeling was observed in the phloem walls (Figures P1-6e and P1-6f), consistent with linkage data (Table P1-3) indicating that heteroxylan is present in *Agave* cell walls. Mannan was detected to a similar extent in cell walls of parenchyma and inner epidermal tissue in both species (Figures P1-6g and P1-6h), again consistent with the linkage data (Table P1-3) that indicated heteromannan in both species.

The soluble fraction contains high levels of fermentable sugars

Sections of whole *Agave* leaves were dried, milled into fine particles, and sequentially extracted with water and ethanol to generate soluble and insoluble fractions. The water soluble carbohydrates (WSC), comprising glucose, fructose, fructans and sucrose, ranged from 15–29% dry weight. In mature *Agave* plants, fructans are the main storage carbohydrate in the stems [10]. Fructans were also the predominant WSC found in

A. tequilana leaves, but *A. americana* leaves were richer in glucose, fructose and sucrose (Table P1-2). Total leaf WSC content was lower than the 36–64% w/w found in 6 year old *Agave* stems [10], which have been traditionally selected and used for tequila production, but was much higher than the 5% and 11% w/w found in the biofuel feedstock switchgrass (*Panicum virgatum*) [51] and fructan-rich chicory (*Cichorium intybus*) [52], respectively.

Other soluble sugars were analyzed by hydrolyzing acid-labile polysaccharides into monosaccharides, which were subsequently identified by HPLC. For both species, these monosaccharides comprised a very small proportion of the total mass (Table P1-2), which is not surprising as the higher molecular weight polymers usually have limited solubility in aqueous solutions [53]. Unhydrolyzed polysaccharides were precipitated with ethanol to create a pectin-enriched fraction [32], which, in *A. tequilana*, comprised over 10% of the dry weight of the leaves (Table P1-2). From a biofuel perspective, pectins play mixed roles: soluble pectins can be hydrolyzed into monosaccharides for fermentation [54], however acetate substituents on pectins can hinder hydrolysis by blocking cleavage sites for lytic enzymes [55] and once liberated from the polymer these compounds can be toxic to susceptible fermenting microorganisms such as *Pichia stipitis* [56]. Alternatively, when thermochemical conversion processes such as catalytic pyrolysis are used instead of fermentation to produce a hydrocarbon based biofuel the amount of non-carbohydrate cell wall components (i.e. acetyl) in the biomass is less important [57].

The insoluble fraction is predominantly cellulose with low levels of lignin

The remaining insoluble residue, largely cell wall material, was dried, milled, and hydrolyzed with concentrated sulfuric acid. The resulting monosaccharide profiles of *A. americana* and *A. tequilana* leaves were similar, with 12–16% w/w glucose, 3–4% w/w xylose, 3–4% w/w galacturonic acid, 1–3% w/w galactose and less than 1% w/w arabinose (Table P1-2). However, acid hydrolysis does not permit identification of cell wall

polysaccharides, so linkage analysis was used to obtain structural information. Linkages were assigned to polysaccharides according to Pettolino *et al.*, 2012 [34] (Table P1-S1).

For both species, the majority of the material was composed of hexose (C6) sugars. Cellulose was the most abundant polysaccharide, comprising 32–45 mol% of the cell walls (Table P1-3). *A. americana* leaf cell walls had higher amounts of pectin-associated polysaccharides such as Type I arabinogalactan and homogalacturonan. There was more heteroxylan in *A. tequilana* than in *A. americana* but the heteroxylan in *A. americana* was less substituted than the heteroxylan in *A. tequilana* (Table P1-S1). Xylans with low degrees of substitution are reported to bind more strongly to cellulose [58]. The amounts of other cell wall polysaccharides were similar between the two species (Table P1-3).

Starch, a (1,4)-α-glucan, was removed from the biomass samples prior to linkage analysis to reduce interference with cellulose quantification. Starch was measured separately using a commercial assay at 1−6% w/w (Table P1-2). The polysaccharide (1,3;1,4)-β-glucan was not detected by enzymatic assays or by linkage analysis.

The total lignin content of the leaves was 9.3–12.7% w/w (Table P1-2). Compared with other biofuel feedstock crops such as corn, sugarcane and poplar, which all have lignin contents >17% w/w (Table P1-2), *Agave* is considered a low lignin feedstock. Lignin is a non-sugar aromatic polymer that binds strongly to cell wall polysaccharides via covalent and non-covalent linkages. This barrier limits enzyme binding sites on the polymers and reduces the rate and efficiency of hydrolysis [59]. Alternatively, lignin can be acid-soluble. High levels of soluble lignin in the hydrolyzate can be an inhibitor to both yeast and bacteria, reducing the yield of ethanol produced [60]. In *Agave*, 28–43% of the total lignin was acid-soluble (Table P1-2). Acid-soluble lignin has been shown to be predominantly composed of syringyl lignin and, to a lesser degree, secondary hydrophilic compounds [61].

Cellulose undergoes 40% saccharification without pre-treatments

The predominant polysaccharide identified in both species of *Agave* using linkage analysis was cellulose (Table P1-3). Due to its recalcitrance, cellulose quantification after hydrolysis with sulphuric acid can be an underestimate [62]. As a result, a method optimized for the isolation and measurement of cellulose was employed [30]. The amount of cellulose in whole tissue was slightly lower in *A. americana (*15.7% w/w) than in *A. tequilana (*16.5% w/w).

Cellulose is embedded *in muro* within a complex matrix of non-cellulosic polysaccharides, lignin and proteins. Saccharification tests were thus performed on the heterogeneous alcohol insoluble residue (removing all free glucose from the matrix) on identical cellulose loadings rather than on purified cellulose. The liberation of glucose was monitored over 48 h of enzymatic digestion using a cellulase cocktail. The extent of saccharification was similar for both species (40–35%) but slightly higher for *A. americana* (Figure P1-7). The efficiency of cellulose breakdown and therefore the total ethanol yield from *Agave* may be increased if the biomass is further processed using pre-treatments, thus loosening the bonds within and between cellulose chains.

Analysis of leaf juice and fiber fractions

Agave leaf juice is rich in fructans

The total moisture content of whole *Agave* leaves is upwards of 89% (Figure P1-2). Pressing released 69% of the fresh weight as a sugar-rich juice that was analyzed for glucose, fructose and sucrose content. The amounts of these directly fermentable sugars were also measured in *A. tequilana* stem juice, which is commonly used for tequila production. *A. americana* leaves and *A. tequilana* stems had similar amounts of free sugars in the juice (38–39 g/L), with a lower level detected in *A. tequilana* leaves (Figure P1-8a).

Glucose was the most abundant sugar in all three samples although stem juice had a similar amount of sucrose. Additional, unidentified oligosaccharides were also detected in the raw juice samples (Figure P1-8b), indicating that these monosaccharide values were likely to be an underrepresentation of the total sugar content.

Two methods were used to hydrolyze the unidentified oligosaccharides into monosaccharides: 1) a non-specific acid hydrolysis using trifluoroacetic acid (TFA); and 2) specific enzymatic cleavage of fructans by a broad specificity fructanase. This fructanase exhibits both *exo*-inulinase activity, which degrades sucrose and kestose (glucose-fructose-fructose), and *endo*-inulinase activity, which liberates fructose from the non-reducing ends of long-chain fructans. Both TFA (Figure P1-8c) and fructanase (Figure P1-8d) cleaved the unidentified oligosaccharides completely into glucose and fructose, confirming that these oligosaccharides were fructans.

The total concentration of fermentable hexose sugars after hydrolysis in leaf samples was 41–48 g/L and increased to 104 g/L in *A. tequilana* stem juice. Fructose accounted for 68% of the stem monosaccharides, comparable to previous studies that found 60% of the total soluble sugars in *A. tequilana* stem to be fructans [10]. Galactose and galacturonic acid were detected in hydrolyzed juice samples at less than 0.5 g/L.

Inorganic elements in leaf juice that may affect fermentation were measured and compared with the inorganic content of whole leaf (Table P1-S2). The concentration of inorganic elements in *A. tequilana* juice was twice as high as in *A. americana* juice, although whole *A. americana* leaves had 20% more inorganic elements than *A. tequilana* leaves. High levels of calcium were observed in both species, particularly *A. americana* whole leaves, which may be attributed to inorganic calcium oxalate crystals detected in the tissue (Figure P1-5). Calcium levels in *A. tequilana* juice and whole leaves were similar to each other, but much higher than *A. americana* juice and much lower than *A. americana* whole leaf. It is possible that the difference in calcium detected between the two *Agave*

species is an artefact of the shredding processes or different growing conditions for the two species.

Agave fibers are predominantly crystalline cellulose

With increasing reliance on synthetic fibers to meet consumer demands, production and markets for *Agave* fibers has been on the decline [14]. In recent years research has begun to investigate *Agave* fibers for emerging markets such as use in thermoplastics [63,64]. However, limited information is available regarding the composition of this waste material.

Crystalline cellulose comprised just under half (47–50% w/w) of the dry weight of fiber-enriched leaf fractions (Table P1-4), lower than the 68.4% w/w previously reported for crystalline cellulose in *A. americana* fibers [65]. The total cellulose in fibers of *A. lechuguilla* and *A. fourcroydes*, species specifically grown for their fibers, accounted for ~80% w/w of dry fiber weight, with the remainder composed mainly of lignin [66].

Non-cellulosic polysaccharides accounted for 22.4% and 15.8% of the dry weight of *A. americana* and *A. tequilana* leaves, respectively. These values are consistent with the values reported in the literature suggesting that *A. tequilana* fibers contain 17% w/w non-cellulosic polysaccharides [67]. Xylose and glucose were the most abundant monosaccharides detected in the fibers after hydrolysis in 1M sulfuric acid, agreeing with linkage analysis that detected heteroxylans and xyloglucan in insoluble leaf fractions. In addition, similar to other studies [67] about ~30% of the fiber mass for both species was unaccounted for which may be attributed to unidentified or unhydrolyzed carbohydrates, lignin, inorganic compounds and protein.

Fermentation of *Agave* juice

A. tequilana leaf juice was used as a substrate to investigate fermentation efficiency using two different strains of *Saccharomyces cerevisiae*. *A. tequilana* juice was autoclaved

to minimize microbial contamination from native organisms and inoculated with one yeast strain. Sugar content of the starting juice was 41.4 g/L of total sugars and 30.0 g/L of readily fermentable WSC. After 96 h, both strains produced ethanol concentrations of 11–14 g/L (Table P1-5). Up to 90% of the monomers were fermented, which represent only 54–66% of the total sugars. Sugars in the *Agave* leaf juice, predominantly the fructans, are therefore being underutilized by these yeast strains.

Historically, *Saccharomyces cerevisiae* is the most readily studied and utilized yeast for alcoholic fermentation assays [68] and can efficiently convert sucrose, glucose and fructose [69]; the main sugars in *Agave* leaf juice. However, alternative microorganisms may be more efficient at fermenting *Agave* juice sugars. For example, microorganisms such *Kluyveromyces marxianus* and *Torulaspora delbrueckii*, isolated from fermenting mezcal (a distilled alcohol made from *Agave*), express enzymes that hydrolyze fructooligosaccharides [70]. Activation of fructanase enzymes was induced by Ca^{2+}, which is present in significant amounts in the leaves and juice of both *A. americana* and *A. tequilana* (Table P1-S2) [71]. In addition, using organisms such as *Escherichia coli* that can catabolise galacturonic acid may be a sensible choice for *Agave* if the pectic sugars in leaf tissue are to be fermented [72]. The use of readily studied *S. cerevisae* strains should thus be considered a benchmark by which to judge other organisms since it may not be optimal for *Agave*. Careful selection of fermenting organisms may obviate the need for expensive pre-treatment processes or use of additional enzymes, which would increase the return on investment of using *Agave* spp. for biofuel production.

Agave ethanol yields rival current biofuel feedstocks

Ethanol yields from three different *Agave* substrates were modelled: 1) the dry mass of the entire *Agave* plant based on leaf sugar composition, thereby underestimating sugar content because the additional sugar in the stem is not accounted for; 2) waste *A.*

tequilana leaves from tequila production, and 3) juice from *A. tequilana* and *A. americana* leaves (Table P1-6). Theoretical ethanol yields were calculated using standard conversion assumptions [73].

The theoretical ethanol yield values for the whole leaf sugars of *A. americana* and *A. tequilana* were 437 L/t and 401 L/t, respectively. These values are comparable to estimates for other lignocellulosic biofuel feedstocks such as corn stover, sugarcane and switchgrass (Table P1-6). However, *Agave* plants may out-perform current biofuel feedstock crops in terms of productivity per hectare. Whole *A. tequilana* plants were predicted to yield 4000–13600 L/ha/yr and *A. americana* plants were predicted to yield 4400–14800 L/ha/yr. At the low end, these values exceed theoretical yields from first-generation feedstocks such as corn, wheat (*Triticum aestivum)* and sugarcane and at the high end, they double the yields of more recently investigated second generation feedstocks such as poplar and sorghum. The current values are consistent with those reported previously in the literature, which estimated that ethanol yields for *Agave* spp. may range from 3000–12000 L/ha/yr [18,20].

Waste *A. tequilana* leaves could generate 2300–7900 L/ha/yr and increase the value of existing *Agave* industries. However, since the majority of the mass of *Agave* plants is water, it may be more economically viable to directly separate and ferment the sugar-rich juice, which could yield 690–4000 L/ha/yr (Table P1-6). Even using a generic *S. cerevisiae* strain unadapted to *Agave* substrates, yields of up to 1500 L/ha/yr from *A. tequilana* leaf juice and 2600 L/ha/yr from *A. americana* leaf juice could be obtained (assuming a fermentation conversion of 66% for both substrates; Table P1-5). More efficient fermenting organisms may increase the value of using *Agave* juice as a biofuel feedstock in terms of yield and revenue returns.

It is worth noting that *Agave* cultivation systems have not yet been optimized to produce sugar for biofuel and biochemical industries. Information about agronomical practices, such as planting density or the optimal age to harvest the plants, is limited. If the plants are harvested at 2–3 years of age rather than the traditional 8–12 years of age, plant spacing could be reduced further, increasing density per hectare. In addition, further information about microorganisms that are naturally found within *Agave* may be beneficial for the industries that grow and commercialize these plants. In a biofuel context, it may be useful to isolate and characterise organisms that naturally grow on *Agave*, as they presumably utilize sugars such as fructans efficiently and are tolerant to a range of environmental conditions. The isolation and use of microorganisms found on or within biomass for the conversion of carbohydrates to biofuel is not novel; grape marc, an agro-industrial waste material, has been found to be a rich source of robust organisms that are economically and productively favourable for second generation bioethanol conversion [79]. Further research is required to identify the microorganisms associated with the *Agave* microbiome.

Conclusion

The leaf tissues of *A. americana* and *A. tequilana* species contain 56–60% (dry weight) of potentially fermentable sugars, over half of which are present in a soluble fraction. These same tissues also contain relatively low amounts of lignin. Ethanol yields (ha/yr) that could be generated from *Agave* leaves and whole plants rival those of the most successful biofuel feedstock crops such as switchgrass and poplar. *Agave* differs from most common feedstocks in its high moisture content, but nearly 70% of plant mass can be extracted with simple mechanical pressing to release a sugar-rich juice. Crushing and fermenting the juice on site without any pre-treatment can produce competitive ethanol yields, with room for improvement by judicious selection of fermenting organisms, and by-

products may be produced from the crystalline cellulose enriched bagasse waste. The comprehensive compositional data for *Agave* leaves and fermentation trials reported herein will be instrumental in the development of agronomic, saccharification and fermentation methods for converting *Agave* raw material into biofuel or biochemical products.

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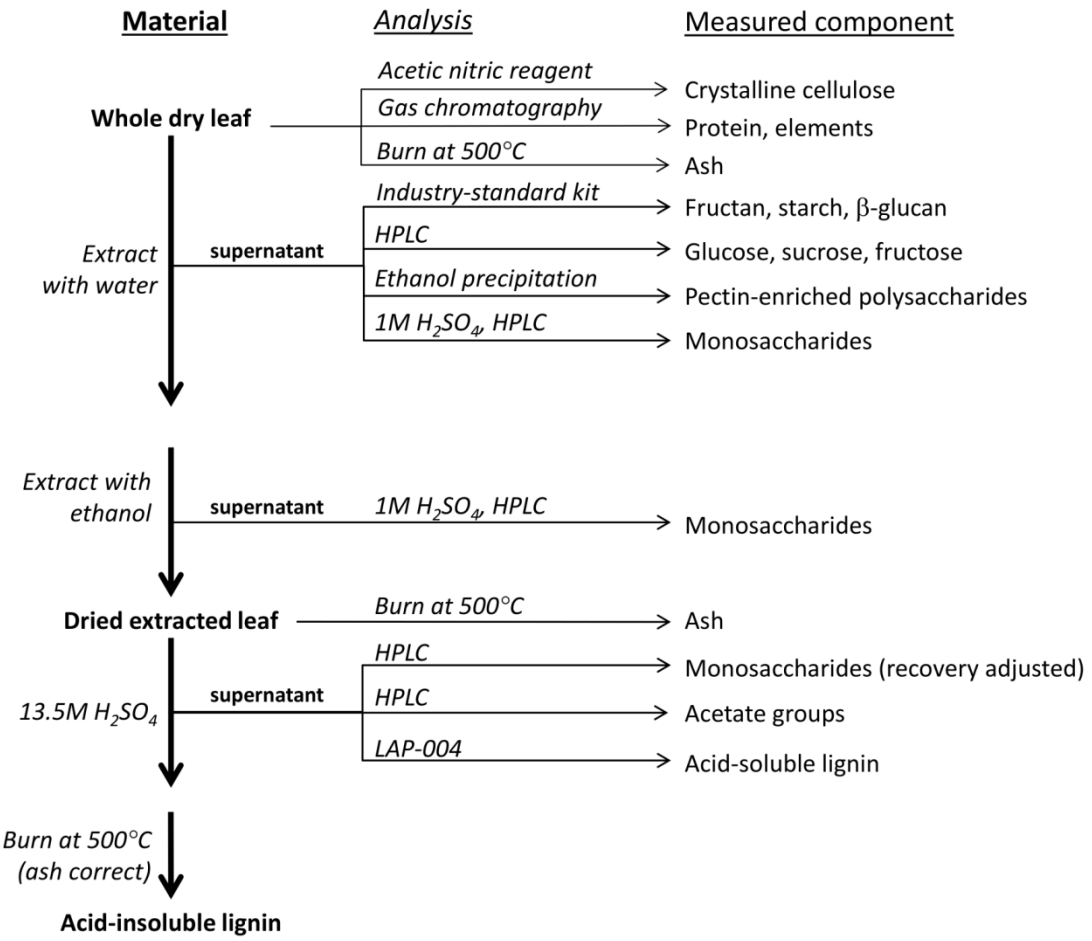

Figure P1-1 Flowchart outlining the steps taken to process and analyze *Agave* leaves

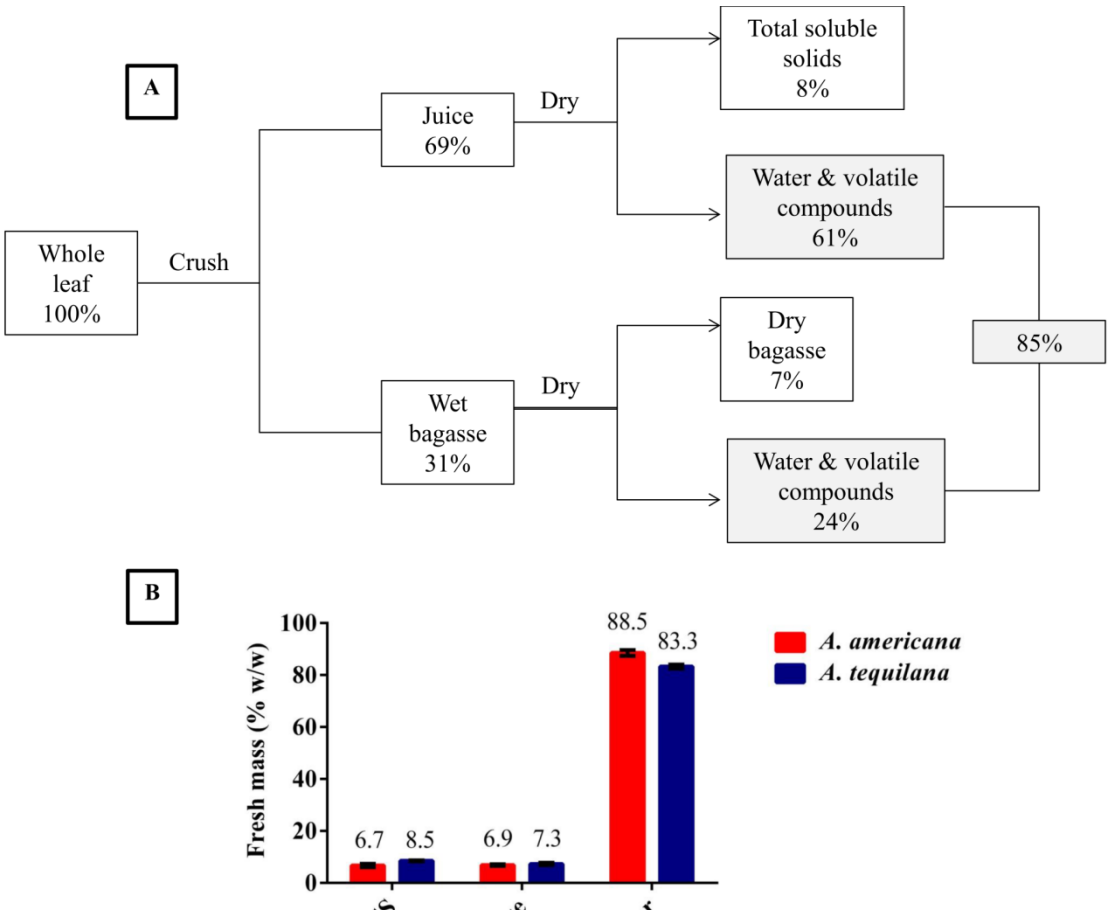

Figure P1-2 *Agave* processing and moisture content

Whole leaves were crushed, producing juice and wet bagasse fractions (a). These fractions were dried separately to calculate moisture content. Data is presented as percentage of fresh (wet) starting mass (% w/w). The values shown in gray are used to calculate total moisture content. The distribution of leaf fresh mass (% w/w) in *A. americana* and *A. tequilana* (b).

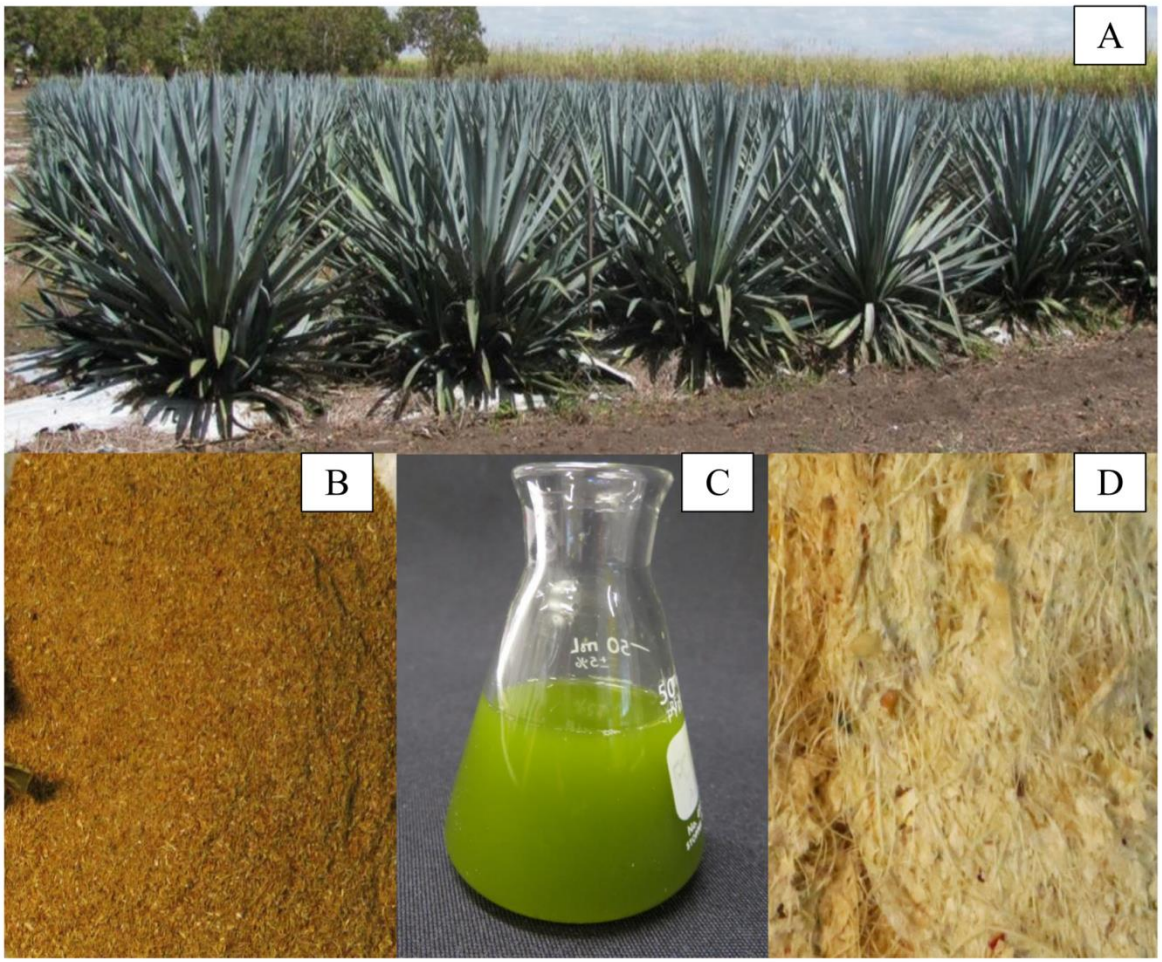

Figure P1-3 Different fractions of *Agave* material

Two year old *A. tequilana* plants in Australia (a). Partially dried leaves reduced to smaller particle sizes using a ball mill (b). Juice extracted from leaves using an experimental shredder (c). Dried fibers after extraction from wet bagasse (d).

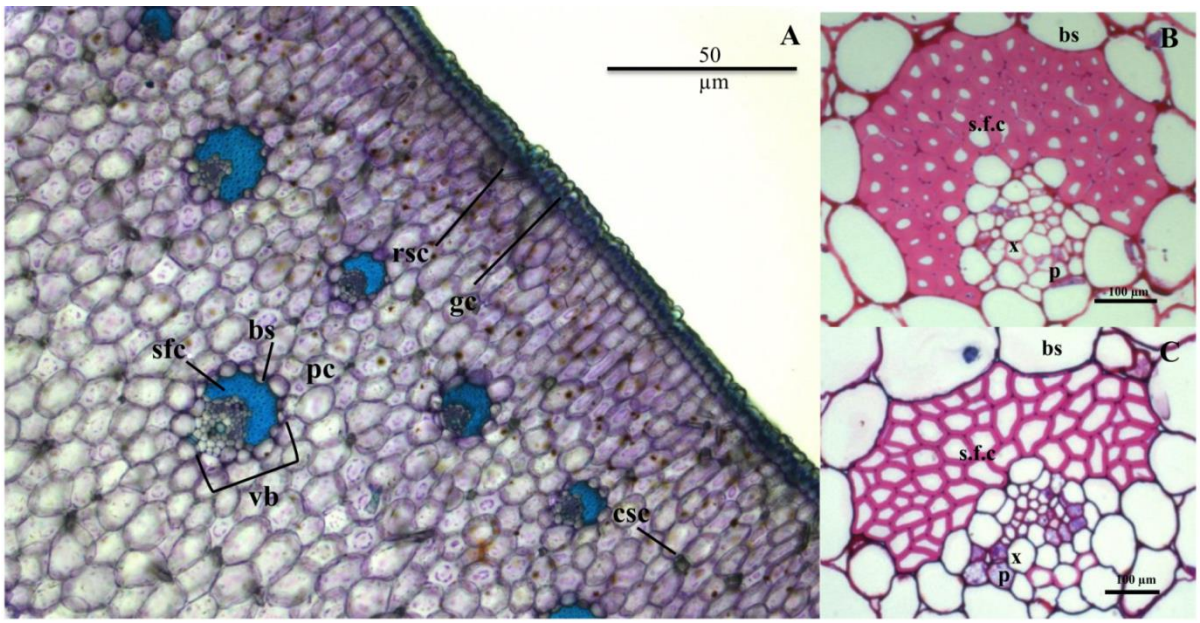

Figure P1-4 *Agave* leaf morphology

Transverse section of *A. tequilana* leaf stained with toluidine blue (a). Crystals are situated at the junction between some parenchyma cells within the tissue and at the site of stomata at the epidermis. Vascular bundles and fibers in *A. tequilana* (b) and *A. americana* leaf (c) stained with basic fuchsin. Sclerenchymatous fiber cap (sfc); bundle sheath (bs); parenchyma cells (pc); guard cells (gc); cubic shaped crystals (csc); rod shaped crystals (rsc); vascular bundle (vb).

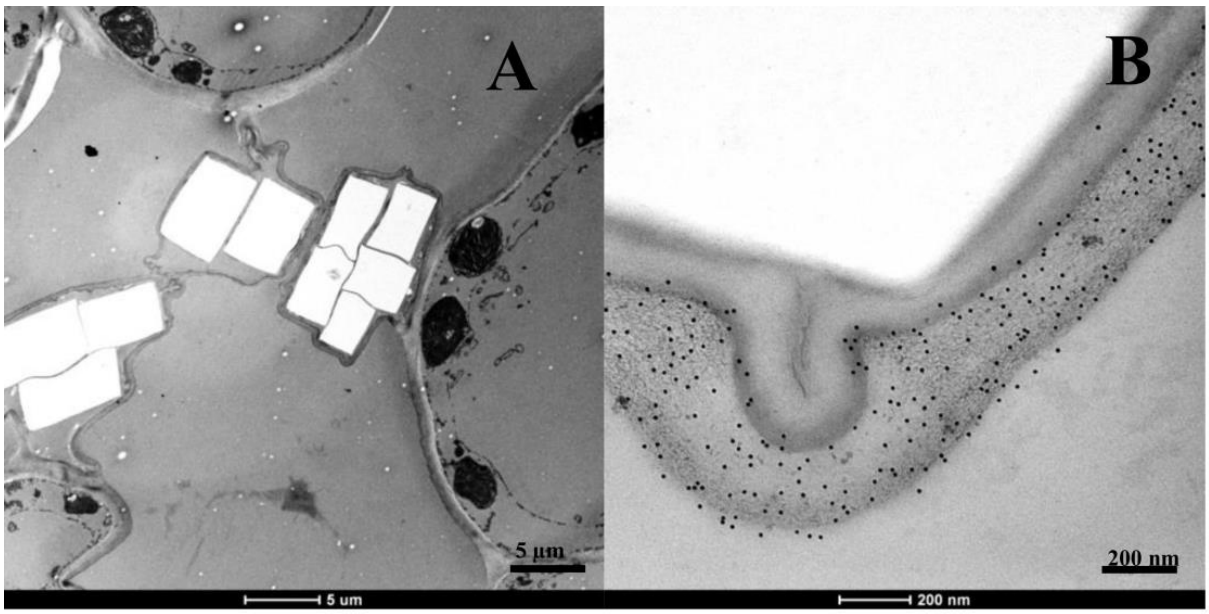

Figure P1-5 *Agave* tissue has pectinaceous crystal clusters localized at cell junctions

Transmission electron microscopy (TEM) image of crystals between junctions of cells (a). Labeling of methyl-esterified homogalacturonan (pectin) with LM20, was identified in outer sheath of the crystals (b).

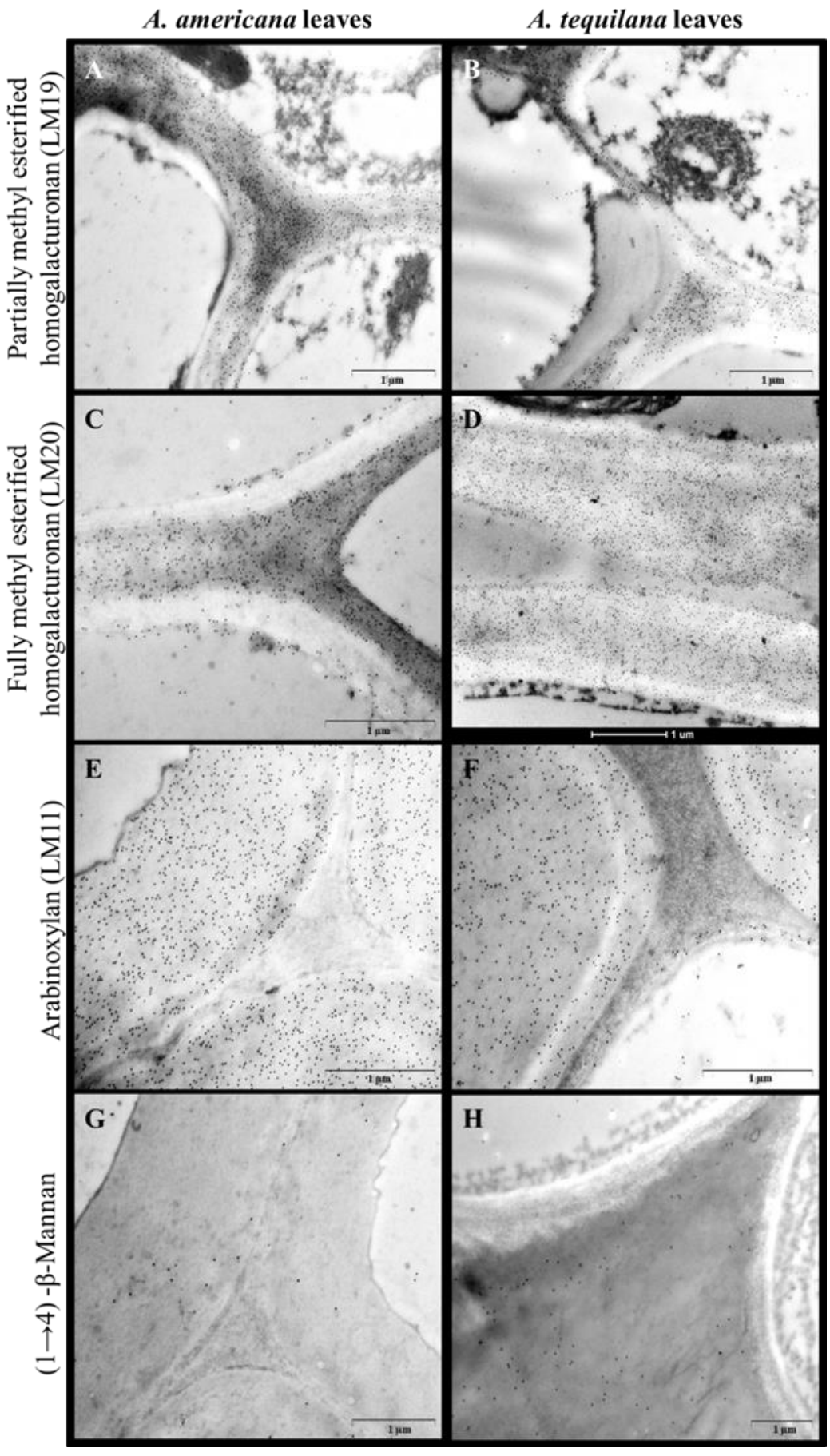
A. americana leaves
A. tequilana leaves
Partially methyl esterified homogalacturonan (LM19)
Fully methyl esterified homogalacturonan (LM20)
Arabinoxylan (LM11)
(1→4) -β-Mannan
A
B
C
D
E
F
G
H
1 µm
1 um

Figure P1-6 Cell wall polysaccharides detected by immunolabelling and transmission electron microscopy

Xylem tissue labeled with LM19, an antibody for partially methyl-esterified homogalacturonan (a-b) (pectin, [44]). Parenchyma cells labeled with LM20, an antibody for methyl-esterified homogalacturonans (c-d) [44]. Phloem tissue labeled with LM11 indicating the presence of arabinoxylan [42] (e-f). Leaf inner epidermal cells labeled with an antibody for (1→4)-β-mannan indicating the presence of mannan (g-h) [43]. Scale bars = 1µm.

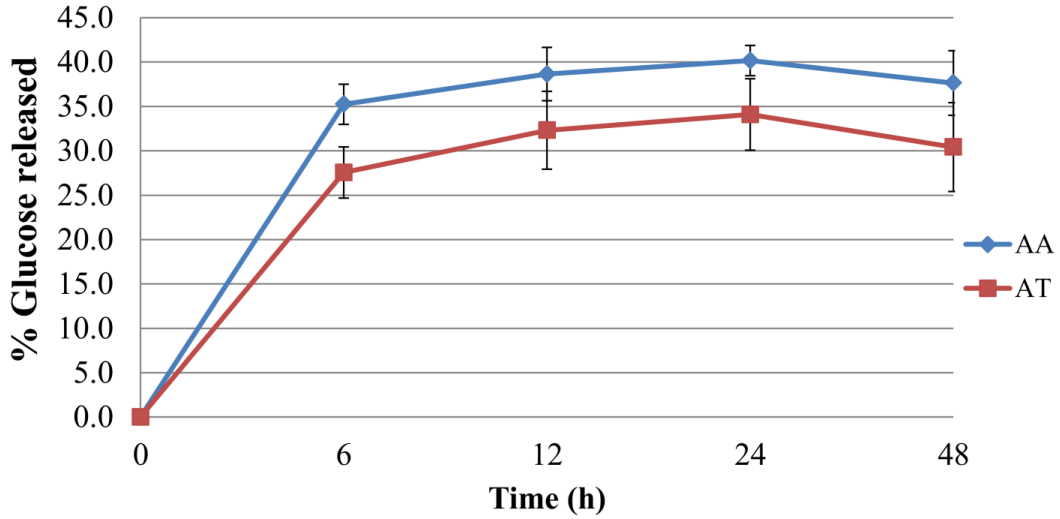

Figure P1-7 Cellulose, the most predominant polymer in *Agave* leaf tissue is degraded by cellulases

Liberation of the monomer glucose in the alcohol insoluble residue of *A. americana* (AA) and *A. tequilana* (AT) was measured over 48 h. The rate of saccharification is expressed as a percentage of cellulose converted into glucose (n=3).

A

Tissue	Type of juice	Fructose (g/L)	Glucose (g/L)	Sucrose (g/L)	Total (g/L)
A. americana leaves	Raw	13.0 ± 1.3	22.7 ± 3.3	3.3 ± 0.7	**39.0**
	Treated	20.8 ± 4.0	26.9 ± 4.7	–	**47.7**
A. tequilana leaves	Raw	10.0 ± 0.4	17.7 ± 0.2	2.3 ± 0.1	**30.0**
	Treated	20.2 ± 1.6	20.9 ± 2.9	–	**41.1**
A. tequilana stem	Raw	9.4 ± 0.6	15.1 ± 0.4	14.2 ± 0.1	**38.7**
	Treated	71.2 ± 5.6	32.8 ± 2.1	–	**104**

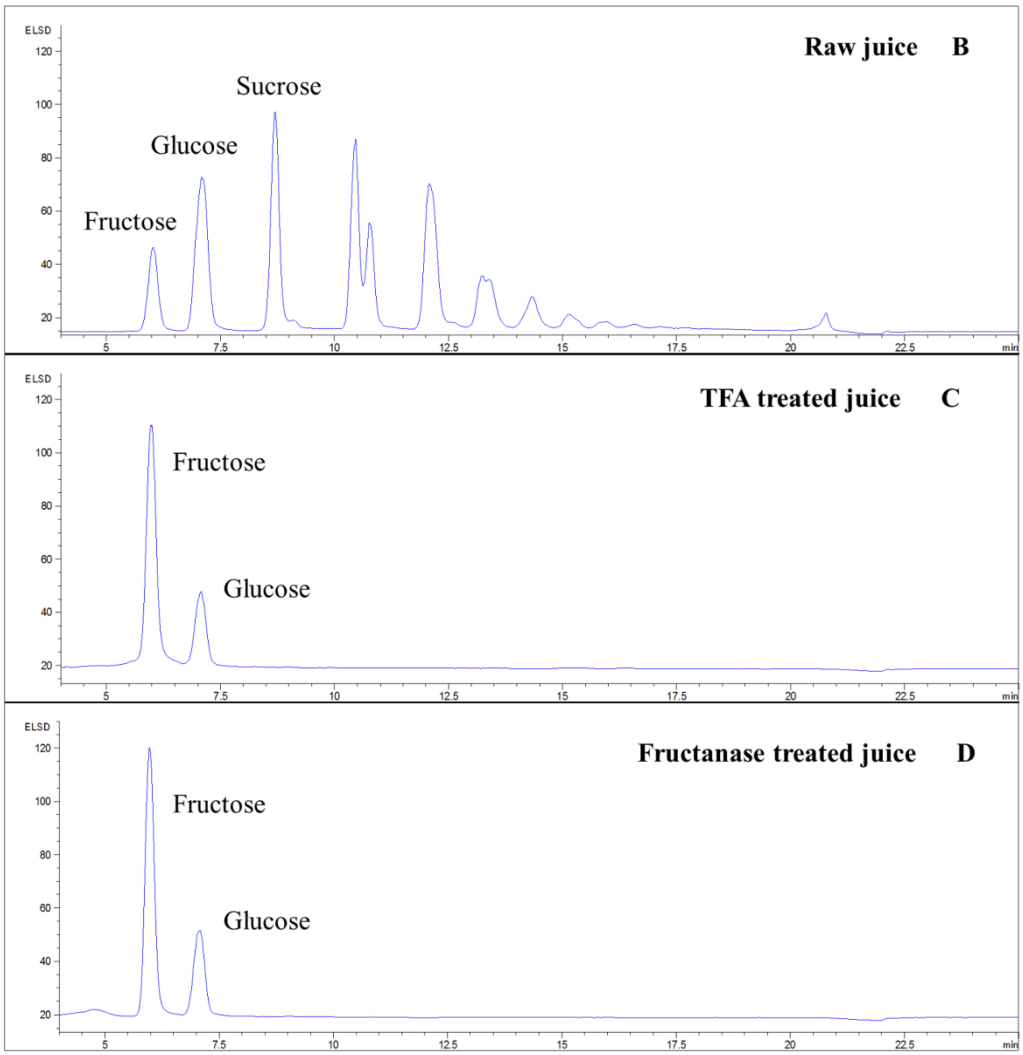

Figure P1-8 Quantification of juice sugars from *A. americana* leaves and *A. tequilana* leaves and stem

The amount of glucose, fructose and sucrose present in both raw and TFA-treated juice samples (a). Data are presented as g/L. Additional peaks for which there are no known standards were detected in the chromatograms of raw juice (b). *A. tequilana* stem juice is used as a representative of all three, very similar, chromatograms for the raw and treated samples. Chromatogram of TFA-treated *A. tequilana* stem juice (c). Chromatogram of fructanase-treated *A. tequilana* stem juice (d).

Table P1-1 Comparison of potential biofuel feedstocks

Species	**Common name**	**Tissue**	**Cellulose** (% w/w)	**Non-cellulosic polysaccharides** (% w/w)	**Lignin** (% w/w)
Zea mays	Corn	Stover without cobs	31–38	19–25	17–21
Triticum aestivum	Wheat	Whole plant	33	23	17
***Saccharum* spp.**	Sugarcane	Bagasse	32–43	12–26	23–28
Sorghum bicolor	Sorghum	Whole plant	23	14	11
Panicum virgatum	Switchgrass	Whole plant	30–35	24–28	17–20
***Populus* spp.**	Hybrid poplar	Whole tree without leaves	41–43	17–20	24–28
***Agave* spp.**	Agave	Whole residue from tequila brewing	31	17	17

Cellulose is the major source of glucose in feedstocks. Non-cellulosic polysaccharides contribute some fermentable hexose (glucose and galactose) and pentose (xylose and arabinose) sugars. Lignin is considered an inhibitory compound in cell wall degradation and fermentation. Data are presented as percentage of dry weight (% w/w). Data may be accessed through the United States Department of Energy, Energy Efficiency & Renewable Energy, Biomass Feedstock Composition and Property Database, 2013 [5].

Table P1-2 Composition of *A. americana* and *A. tequilana* leaves

	A. americana **(% w/w)**	***A. tequilana*** **(% w/w)**
Soluble extracts	**55.5 ± 2.9**	**45.8 ± 2.5**
*WSC	29.1 ± 5.9	15.3 ± 3.0
Glucose	13.5 ± 3.6	4.6 ± 0.8
Fructose	7.8 ± 1.4	2.8 ± 0.6
Fructan	3.4 ± 2.5	4.9 ± 2.5
Sucrose	4.4 ± 0.5	3.0 ± 1.1
*Polysaccharides	4.0 ± 0.2	12.6 ± 1.1
Hydrolyzed monosaccharides	2.2 ± 0.3	2.4 ± 0.2
Ethanol-insoluble (pectin-enriched)	1.8 ± 0.4	10.2 ± 1.1
*Ethanol-soluble monosaccharides	6.0 ± 1.6	1.3 ± 0.2
Ash (non-structural inorganics)	*6.4 ± 1.4*	*15.1 ± 1.6*
Other	*10.0*	*1.5*
Insoluble components	**44.5 ± 2.9**	**54.1 ± 2.5**
*Monosaccharides	21.3 ± 1.7	26.1 ± 3.6
Glucose	12.0 ± 1.8	16.4 ± 2.3
Starch	5.7 ± 1.4	1.4 ± 0.3
Xylose	2.9 ± 0.7	4.4 ± 0.7
Galacturonic acid	2.8 ± 0.2	3.1 ± 0.7
Galactose	2.7 ± 0.6	1.4 ± 0.1
Arabinose	0.9 ± 0.1	0.8 ± 0.1
Lignin	8.3 ± 0.9	12.7 ± 1.1
Acid-insoluble	4.3 ± 1.0	9.1 ± 1.4
Acid-soluble	4.0 ± 0.7	3.6 ± 0.3
Protein	6.2 ± 2.0	5.8 ± 0.7
Acetate groups	1.0 ± 0.2	0.7 ± 0.2
Ash (structural inorganics)	2.1 ± 1.0	5.5 ± 1.1
Other	*5.6*	*3.3*

The soluble extracts and insoluble residue, comprising structural carbohydrates and other cell wall components, were quantified (n=6). Data are presented as percentage of dry weight (% w/w). * indicates the values used to calculate total sugar content: 60.4% w/w for *A. americana* and 55.3% w/w for *A. tequilana*. Italics indicate values derived from calculation rather

than direct measurement. Components of ‘Other’ (otherwise unaccounted for mass) are likely to be lipids, waxes and organic acids in the soluble fraction or unhydrolyzed crystalline cellulose and pectin in the insoluble fraction.

Table P1-3 Polysaccharides detected by linkage analysis in *Agave* leaf

Polysaccharide	***A. americana*** **(mol %)**	***A. tequilana*** **(mol %)**
Arabinan	5.5	4.7
Type I arabinogalactan	7.4	2.3
Type II arabinogalactan	2.4	1.5
Arabinoxylan	13.4	16.4
Cellulose	31.9	45.3
Heteromannan	6.6	6.0
Homogalacturonan	17.6	6.5
Rhamnogalactan I/II	0.7	0.3
Xyloglucan	10.6	12.7
Unassigned	3.9	4.3
Total	**100.0**	**100.0**

Polysaccharides detected in alcohol-insoluble residues (AIR) of *A. americana* and *A. tequilana* leaves (n=3). Data are presented as relative percent molarity (mol %). Individual linkages were classified as described in Table P1-S1. Unassigned linkages include the linkages measured where the polysaccharide of origin was not clear.

Table P1-4 Carbohydrates in fiber-enriched fractions from *Agave* leaves

Component	***A. americana*** **(% w/w)**	***A. tequilana*** **(% w/w)**
Crystalline cellulose	47.2 ± 2.3	49.5 ± 1.9
Non-cellulosic polysaccharides	22.4 ± *0.8*	15.8 ± 1.3
Arabinose	*0.6 ± 0.1*	*0.3 ± 0.1*
Glucose	*8.6 ± 0.3*	*2.7 ± 0.6*
Xylose	*9.4 ± 0.9*	*11.4 ± 1.0*
*Other monosaccharides**	*3.8 ± 0.1*	*1.4 ± 0.1*

Data are presented as a percentage of dry weight (% w/w). *Includes mannose, rhamnose, glucuronic acid, galacturonic acid and galactose.

Table P1-5 Fermentation of *Agave tequilana* juice using *Saccharomyces cerevisiae*

	Ethanol yield (96 hr)		
***S. cerevisiae* strain**	**Yield** (g/L)	**Conversion** (% of total sugars)	**Conversion** (% of monomers)
139	11.4 ± 0.6	54%	74%
636	13.8 ± 0.5	66%	90%

Two strains of *S. cerevisiae* were used to ferment untreated *Agave* leaf juice with a starting sugar concentration of 41.4 g/ L and WSC concentration of 30.0 g/L. Conversion efficiencies are based on a maximum conversion rate of sugar to ethanol of 51.1% w/w.

Table P1-6 Theoretical ethanol yields for lignocellulosic feedstocks

Biomass	**Source of sugars**	**Ethanol yield** (L/t)	**Productivity** (t/ha/yr)	**Ethanol yield** (L/ha/yr)
Corn	Stover without cobs	362−456[*]	3[18]	1086−1369
Wheat	Straw	406[*]	2.6[85]	1055
Sugarcane	Bagasse	318−500[*]	10[18]	3179−4996
Sorghum	Whole plant	268[*]	24−32.5[86,87]	6430−8708
Switchgrass	Whole plant	392−457[*]	5.2−23[88,89]	2036−10508
Poplar	Whole tree, no leaves	419−456[*]	5−11[18]	2096−5011
Agave	Whole residue	347[*]	10−34[18]	3474−11811
A. americana	Whole plant, extrapolated from leaf sugar content	437[^]	10−34[18]	4368−14851
A. tequilana	Whole plant, extrapolated from leaf sugar content	401[^]	10−34[18]	4009−13636
A. tequilana leaves	Whole leaf	401[^]	5.7−19[#]	2273−7728
A. americana leaves	Juice[†]	34[^,‡]	34−115.7[‡]	1165−3961
A. tequilana leaves	Juice[†]	30[^,‡]	23.4−79.7[‡]	691−2350

*****Calculations were based on the compositional values listed in Table P1-1 [5]. [^]Calculations based on data obtained in this study. [#]Assumes that 56.7% dry w/w of the whole 3 year old plants is leaf material [8]. [†]Assumes that juice accounted for 69% of plant wet weight; *A. americana* leaf was 88.5% w/w water; and *A. tequilana* leaf was 83.3% w/w water. [‡] Tonnes of wet weight rather than dry weight. Units for data are given in table headings. Constants for ethanol calculations are consistent with the National Renewable Energy Laboratory Theoretical Ethanol Yield Calculator [73]: 1.111 kg

monomeric C6 sugar per 1 kg polymeric C6 polymer (glucan, fructan); 1.1363 kg monomeric C5 sugar per 1 kg polymeric C5 polymer (xylan, arabinan); 0.51 kg of ethanol produced from 1 kg of sugar; 0.7892 g/L relative density of ethanol. Productivity per hectare is based on previous studies [18,74-78].

Supporting Information

Table P1-S1 Monosaccharide linkage analysis data for *Agave* leaves (mol %)

Polysaccharide	Derivative linkage	*A. americana* (mol %)	*A. tequilana* (mol %)
Arabinan	*1,5 - Ara (f)* *1,2,5- Ara (f)*	4.8 ± 0.8 0.7 ± 0.2	4.4 ± 0.2 0.3 ± 0.2
Type I arabinogalactan	*1,4-Gal (p)* *1,4,6-Gal (p)*	6.5 ± 2.1 0.9 ± 0.5	2.3 ± 0.7 0.0
Type II arabinogalactan	*1,6-Gal (p)* *1,3,6-Gal (p)* *t-Gal*	0.2 ± 0.1 1.1 ± 0.4 1.1 ± 0.2	0.2 ± 0.0 0.7 ± 0.3 0.7 ± 0.2
Glucuronarabinoxylan	*1,4-Xyl (p)* *1,2,4-Xyl (p)* *1,3,4-Xyl (p)* *1,2,3,4-Xyl (p)* *t-Ara*	9.9 ± 2.3 0.6 ± 0.2 0.2 ± 0.1 0.6 ± 0.4 2.1 ± 1.2	11.6 ± 2.0 1.1 ± 0.2 0.5 ± 0.2 1.2 ± 0.6 1.9 ± 0.2
Cellulose	*1,4-Glc(p)*	31.9 ± 2.1	45.3 ± 4.2
Heteromannan	*1,4-Man (p)* *1,4-Glc (p)*	3.3 ± 0.3 3.3 ± 0.4	3.0 ± 0.3 3.0 ± 0.3
Homogalacturonan	*1,4-Gal A (p)* *t-Gal A (p)*	16.8 ± 1.1 0.9 ± 0.4	5.9 ± 1.9 0.6 ± 0.1
Rhamnogalactan I/II	*1,2,4-Rha (p)*	0.7 ± 0.3	0.3 ± 0.0
Xyloglucan	*1,4,6-Glc (p)* *1,4-Glc (p)* *1,2-Xyl (p* *1,2-Gal (p)* *t-Fuc (p)* *t-Xyl (p)*	3.1 ± 0.3 3.1 ± 2.1 1.1 ± 0.3 0.7 ± 0.1 0.7 ± 0.5 2.0 ± 0.6	3.7 ± 0.4 3.7 ± 4.2 1.3 ± 0.2 0.6 ± 0.1 0.9 ± 0.3 2.6 ± 0.2

Analysis completed on alcohol insoluble residues (AIR). Data are presented as relative percent molarity (mol %).

Table P1-S2 Elemental analysis of *Agave* juice and whole leaf

Element	Whole leaf		Juice	
	A. americana[2] (mg/kg)	*A. tequilana*[2] (mg/kg)	*A. americana*[2] (mg/kg)	*A. tequilana*[1] (mg/kg)
Aluminium (Al)	5	24	0	1
Calcium (Ca)	16733	3400	873	3800
Iron (Fe)	36	70	1	47
Magnesium (Mg)	10667	10007	883	1190
Phosphorus (P)	1073	4767	54	720
Potassium (K)	20700	20400	2033	2500
Sodium (Na)	20	81	4	17
Sulfur (S)	657	693	42	72
Zinc (Zn)	18	21	4	6
Total	**49909**	**39463**	**3895**	**8353**

Data are presented as mg/kg of material. [1]Average of two biological replicates. [2]Average of three biological replicates.

11.2 Paper II: Highlights

The chemical composition of white and red grape marc was determined

Marc carbohydrates were characterized using enzymes, HPLC and MALDI-TOF-MS

Dilute acid pre-treatments liberated glucose more efficiently than thermal treatments

White marc contains 40% fermentable water-soluble carbohydrates

Theoretical ethanol yields were calculated based on chemical composition

Statement of Authorship

Title of Paper	Grape marc as a source of carbohydrates for bioethanol: chemical composition, pre-treatment and saccharification
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Kendall Corbin
Contribution to the Paper	Completed the majority of the experimental work: including compositional analysis, pre-treatment and saccharifications. Data analysis and wrote majority of the manuscript.
Signature	Date: 13/05/15

Name of Co-Author	Seth DeBolt
Contribution to the Paper	Helped to evaluate and edit the manuscript and saccharification efficiency
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Name of Co-Author	Yves SY Hsieh
Contribution to the Paper	Preparation of samples, interpreted data
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Name of Co-Author	Natalie Betts
Contribution to the Paper	Supervised development of work Helped interpret data Edited and helped evaluate manuscript
Signature	Date: 8/5/15

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Contribution to the Paper	Supervision, concept development, help drafted manuscript
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Grape marc as a source of carbohydrates for bioethanol: chemical composition, pre-treatment and saccharification

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Abstract

Global grape production could generate up to 13 Mt/yr of wasted biomass. The compositions of Cabernet Sauvignon (red marc) and Sauvignon Blanc (white marc) were analyzed with a view to using marc as raw material for biofuel production. On a dry weight basis, 31−54% w/w of the grape marc consisted of carbohydrate, of which 47−80% was soluble in aqueous media. Ethanol insoluble residues consisted mainly of polyphenols, pectic polysaccharides, heteroxylans and cellulose. Acid and thermal pre-treatments were investigated for their effects on subsequent cellulose saccharification. A 0.5 M sulfuric acid pre-treatment yielded a 10% increase in the amount of liberated glucose after enzymatic saccharification. The theoretical amount of bioethanol that could be produced by fermentation of grape marc was up to 400 L/t. However, bioethanol from only soluble carbohydrates could yield 270 L/t, leaving a polyphenol enriched fraction that may be used in animal feed or as fertilizer.

Keywords: bioethanol; grape marc; polysaccharide; pre-treatment; saccharification

Abbreviations

A:X; arabinose:xylose ratio; AIR: alcohol-insoluble residue; Ara: arabinose; ASE: accelerated solvent extractor; CDTA: Cyclohexane-1,2,-diamine tetraacetate; Fuc: fucose; Gal: galactose; GalA: galacturonic acid; Glc: glucose; GlcA: glucuronic acid; HILIC: hydrophilic interaction chromatography; HMF: 5-(Hydroxymethyl)furfural; HPLC: high-performance liquid chromatography; Man: mannose; mol%: relative percent molarity; MS: mass spectrometry; Na_2CO_3: sodium carbonate; NCPs: noncellulosic polysaccharides; Rha: rhamnose; WSC: water soluble carbohydrates; Xyl: xylose

Introduction

The carbohydrates in plant biomass can be used as a raw material for producing liquid biofuels and valuable biochemicals. However, plant material is heterogeneous and recalcitrant to degradation. Further, the carbohydrates may take a variety of chemical forms, including polysaccharides, oligosaccharides, monosaccharides, or form glyco-conjugates like glycoproteins and glycolipids. To overcome the structural complexity of plant biomass, research has focused on modifying plants to make the carbohydrates more available [1]. Alternatively, physical or chemical pre-treatments combined with enzymatic hydrolysis have been used to facilitate the liberation of fermentable sugars [2]. However, these additional processing steps are energetically and financially costly, and potentially create bottlenecks in large scale production [3]. Thus the need to identify dedicated biomass sources that are structurally favorable in their native form, require negligible processing, and are socially and environmentally advantageous is essential to the progression and prosperity of the biofuels industry.

One way to circumvent the issues imposed by using classic lignocellulosic feedstocks is to identify waste materials that are produced in abundance, have been accumulated for other uses, can be sourced cheaply and have carbohydrates in a form amenable to fermenting microorganisms. One such potential source of raw material is grape marc. Grape marc (or pomace) is the waste that remains after the juice is collected from the pressing of grapes for wine production; it includes grape skin, pulp, seeds, stems and residual juice. The composition of grape marc is related to the grape variety, the method of processing, environmental conditions and the ratio of skin:seeds:stem.

The global production of grapes is 67.1 Mt/yr with China, the United States of America and Italy being the leading producers [4]. It has been estimated that 18–20% of

the grapes used for wine making remains as waste marc, which could generate up to ~13.4 Mt (fresh weight) of waste biomass [5]. Currently, the global accumulation of grape marc is lower at 4.8 Mt/yr (average of last ten years; [4]) and is considered to have limited economic value. Grape marc is generally disposed of at a cost to the winery but may be utilized as filler in livestock feed, fermented to make the alcoholic beverage grappa, reapplied as fertilizer or used as a source of phenolic compounds.

In recent years, grape marc has been proposed as a raw material for bioenergy production [6] and has been used to generate butanol and biogas [7,8]. Grape marc may also be used to produce biofuel at the winery site and the evolution of local biofuel processing plants will obviate costs associated with transport of the biomass. However, further information about the composition of grape marc is required to allow estimates to be made of biofuel yields and hence facilitate cost-benefit analyses of different types of marc for biofuel production.

Here the composition–of grape marc derived from two grape varieties, Cabernet Sauvignon and Sauvignon Blanc was investigated. Various pre-treatments were analyzed for their effect on depolymerisation of non-cellulosic polysaccharides, and the residual cellulosic biomass was subsequently hydrolyzed using crude cellulase preparations. Finally, the chemical composition of grape marc was compared with other known agro-industrial waste materials, and these data were extrapolated to estimate theoretical ethanol yields.

Material and Methods

Collection and preparation of plant material

Material was collected after the pressing of Cabernet Sauvignon and Sauvignon Blanc grapes in both April 2012 and March 2013 at the University of Adelaide Waite Campus vineyards (Adelaide, Australia), n=2 for each variety. Amounts of 30 g (three

technical replicates) were separated into seed, skin and stem fractions. The weight of each fraction was recorded and the mass distribution calculated.

Whole grape marc was lyophilized (Labconco-Freezone, Kansas City, MO, USA) to determine moisture composition. Lyophilized grape marc was ground in a Retsch mill MM400 (Retsch GmbH; Haan, Germany) at 30 Hz for 3 min for compositional analyses.

Extraction and analysis of soluble and insoluble components

Grape marc samples were extracted with three water cycles followed by three 95% v/v ethanol cycles at 100°C using an Accelerated Solvent Extractor (ASE300, Dionex) [9]. Briefly, ASE (accelerated solvent extractor) stainless steel extraction cells (11 mL) were fitted with dried (105°C) and pre-weighed Whatman GF/C 55 mm glass microfiber filters (Sigma-Aldrich, United States) and 1 g dried marc was added. Extraction settings were modified to 60 s nitrogen purges following extraction, 5 min static time and 120% rinse volume. Following extraction, the biomass was dried overnight at 105°C. The percentage of material extracted was calculated based on the difference between the initial weight (before the water and ethanol extractions) and final weight (after extraction).

The carbohydrates and lignin content in the ASE extracted biomass was quantified according to [10]. Briefly, the material (30 mg) was incubated with 13.5 M H_2SO_4 at room temperature for 1 h, followed by dilution to 0.75 M H_2SO_4 and autoclaving at 121°C (Tuttnauer 3850 ELC Benchtop Sterilizer). The solubilized material and the residual biomass were separated by centrifugation for 10 min at 10 000 g. The supernatant was analyzed for monosaccharides [11]. A sugar recovery standard for monosaccharides was carried through the acid hydrolysis to compensate for degradation of monomers during the hydrolysis step. The acetate content in the supernatant was analyzed at 60°C using an Aminex HPX-87H column (300 × 7.8 mm) (Bio Rad; California, United States) on an 1100 series liquid chromatography instrument. Elution was performed isocratically with

2.5 mM H_2SO_4 at a rate of 0.5 mL/min. The residual biomass was assayed for acid-insoluble lignin [10].

Small scale extractions of grape marc were conducted to determine the water soluble carbohydrate content. Briefly, 50 mg of dried, ball milled grape marc was extracted with equal volumes (1.0 mL) of water, 95% v/v ethanol, and 70% v/v ethanol. Extraction for each solvent was conducted at 80°C for 15 min followed by centrifugation at 10 000 g for 10 min and the supernatant collected. Glucose and fructose in water extracts were measured by hydrophilic interaction chromatography (HILIC), using a Prevail Carbohydrate ES column (150 × 4.6 mm) (Alltech; Illinois, United States) on an Agilent 1200 series liquid chromatography instrument equipped with an evaporative light scattering detector (Alltech ELSD 800). The mobile phase conditions were modified from those used by [12] and consisted of water (A) and 90% acetonitrile (B) at a flow rate of 1.0 mL/min at 20°C. Soluble pectins in the water extracts were precipitated and quantified according to [13]. Additional monosaccharides present in the water and ethanol extracts were analyzed using HPLC [11], following centrifugal evaporation (Savant SC110 Speed Vac, Thermofisher; Massachusetts, United States) and hydrolysis using 1 M sulfuric acid (H_2SO_4) for 3 h at 100°C.

Starch was measured in samples using a commercial assay (Total Starch-Megazyme: AOAC Method 996.11; International Ireland Ltd., Wicklow, Ireland), following the method for samples that contain glucose and/or maltodextrins. The elemental content of ball milled grape marc (300 mg) was measured using a closed tube nitric acid/hydrogen peroxide digest and radial view inductively coupled plasma-optical emission spectrometry method [14]. The nitrogen value was converted to an estimate of the protein content using the nitrogen factor (NF) 6.25.

Isolation of polysaccharides

Raw grape marc was extracted overnight at 37ºC with 70% aqueous ethanol. The homogenates were filtered using Miracloth (Calbiochem), and alcohol-insoluble residues (AIRs) were washed with 70% v/v ethanol and 100% v/v acetone. The AIR material was collected after drying at room temperature overnight under constant airflow. To isolate polysaccharides from the marc, the AIR material was fractionated by sequential extraction (Figure P2-1) with 50 mM CDTA (pH 6.5) (cyclohexane-1,2,-diamine tetraacetate) and Na_2CO_3 (sodium carbonate) + 25 mM $NaBH_4$ (sodium borohydride) according to [15]. Both of these extraction steps were used to extract pectic polysaccharides. Next, 1 M KOH + 25 mM $NaBH_4$ was used to release heteroxylans and other non-cellulosic cell wall polysaccharides. Finally, 4 M KOH + 25 mM $NaBH_4$ resulted in the extraction of a xyloglucan fraction. Following polysaccharide isolation, the fractions were dialyzed and lyophilized (Labconco-Freezone, Kansas City, MO, USA) and hydrolyzed with 1 M H_2SO_4 at 100°C for 3 h. The liberated monosaccharides were quantified by HPLC (Figure P2-1) [11].

Enzymatic hydrolysis and MS profiling

Three of the whole marc fractions were treated with different enzymes for cell wall analysis (Figure P2-1). The CDTA fraction (1 mg) was treated with *endo*-polygalacturonanase M2 (50U) from *Aspergillus aculeatus* (Megazyme, Ireland) with 100 mM NaOAc (pH 4) for 16 h at 37° C. The 1 M KOH fraction (1 mg) was treated with *endo*-1,4-β-xylanase M6 (45U) from rumen microbacteria (Megazyme) with 100 mM NaOAc (pH 6) for 16 h at 37°C. The 4 M KOH fraction (1 mg) was treated with recombinant xyloglucan-specific *endo*-β-glucanase (10U) from *Paenibacillus* spp (Megazyme) with 100 mM NaOAc (pH 5.5) for 16 h at 37°C, or *endo*-1,4-β-xylanase M6 (45U) (as described earlier). The enzymes were inactivated by boiling for 2 min.

The molecular weights of the sodium adducts of oligosaccharides $[M+Na]^+$ or $[M-H]^-$ were determined using a BioTOF UltraFlex II (Bruker Daltonics) mass spectrometer (MS), in positive or negative ion modes. The enzyme hydrolyzates containing oligosaccharides were mixed with 2,5-dihydroxybenzoic acid (10 mg/mL) and 10 mM NaCl in the ratio of 5:5:3 (v/v/v). In MS mode, the spectra were accumulated over an average of 2000 laser shots [16].

Crystalline cellulose

Crystalline cellulose was measured in non-pre-treated and pre-treated grape marc using a modified [17] method as described in [18]. For non-pre-treated samples, the soluble carbohydrates were removed prior to cellulose analysis: dried homogenized grape marc (50 mg) was incubated three times with 70% v/v ethanol at 55°C for 1 h. The material was washed with 1 mL acetone for 2 min at room temperature and dried overnight under vacuum.

The cellulose content was determined in triplicate using 5 mg of dry non-pre-treated grape marc or pre-treated grape marc boiled in 3 mL acetic-nitric reagent for 30 min. Samples were cooled to room temperature, centrifuged (2 500 rpm/10 min) and the supernatant discarded. The residual material was washed twice with 8 mL water, followed by 4 mL acetone, and dried under vacuum. To hydrolyze the cellulose, 1 mL H_2SO_4 (12.5 M) was added, and incubated for 1 h at room temperature. The liberated glucose content was quantified using the anthrone method outlined in [18] using a spectrophotometer (Thermo Fischer, Waltham, MA, USA) set at a wavelength of 620 nm. Filter paper (20 mg) was carried through the experiment as a control and a standard curve was calculated from known concentrations of glucose (0–50 µg). The cellulose content was calculated by multiplying the glucose value for each sample by the total reaction volume and the hydration factor of 0.9.

Pre-treatment conditions

Whole marc samples (1 g) were homogenized using an Arthur H Thomas Co Scientific grinder (Philadelphia, PA, USA) equipped with a 2 mm sieve. Dried, milled grape marc was added to acid or water in a 1:10 (solid:liquid) ratio. Pre-treatments at 100°C were conducted in an oven and pre-treatments at 121°C were completed in an autoclave at a maximum pressure of 210 kPa (Tuttnauer 3850 ELC Benchtop Sterilizer). The four different pre-treatment conditions were: 1) 1 h, 121°C, 0.5 M H_2SO_4; 2) 3 h, 100°C, 1 M H_2SO_4; 3) 1 h, 121°C, H_2O; 4) 3 h, 100°C, H_2O. Following pre-treatment, the slurry was cooled to room temperature and filtered using Whatman GF/C 55 mm glass microfiber filters (Sigma-Aldrich, United States). The undissolved biomass was washed to neutral pH, the cellulose content measured and the material was carried through the saccharification procedure. For comparison, non-extracted (raw, dried and ball milled) grape marc was carried through the NREL compositional analysis method [10].

Enzymatic saccharification

The enzyme activity of a Celluclast 1.5 L (cellulase from *Trichoderma reesei;* endoglucanase activity ≥ 700 units/g) and Novozyme 188 (cellobiase from *Aspergillus niger*; cellobiase activity ≥ 250 unit/g) (Sigma-Aldrich; St Louis, MO, USA) cocktail mixed 1:1 was measured according to [19]. Microscale saccharifications were completed using an enzyme concentration of 60 filter paper units and 0.02 g cellulose for non-treated (AIR) and pre-treated samples [18]. The glucose concentration was measured over 48 h using a Yellow Springs Instrument (YSI) glucose analyzer (Yellow Springs, OH, USA).

Assumptions for calculating theoretical ethanol yields

Standard constants for ethanol calculations were used: 1.111 kg monomeric C6 sugar per 1 kg polymeric C6 polymer (glucan); 1.1363 kg monomeric C5 sugar per 1 kg polymeric C5 polymer (xylan, arabinan); 0.51 kg of ethanol produced from 1 kg of sugar.

Results and Discussion

Characterization of grape marc raw materials and soluble carbohydrates

After the grapes are crushed and processed for winemaking, approximately 20% of the starting material remains as a moisture-dense waste material [5]. The composition of this heterogeneous material is affected by the percentage of seed, skin and stem in the total mass (Figure P2-2). For both varieties, the majority of the weight (up to 80% in Sauvignon Blanc grape marc, Figure P2-2) is attributable to grape skins, with seeds and stems present to a lesser degree. The moisture content of this waste material was 59−67% w/w (Figure P2-2).

The residual carbohydrates that remain in the marc after crushing the grapes are predominantly water soluble monosaccharides, oligosaccharides and polysaccharides and water insoluble structural polysaccharides from the cell wall. The soluble carbohydrates can be extracted with minimal energetic input and may be directly used as a raw substrate for fermentation, whereas cell wall polysaccharides need to be liberated through pre-treatment and saccharification. The water soluble carbohydrates (WSC) in white marc accounts for approximately one-third of the dry weight (37.6% w/w) and 70% of the total carbohydrate content (Table P2-1). Red grape marc has a much lower percentage of soluble carbohydrates, at 4.6% w/w. The comparatively low amount of WSC detected in the red marc may be due to the processing of the grapes during wine making, as red marc stays in contact with the juice for several days (maceration period) to enhance color and sensory attributes of the wine. During this period, the grapes are subjected to a mild, but prolonged, extraction in which phenolic compounds are released and carbohydrates are partially fermented.

Grape marc is also enriched in soluble pectins. Previous studies have shown that the galacturonan content in the mesocarp of grape berries increases from 26–46% of cell wall polysaccharides and becomes more soluble as ripening progresses [20]. Soluble pectins were quantified in the water extracts of the grape marc and contributed 4% and 9% w/w of the mass of Sauvignon Blanc and Cabernet Sauvignon, respectively (Table P2-1).

Cell wall polysaccharides: characterization, abundance and spatial distribution

Following the extraction (ASE) of soluble components, an insoluble residue (largely cell wall material) remained. This extracted material contains predominantly polysaccharides and the phenolic polymer lignin. The hydrolysis of the polysaccharides was achieved using concentrated sulfuric acid (13.5 M), resulting in liberated monosaccharides, and acetyl groups; the remaining biomass was classified as acid-insoluble lignin [10].

Monosaccharides liberated from the insoluble material represent a small proportion of the total mass for grape marc at 11−17% w/w (Table P2-1). Glucose (Glc) is the most predominant monosaccharide (5–6% w/w). Xylose (Xyl), galacturonic acid (GalA), mannose (Man), galactose (Gal) and arabinose (Ara) are detected to lesser extents. Red grape marc has a higher proportion of cell wall polysaccharides (53% of total carbohydrates measured) compared with white marc (20%) (Table P2-1).The structural complexity of polysaccharides impacts processing methods and costs, but monosaccharide profiling alone does not provide structural information about the polysaccharides present. Intact polysaccharides from the alcohol-insoluble grape marc residue (AIR) were therefore fractionated [15] and the relative proportion of monomers quantified (Table P2-2).

The CDTA and Na_2CO_3 pectic polysaccharide-enriched fractions contained predominantly GalA, Ara and Gal with a minor proportion of Rha. These components could be derived from pectic polysaccharide, such as rhamnogalacturonans. However, the

MALDI-TOF-MS analysis of the galacturonanase digest provided no molecular ion detection in the CDTA and Na_2CO_3 fractions (data not shown). The lack of detection for oligomers may be attributed to a highly branched rhamnogalacturonan structure in grape marc causing a steric hindrance of the enzyme, or alternatively, the size of digest fragments being above the MALDI-TOF-MS detection threshold. Also, higher levels of glucose and mannose were detected in the Cabernet Sauvignon fractions (Table P2-2), which may be attributed to contamination of Glc and Man enriched yeast cell wall polysaccharides, as the red marc has gone through a partial fermentation step.

The other two fractions (KOH) were enriched in heteroxylans and xyloglucans. The high abundance of Ara, Glc and Xyl in the 1 M KOH fraction was attributed to heterogenous polymers such as xyloglucan or arabinoxylan (Table P2-2). The Ara may have originated from arabinoxylan, in which case the arabinose:xylose (A:X) ratio for red marc is 1.3 and 0.7 for white marc. The variation in A:X ratio between varieties has been observed in previous studies, where the A:X ratio was 0.7 for Muscat Gordo Blanco and 1.3 for Ohanez grape mesocarp [21]. However, when 1 M KOH fractions were treated with *endo*-1,4-β-xylanase M6, no molecular ions were detected using MALDI-TOF-MS (data not shown). Xylanase M6 enzymatically cleaves between two consecutive unsubstituted xylose residues along the xylan backbone, thus the lack of cleavage indicates that these heteroxylans are highly substituted, consistent with the A:X ratios of 1.3 and 0.7. It is unlikely that (glucurono)arabinoxylan or glucuronoxylan is present in this fraction as no glucuronic acid (GlcA) was detected. Whereas in contrast, the 4 M KOH fractions releases two oligosaccharides with *m/z* 759 and *m/z* 1023, putatively assigned as $Xyl_4MeGlcA_1$ and $Xyl_6MeGlcA_1$, respectively (data not shown).

The polysaccharides isolated in the 4 M KOH fraction were composed of 52–56% mol xylose and 23% mol glucose, with lesser amounts of galactose, mannose, fucose and arabinose. These monosaccharides are characteristic of xyloglucans with the exception of

mannose, which may be an artifact from yeast mannan contamination [22]. Previous reports indicate the predominant non-cellulosic polysaccharide in grape berries are xyloglucans, accounting for 10% of the polysaccharides in grape berry mesocarp [21].

The xyloglucan extracted in the 4 M KOH fraction of the marc was treated with xyloglucan-specific *endo*-(1,4)-β-D-glucanase and analyzed using MALDI-TOF-MS for structural differences in xyloglucan fine structure [16]. The structure of the hydrolyzate products are similar to those found in xyloglucans from other dicots: XXG (*m/z* 791), XXGG (*m/z* 953), XXXG (*m/z* 1085); XLXG/XXLG (*m/z* 1247); XXFG (*m/z* 1393); XLLG (*m/z* 1409); XLFG (*m/z* 1555), where X represents the Xyl(α1,6)Glc unit, L represents Gal(β1,2)Xyl(α1,6)Glc, F represents Fuc(α1,2)Gal(β1,2)Xyl(α1,6)Glc and G represents the backbone Glc in β1,4-linkage [23].

The isolated grape marc xyloglucans examined here consisted predominantly of XXXG backbones with a lower proportion of XXGG and XXG oligomers, which may have been generated from XXXG during cell wall maturation and restructuring [22]. The backbones also contained the fucosylated oligomers XLFG and XXFG, with XLXG/XXLG and XLLG substituents present at a lower intensity.

Analysis of non-fermentable cell wall components in grape marc

Compared with other dedicated biofuel or waste materials, the amount of lignin present in grape marc is relatively high [24,25]. The acid- insoluble lignin content contributes 11% and 32% w/w of white and red marc mass, respectively (Table P2-1). However, the high lignin content of grape marc has been attributed to the presence of condensed tannins (22% dry matter in red marc) and resistant proteins present as insoluble protein-tannin complexes or as Maillard products [26]. Thus presence of these compounds could interfere with the quantification of insoluble lignin, resulting in overestimations, but may still be informative when making processing decisions.

In a biofuel context, mineral content is an important consideration, for example, specific elemental levels are essential in the maintenance and progression of fermentation as yeast cannot function effectively outside a narrow range of environmental conditions [27]. The total concentration of elements in red marc is 27% w/w higher than that measured in white marc. Potassium was detected in the highest abundance in both varieties and represented 75% w/w of the total elemental content (Table P2-3). Phosphorus, calcium, and sulfur are the next most abundant and collectively contribute an additional 20% w/w.

Comparison of acid and thermal pre-treatments

Pre-treatment is the initial step to convert lignocellulosic biomass from its native recalcitrant state into a form that can be more readily hydrolyzed by enzymes [2]. In this study, the effectiveness of four pre-treatments was assessed by comparison with grape marc hydrolyzed following [10].

Pre-treatment can generate a number of compounds inhibitory to fermenting organisms, such 5-(Hydroxymethyl)furfural (HMF; from hexose carbohydrate degradation) and formic or levulinic acids (from the breakdown of HMF) [28]. As inhibitory compounds were not quantified in this study, moderate pre-treatment conditions were chosen that would minimize the formation of these secondary byproducts [29]. The four pre-treatments conditions were: Acid/autoclave (1 h, 121°C, 0.5 M H_2SO_4); Acid/oven (3 h, 100°C, 1 M H_2SO_4); Water/autoclave (1 h, 121°C, H_2O; and Water/oven (3 h, 100°C, H_2O).

Glucose was the most abundant monosaccharide liberated in all pre-treatments (Table P2-4). Only acid treatments liberated other monomers, predominantly xylose and arabinose, from non-cellulosic polysaccharides (NCPs), consistent with previous studies in which heated water treatment was not severe enough to facilitate NCP solubilization [30]. The acid/autoclave treatment liberated the highest proportion of monosaccharides for both

varieties: 58% of the total carbohydrates measured using the NREL method were liberated from the red marc and 84% from the white marc (Table P2-4). The dilute acid (0.5 M) at higher temperature and pressure was the most effective pre-treatment.

There are advantages to using thermal rather than acid pre-treatments such as lower chemical costs, no need to neutralize the resultant hydrolyzate, and reduced production of inhibitory compounds [2]. However, there is a trade off in the increased energy consumption and lower recovery of fermentable sugars. In red marc, only 15−18% of the total glucose was liberated using thermal pre-treatments (2.1% w/w; Table P2-1) and 85−87% in white marc, indicating that the cellulose in white marc may be less structured and more amenable to hydrolysis. No NCPs were hydrolyzed using thermal pre-treatments, possibly due to the complexity of grape rhamnogalacturonan and xyloglucan. These data suggest that little or no cell wall breakdown occurred with thermal pre-treatments, rendering 35–93% of the carbohydrates in a structural, unfermentable form, thus significantly decreasing ethanol yields.

Effects of pre-treatment on the saccharification of cellulose

Pre-treatment not only liberates fermentable monomers but also weakens hydrogen bonding within and between glucan chains, which enhances the rate of polysaccharide breakdown into fermentable sugars in the presence of enzymes. Saccharification tests were performed on non-treated AIR, acid/autoclaved and water/autoclaved grape marc based on identical cellulose loadings following pre-treatment. The liberation of glucose from cellulose was monitored over 48 h of enzymatic digestion using a cellobiase preparation (cellobiase activity ≥ 250 units/g) from *Aspergillus niger* and a commercial cellulase cocktail (endoglucanase activity ≥ 700 units/g) isolated from *Trichoderma reesei*.

The acid/autoclave-treated grape marc exhibited the highest conversion of cellulose to glucose and for all pre-treatments, the amount of glucose released was higher for white

marc than for red marc at all time points (Figure P2-3). The greatest amount of glucose liberated was observed for acid/autoclave-treated Sauvignon Blanc samples, with 28% of cellulose hydrolyzed. Thermal pre-treatment did not increase the rate of glucose liberation from white marc compared with non-treated samples, with only 18% glucose present in the hydrolyzate (Figure P2-3a). Similar findings have been recorded for sweet sorghum bagasse, in which water did not significantly improve the rate of cellulose hydrolysis yield (11.8%) over 96 h compared with the control (12.6%) [31].

However, for red marc, the amount of glucose released was correlated to the severity of the pre-treatment, and both pre-treatments resulted in higher saccharification rates than untreated samples. The maximum glucose liberated from Cabernet Sauvignon was 17% in acid-treated samples, 15% in thermal-treated samples and 13% in non-treated samples over 48 h (Figure P2-3b).The slower and lower rate of glucose release observed in Cabernet Sauvignon samples compared to Sauvignon Blanc may be attributed to a higher ratio of fucosylated xyloglucan oligomers (XXFG; 1393 and XLFG; 1555) present in this tissue. The presence of fucosylated xyloglucans has been proposed to facilitate binding to cellulose, thus reinforcing the recalcitrant nature of the cell walls and making the cellulose polymers less accessible to enzymatic hydrolysis [32].

Overall, the acid pre-treatments were more efficient at degrading grape marc polysaccharides (Table P2-4) as well as modifying or weakening the hydrogen bonding of the crystalline cellulose polymers, making them more accessible to enzyme attack. Pre-treatments that increase the number of enzyme binding sites on cellulose microfibrils may increase biofuel yields, although studies have shown that saccharification may be a limiting factor when converting from small to large scale production [3]. As a result, it may be more economically favorable to identify and capitalize on the WSC present in dedicated sources of biomass for biofuel production, such as grape marc.

Theoretical ethanol yields from grape marc based on compositional data

The agro-industrial waste grape marc has been compared with other lignocellulosic waste materials based on global production, crop/residue ratio, chemical composition, and yield of ethanol per tonne (Table P2-5). On a dry w/w basis, 31% of the marc from Cabernet Sauvignon is carbohydrates (15% soluble and 17% insoluble) with more from Sauvignon Blanc at 54% w/w (43% soluble and 11% insoluble) (Table P2-1).

The global production and subsequently the residue:crop ratio for grapes are lower than feedstocks such as *Zea mays* (corn), *Oryza sativa* (rice) and *Triticum aestivum* (wheat) (Table P2-5). However, grape marc is a discarded waste residue from an established industry, and would not be grown as a dedicated biofuel feedstock. Thus the utilization of grape marc for bioethanol production is still value-adding, even if lower yields (biomass and ethanol) are achieved. Ethanol yields of 211 L/t can potentially be generated from red marc and 400 L/t from white marc, which is slightly lower than yields predicted for other lignocellulosic biofuel feedstocks such as sugarcane bagasse (*Saccharum* spp.; 463 L/t), rice straw (370−536 L/t) and sorghum straw (*Sorghum bicolor*; 428 L/t) (Table P2-5). Alternatively, just the WSC in white marc can be extracted and fermented directly, yielding up to 270 L/t. The utilization of only WSC can minimize processing costs associated with liberating fermentable sugars from the cell wall, leaving a polyphenol enriched fraction that may be used in animal feed, applied as a fertilizer or exploited as a source of phenolic compounds. An integrated processing system for grape marc would not only enable multiple products to be refined from the same waste material, increasing its value, but would be environmentally, socially and economically advantageous.

Conclusions

Grape marc is a waste material that is rich in carbohydrates. The majority of these carbohydrates are soluble monosaccharides (glucose and fructose) and structurally complex polysaccharides (pectins, heteroxylans, xyloglucan and cellulose). The soluble carbohydrates can be directly converted to ethanol through fermentation, yielding up to 270 L/tonne. Alternatively, ethanol yields may be increased by utilizing the whole material, which benefits from acid pre-treatment followed by enzymatic hydrolysis. Overall, these data suggest that on a weight for weight basis, grape marc has the potential to be a competitive, value-adding waste material for biofuel production, contributing up to 400 L/t ethanol.

Acknowledgements

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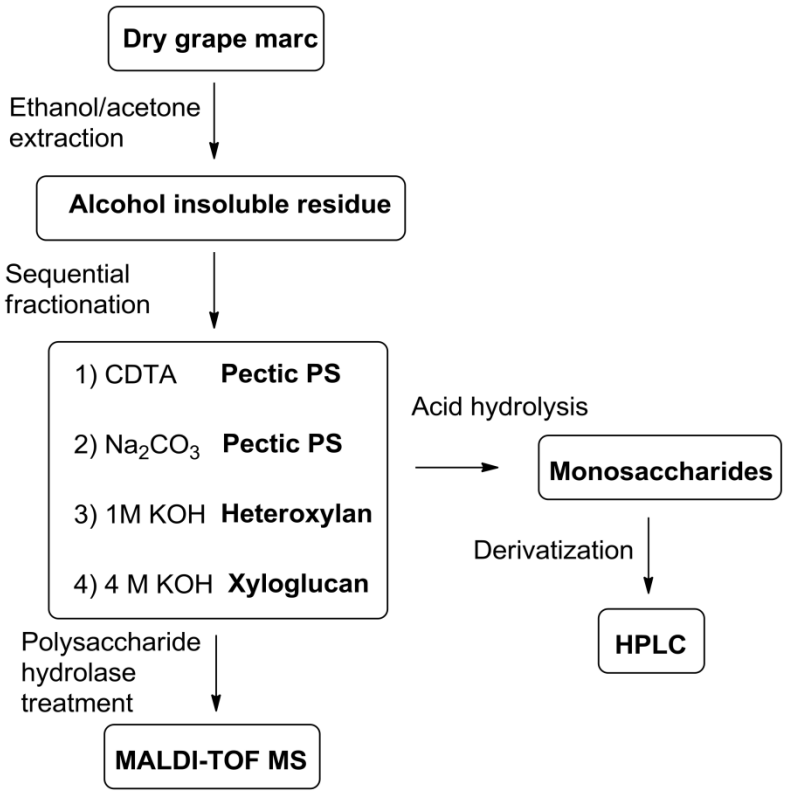

Figure P2-1 Flowchart outlining the sequential fractionation of grape marc (AIR) to isolate polysaccharides for characterization

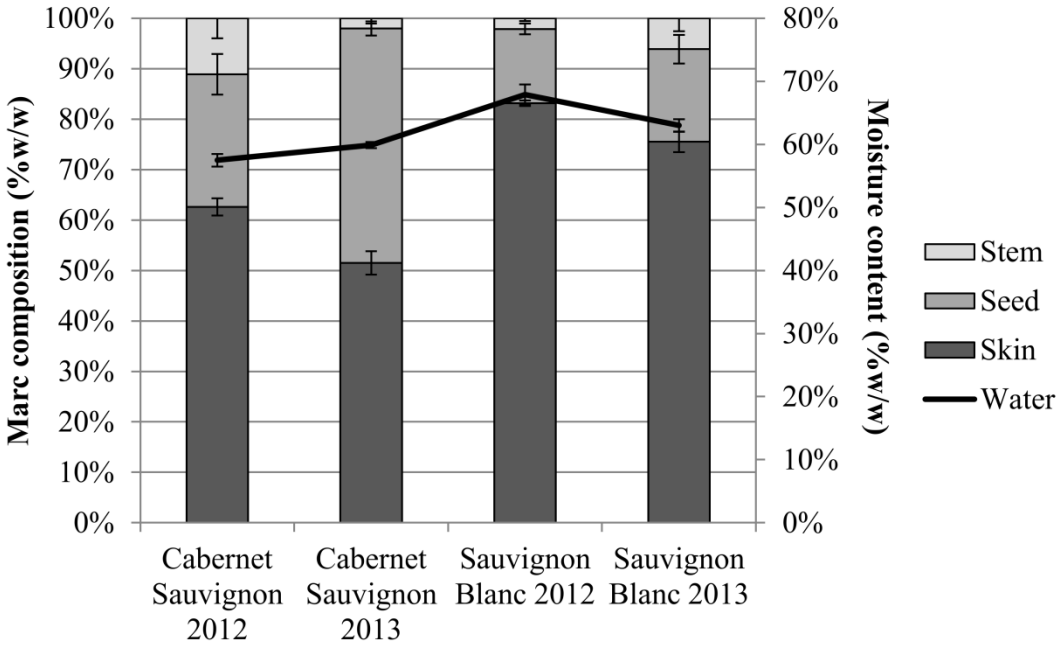

Figure P2-2 Grape marc is a heterogeneous material composed predominantly of grape skin

Fresh grape marc from the varieties Cabernet Sauvignon and Sauvignon Blanc (collected in 2012 and 2013) were separated into three fractions. The water content of whole marc was measured for each sample. Data is presented as percent fresh weight (w/w), n=3.

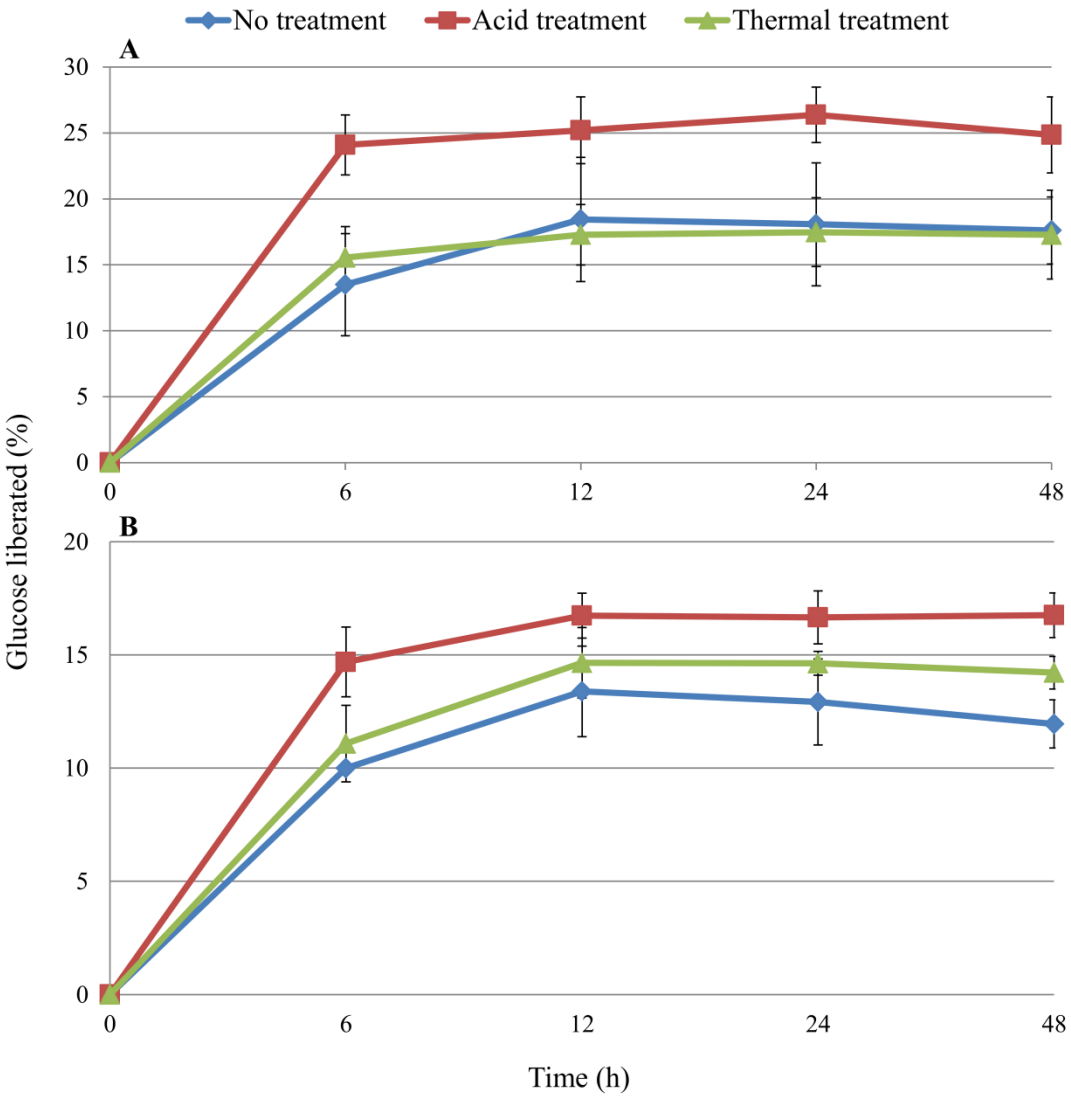

Figure P2-3 Pre-treatment of grape marc increases the biochemical conversion of cellulose in the presence of cellulases

Liberation of the monomer glucose in Sauvignon Blanc marc (A) and Cabernet Sauvignon (B) was measured over 48 h. Grape samples were treated with 0.5 M sulfuric acid or water at elevated temperatures (1 h, 121°C). The rate of saccharification of pre-treated grape marc is compared to non-pre-treated AIR grape marc and expressed as a percentage of cellulose converted into glucose.

Table P2-1 Mass balance of Cabernet Sauvignon and Sauvignon Blanc grape marc

	***Cabernet Sauvignon* (% w/w)**	***Sauvignon Blanc* (% w/w)**
Extractables	20.7 ± 1.0	60.5 ± 4.7
Soluble carbohydrates	**14.8**	**43.0**
*WSC	4.6 ± 1.5	37.6 ± 6.8
Glucose	2.1 ± 0.7	18.6 ± 3.7
Fructose	2.5 ± 0.8	19.0 ± 3.2
*Polysaccharides	8.7 ± 0.2	3.7 ± 1.1
Hydrolyzed monosaccharides	0.2 ± 0.3	0.2 ± 0.2
Ethanol insoluble (pectin-enriched)	8.5 ± 0.5	3.5 ± 0.4
*Ethanol soluble monosaccharides	1.5 ± 1.6	1.7 ± 0.2
Insoluble components (AIR)	**63.9**	**30.6**
*Monosaccharides	16.6 ± 0.3	10.9 ± 0.9
Glucose	6.0 ± 0.4	5.2 ± 0.5
Starch	2.7 ± 0.3	4.0 ± 1.3
Xylose	4.3 ± 0.9	2.4 ± 0.3
Galacturonic acid	1.9 ± 0.2	1.4 ± 0.4
Mannose	1.6 ± 0.3	0.6 ± 0.1
Galactose	1.0 ± 0.1	0.6 ± 0.1
Arabinose	1.8 ± 0.1	0.7 ± 0.1
Acid-insoluble lignin	32.5 ± 2.1	10.5 ± 2.3
Protein	10.8 ± 0.3	7.2 ± 0.8
Acetate groups	1.0 ± 0.1	0.8 ± 0.1
Ash (structural inorganics)	3.0 ± 0.8	1.2 ± 0.2
Total mass balance	**85.0**	**91.0**
Total carbohydrates	**31.4**	**53.9**

The composition of the soluble extracts and alcohol-insoluble residues (n=2). Data are presented as percentage dry weight (% w/w). The soluble carbohydrates include water and ethanol soluble sugars and minerals. Other extractables may include lignin, waxes or soluble proteins. The alcohol-insoluble residue is separated into structural carbohydrates (detected as

monosaccharides), total polyphenols, protein, acetate groups and ash. *Indicates values used to calculate carbohydrate sugar content: 31.4% w/w for Cabernet Sauvignon and 53.9% w/w for Sauvignon Blanc. Bold text indicates values derived from calculation. Unaccounted- for mass is likely to include volatile compounds, lipids, waxes and unhydrolyzed polysaccharides (pectin or crystalline cellulose).

Table P2-2 Composition of extracted fractions from grape marc

	Neutral monosaccharides (mol %)[1]								Uronic acids (mol %)[1]		
	Ara	Fuc	Gal	Glc	Man	Rha	Xyl	TOTAL	GalA	GlcA	TOTAL
Cabernet Sauvignon											
CDTA[2]	21.2	0.0	15.5	10.7	19.9	3.8	7.7	78.8	21.2	0.0	21.2
Na_2CO_3[3]	34.2	0.0	13.8	17.0	8.0	6.4	6.6	86.0	14.0	0.0	14.0
1 M KOH[4]	26.7	0.0	9.1	28.7	13.3	0.0	20.2	98.0	2.0	0.0	2
4 M KOH[5]	7.1	2.3	6.8	22.3	7.2	0.0	52.1	97.8	0.0	2.2	2.2
Sauvignon Blanc											
CDTA[2]	30.1	0.0	21.6	2.5	5.6	5.6	7.7	73.1	24.8	2.1	26.9
Na_2CO_3[3]	48.6	0.0	15.8	6.1	0.0	8.6	5.3	84.4	15.6	0.0	15.6
1 M KOH[4]	25.7	0.0	8.2	22.9	1.5	0.0	36.5	94.8	5.2	0.0	5.2
4 M KOH[5]	6.0	1.7	7.5	22.6	6.5	0.0	55.7	100.0	0.0	0.0	0

[1]Average of duplicate determinations presented as relative mol %.

[2]Acid hydrolyzed trans-cyclohexane-1,2-diamine-tetraacetate (CDTA) extract.

[3]Acid hydrolyzed Na_2CO_3 + 25 mM $NaBH_4$ extract.

[4]Acid hydrolyzed 1 M KOH + 25 mM $NaBH_4$ extract.

[5]Acid hydrolyzed 4 M KOH + 25 mM $NaBH_4$ extract.

Table P2-3 Elemental analysis of grape marc

	Cabernet Sauvignon (mg/kg)	**Sauvignon Blanc (mg/kg)**
Aluminium (Al)	31 ± 17	13 ± 14
Calcium (Ca)	3867 ± 611	2170 ± 549
Iron (Fe)	85 ± 21	60 ± 27
Magnesium (Mg)	987 ± 76	710 ± 46
Phosphorus (P)	2733 ± 351	2367 ± 58
Potassium (K)	27333 ± 2309	20267 ± 3164
Sodium (Na)	58 ± 15	61 ± 15
Sulfur (S)	1370 ± 44	1027 ± 60
Zinc (Zn)	15 ± 3	9 ± 4
Total	**36479 ± 2267**	**26684 ± 2567**

Data is presented as mg/kg of dry material, n=2.

Table P2-4 Compositional changes in hydrolyzate after pre-treatment

Biomass	Method	Glucose	Xylose	Arabinose	Other[1]	Total
Cabernet Sauvignon	NREL	11.0 ± 2.0	4.0 ± 0.9	1.8 ± 0.2	6.4 ± 0.9	23.2
	Acid/ autoclave	5.3 ± 1.6 48%	3.1 ± 0.4 78%	1.6 ± 0.1 89%	3.4 ± 0.4 53%	13.4 58%
	Acid/oven	4.1 ± 1.5 37%	3.1 ± 0.6 78%	1.6 ± 0.1 89%	3.9 ± 0.6 61%	12.7 55%
	Water/ autoclave	2.0 ± 1.2 18%	0 ± 0.0 0%	0 ± 0.0 0%	0 ± 0.0 0%	2.0 9%
	Water/oven	1.7 ± 1.1 15%	0 ± 0.0 0%	0 ± 0.0 0%	0 ± 0.0 0%	1.7 7%
Sauvignon Blanc	NREL	24.5 ± 2.6	2.3 ± 0.3	1.1 ± 0.2	3.9 ± 0.4	31.8
	Acid/ autoclave	22.1 ± 4.2 90%	1.3 ± 0.4 57%	1.0 ± 0.3 91%	2.2 ± 0.4 56%	26.6 84%
	Acid/oven	19.0 ± 2.5 78%	1.6 ± 0.2 70%	1.0 ± 0.3 91%	1.9 ± 0.2 49%	23.5 74%
	Water/ autoclave	21.3 ± 4.9 87%	0 ± 0.0 0%	0 ± 0.0 0%	0 ± 0.0 0%	21.3 67%
	Water/oven	20.8 ± 4.6 85%	0 ± 0.0 0%	0 ± 0.0 0%	0 ± 0.0 0%	20.8 65%

Four pre-treatments were used and compared to the standard NREL acid hydrolysis method [10]. Data are presented as percent dry weight (% w/w), n=2. The pre-treatment conditions were: Acid/autoclave (1 h, 121°C, 0.5 M H_2SO_4); Acid/oven (3 h, 100°C, 1 M H_2SO_4); Water/autoclave (1 h, 121°C, H_2O); and Water/oven (3 h, 100°C, H_2O).[1]Other includes additional monosaccharides (galactose, mannose, rhamnose, glucuronic acid and galacturonic acid).

Table P2-5 Theoretical yields of ethanol for agro-industrial waste

Feedstock	Global production (Mt)[a]	Residue/ crop ratio[b]	Waste biomass	Composition (dry % w/w)			Ethanol yield (L/t)
				Cellulose (C6)	NCPs (C5)	Lignin	
Corn (*Zea mays*)	768	1	Stover	40−43[c,d]	21−25[c,d]	17−19[b,c]	441−492
Barley (*Hordeum vulgare*)	140	1.2	Straw	38[e]	37[e]	9−16[b,e]	544
Rice (*Oryza sativa*)	655	1.4	Straw	32−47[d]	19−27[d]	7−12[b,c]	370−536
Wheat (*Triticum aestivum*)	636	1.3	Straw	30−45[c,d]	20−30[d]	16−20[b,c]	362−543
Sorghum (*Sorghum bicolor*)	59	1.3	Straw	32[f]	27[f]	7−15[b,f]	428
Sugarcane (*Saccharum* spp.)	1560	0.6	Bagasse	40[c]	24[c]	15−25[b,c]	463
Cabernet Sauvignon (*Vitis vinifera*)	67	0.3[g]	Red marc: whole	20.8	8.4	32.5	211
Sauvignon Blanc (*Vitis vinifera*)		0.3[g]	White marc: whole	48.2	7.3	10.5	400
		−	White marc: WSC	37.6	−	−	270

Average of world production from 2002-2012 [a][4] expressed as Mt (megatonnes) and the ratio of residue/crop [b][24] indicate the global availability of raw waste material for bioethanol production. *67 Mt of grapes (red and white) is produced annually. The theoretical ethanol yields for agricultural industrial waste materials were calculated based on compositional data from other studies: [c][25]; [d][33]; [e][34]; [f][35]; [g][5].

Supporting Information

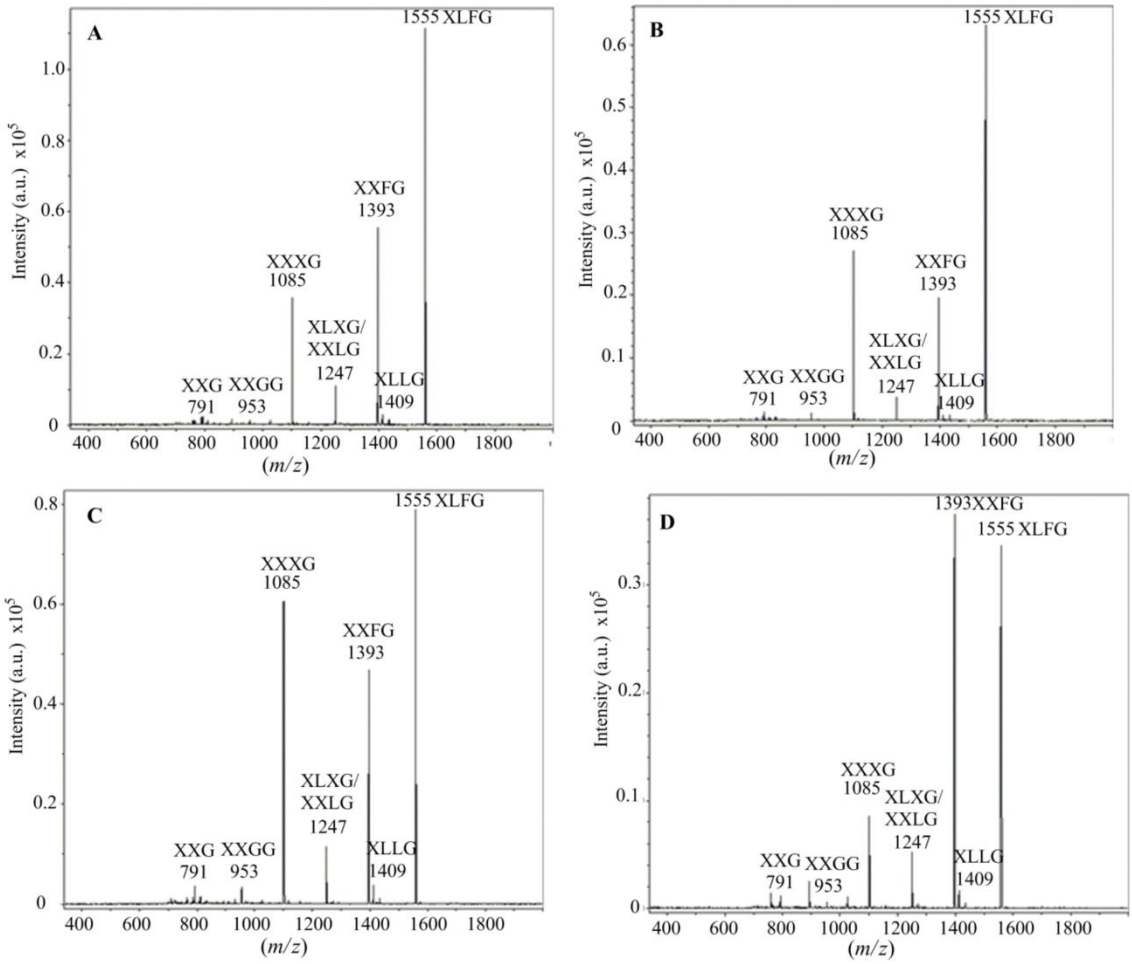

Figure P2-S1 Maldi-TOF-MS spectra of xyloglucan oligomers from grape marc

The $[M+Na]^+$ ions in the spectrum correspond to xyloglucan oligosaccharides that were liberated from 4 M KOH fractions using xyloglucan *endo*-(1,4)-β-D-glucanase: (**A**) Cabernet Sauvignon 2012; (**B**) Cabernet Sauvignon 2013; (**C**) Sauvignon Blanc 2012; (**D**) Sauvignon Blanc 2013. G, Glucose; X, Xylose; L, Galactose-Xylose-Glucose; F, Fucose-Galactose-Xylose-Glucose.

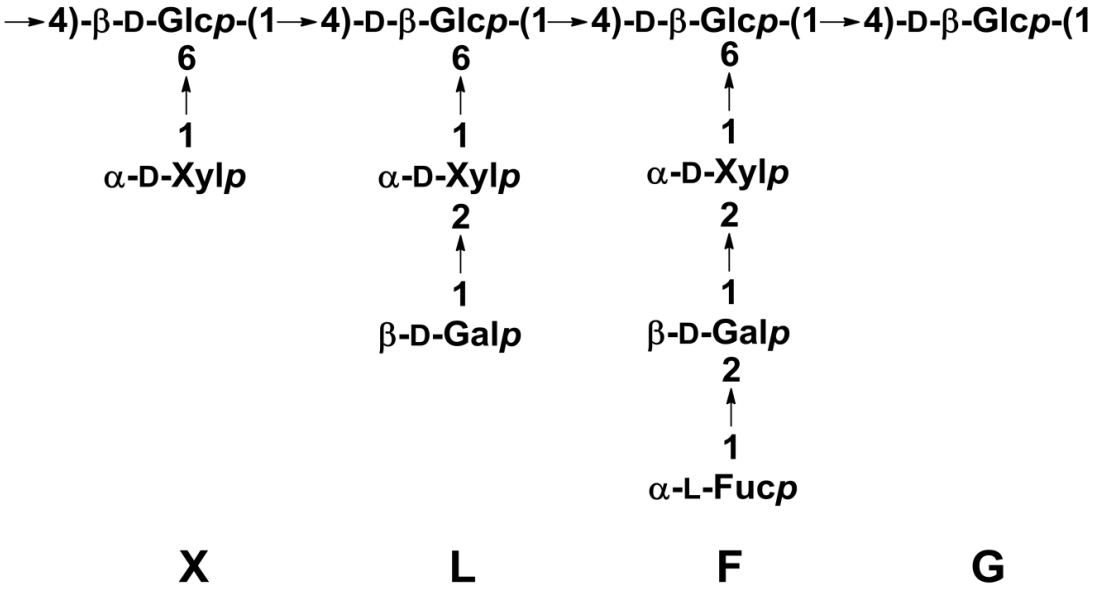

Figure P2-S2 Basic repeating unit of xyloglucans

Nomenclature of the xyloglucan sidechains are as follows, X represents the Xyl(α1,6)Glc unit, L represents Gal(β1,2)Xyl(α1,6)Glc, F represents Fuc(α1,2)Gal(β1,2)Xyl(α1,6)Glc and G represents the backbone Glc in β1,4-linkage [23].

11.3 Paper III: Highlights

A. tequilana leaf juice is rich in soluble carbohydrates yielding 4729 kg/ha/y

The standard method of autoclaving *Agave* juice does not significantly increase yields

Non-*Saccharomyces* yeast converts leaf carbohydrates to ethanol at 78% efficiency

Co-fermentation of leaf and stem juice benefits from using native yeast, *K. marxianus*

Predicted ethanol yields are up to 3411 L/ha/y for *A. tequilana* (4.5 y old)

Statement of Authorship

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Signature	Date: 12/05/15

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Contribution to the Paper	Supervision, concept development, helped with ms.
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Signature		Date: 10/05/2015

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Contribution to the Paper	Development of project. Project guidance Revision of manuscript.	
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Contribution to the Paper	Development of project Revision of manuscript.	
Signature		Date: 12/5/15

Low-input fermentations of Agave tequilana leaf juice generate high returns on ethanol yields

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Abstract

During tequila production, up to 75% w/w of the *Agave* plant is discarded when leaves are removed from the stem. The discarded leaves represent an extensive amount of unexploited biomass that was used here for bioethanol production in no-input fermentations, where no acid or enzymatic hydrolysis, supplementation of nutrients or standardization of carbohydrate content occur. Conversion rates of the carbohydrates in juice extracted from *Agave* leaves are unaffected by sterilization, but ethanol yields are reduced if fermentation is reliant solely on endogenous microorganisms. Non-*Saccharomyces* yeasts, including *Kluyveromyces marxianus* and *Candida akabanensis*, proved to be more robust than standard *Saccharomyces spp* and converted up to 78% of the carbohydrates in the leaf juice to ethanol. This conversion efficiency could be further increased to 85% by combining leaf and stem juice, as when a whole plant is crushed and directly fermented, and was estimated to yield up to 15 350 L/ha of ethanol.

Keywords: *Agave tequilana*, ethanol, fermentation, non-*Saccharomyces*

Abbreviations:

CAM, Crassulacean acid metabolism; TSS, total soluble solids; YPD, yeast extract-peptone-dextrose

Introduction

The advent of plant-based ethanol production using fermentation can be traced back to as early as 6000BC [1]. Alcoholic fermentation involves the release of energy from carbohydrates by microorganisms, usually under low oxygen conditions, and yields ethanol and carbon dioxide. Historically, the production of alcoholic beverages has been reliant on a consortium of commensal microflora to convert the plant-derived carbohydrates into ethanol [2]. However, these spontaneous (or natural) fermentations often yield unreproducible and unpredictable end-products. In more recent times, industry has optimized the fermentation process to generate more predictable and consistent high value end-products, such as wine, beer and spirits.

In principle the same techniques apply for the production of alcohol for transportation fuel, however the goal is not to produce a high value end-product but rather a cheaply produced "drop-in" fuel, which is both profitable and cost competitive with fossil fuels. In recent years, waste from feedstocks used for the manufacture of various beverages has been attracting attention as potential biomass sources for bioethanol and biochemical production. *Agave tequilana* plants, which are traditionally used for making tequila in Mexico, produce large volumes of juice that is rich in fermentable carbohydrates. At the time of harvest, the leaves are discarded and extensive processing is performed upon the stem biomass to generate a fermentable juice. Such processing steps include cooking the stem for extended periods to hydrolyse polymers, milling the cooked stems to extract the juice or "honey", converting the sugars in the honey to alcohol by fermentation and finally double distilling the fermentation broth to produce pure tequila, which may be aged for a further three to twelve months in oak barrels [3]. The fibrous biomass that remains following milling of the stems is commonly referred to as the bagasse. This discarded bagasse contributes 40% (wet w/w) of the stem mass [3]. Thus, under this processing

regime only about 25% of the above-ground biomass of the *Agave* plants is utilized. In a biofuel context, this processing scheme provides opportunities for converting a substantial amount of aggregated vegetative tissue to ethanol or other low molecular mass alcohols.

To date, available information is related to the fermentation of juice extracted from cooked *Agave* stems, which is reflective of the processes used in the tequila industry (Table P3-1). *Agave* stem tissue is enriched with fructans, which are soluble polymers of fructose with mainly fructosyl-fructose linkages linked to a terminal glucose molecule that acts as storage polymers in 15% of flowering plant species [4]. In these published studies the effects of a range of parameters, including incubation temperatures and differing fermenting microorganisms, has been investigated (Table P3-1). Standard methods for *Agave* fermentation include sterilizing the juice, spiking it with monosaccharides and nutrients (usually to boost nitrogen content) and/or adding polysaccharide-specific enzymes to optimize fermentation conditions (Table P3-1). However, the costs incurred by optimizing the nutrient content of the juice is frequently overlooked [5] and may not be economically viable for the production of cheap biofuel. In addition, little is known about the composition and fermentation of juice extracted from the discarded *Agave* leaves. To obtain a realistic, base-line view of utilizing novel fructan-enriched biomass such as *Agave* for potential large-scale bioethanol production, information about fermentations using no or minimal inputs other than leaf and stem juice is required.

In the present study ethanol yields achieved from *Agave* leaf juice on a small scale was investigated using different processing methods and fermenting microorganisms. Four different fermentation schemes were used for *Agave* leaf juice, none of which included supplementation or standardization of the carbohydrate or nutrient content. The production of ethanol by native or commercially available fermenting organisms was assayed from raw and sterilized leaf juice substrates and compared with fermentation rates of either pure stem juice or combined stem and leaf juice. The observed rates of carbohydrate to ethanol

conversion (%) based on this fermentation data were extrapolated to predict the maximum ethanol yields that could be achieved using *Agave* waste on a large scale. Thus, a toolset of methods and information specific to the small-scale fermentation of *Agave* juice has been developed and is likely to be useful in a commercial context for forecasting the potential yields of bioethanol from novel biomass sources such as *Agave* leaves.

Material and Methods

Collection of Agave tequilana plants

A. tequilana plants (n=3) were harvested in Ayr, Queensland, Australia (19° 31′ 49.9″ S; 147° 24′ 51.5″ E). At the time of harvest, the 4.5 y old plants were separated into leaves, roots, stem and offshoots, and the fractions were weighed. A commercial shredder (Cutter-Grinder CG03; South Australia, Australia) was used to extract the juice from a subset of each fraction. The juice was collected and transported to the University of Adelaide on dry ice and stored at -20°C.

Total carbohydrate content in *Agave* juice

The carbohydrate content in raw and processed (autoclaved, triflouroacetic acid (TFA)- or fructanase-treated; Fructan HK-Megazyme: AOAC Method 999.03; International Ireland Ltd., Wicklow, Ireland) *A. tequilana* juice from leaves, stem and offshoots was quantified by hydrophilic interaction chromatography (HILIC) as previously described [6]. A sugar recovery standard for water-soluble carbohydrates (fructose, glucose and sucrose) was carried through the acid hydrolysis to calculate the degradation rates of released monosaccharides, which were used to correct HILIC data.

pH, Brix, total soluble solids (TSS) and mineral content

The pH of the juice was determined using a 900-P pH-mv-Temperature meter (TPS Pty Ltd, Australia). Brix readings of the juice samples were measured with a refractometer (Atago Co., Japan). The total soluble solids (TSS) and mineral content of juice samples were determined as previously described [6].

Selection and screening of microorganisms

Fermenting microorganisms were obtained from the United States Department of Agriculture Agricultural Research Service (ARS) Culture Collection, National Center for

Agricultural Utilization Research (Peoria, IL, USA) [7]. The strains were streaked on 2% w/v agar (YPD) plates containing 1% w/v yeast extract, 2% w/v peptone and 2% w/v D-glucose, and incubated at 28°C for 48 h. Single colonies were picked and inoculated into YPD medium and shaken (120 rpm) overnight at 28°C. Cultures were diluted to an OD_{600} of 0.5 for thermo-tolerance spot assays. Serial 5-fold dilutions were spotted on YPD plates and incubated for 24 h at 28°C, 32°C, 37°C or 42°C, n=2.

Fermentation conditions

Juice samples were centrifuged at 5000 rpm for 5 min to remove excess leaf tissue. Autoclaving of juice was completed at 121°C at a maximum pressure of 210 kPa (Tuttnauer 3850 ELC Benchtop Sterilizer; Brinkmann Instruments, New York, USA). Leaf and stem juice were combined in a volume ratio of 3:1 (leaf:stem), to a final volume of 100 mL, representative of the whole plant being fermented simultaneously.

Native microorganisms in raw *Agave* leaf and stem juice were streaked (0.05 mL aliquots) on solid YPD plates and grown at 28°C. A representative microbial culture of the endogenous species found within the *Agave* juice was made by harvesting these plates using a sterile loop. This formed an inoculum that was used to re-inoculate 100 mL of autoclaved juice samples, representative of juice samples that had become contaminated following sterilization.

The yeasts obtained from the ARS Culture Collection grown in YPD cultures were also used to inoculate 100 mL of raw juice samples at a cell density of 5×10^6 cells/mL. All fermentations were completed in sterile Erlenmeyer flasks (250 mL in size) with side arm sampling ports sealed with water-filled airlocks. The fermentation flasks were shaken at 150 rpm and at 28°C. The yeast cells were removed from the fermentation broth by centrifugation (1m / 10 000 g) and the supernatant stored at -20°C until analysis.

Analysis of substrate and fermentation products

The concentrations of organic acids, glycerol and ethanol in the fermentation broth were quantified by High Performance Liquid Chromatography (HPLC) as previously described [8]. Analysis was completed using an Aminex HPX-87H cation exchange column (Bio-Rad Laboratories) on a HPLC 1100 series (Agilent Technologies, Germany) with a mobile phase of 2.5 mM sulphuric acid (H_2SO_4). Calibration curves relating concentration to optical density or refractive index were fitted using ChemStation software (Agilent, California, USA).

Maximum ethanol yields

Maximum ethanol conversion yields were calculated using the assumption that 0.511 kg of ethanol is produced from 1 kg of sugar [9]; and that *Agave* juice has a density of 1.25 g/L.

Statistical analysis

Two-way ANOVA and Tukey's multiple comparisons tests were completed using the program GraphPad Prism (GraphPad Software, CA, USA).

Results and Discussion

Composition of *Agave tequilana*

A whole *Agave* plant, including the main unit (mother plant) and its attached offshoots, generated 360 kg of raw biomass that could be used for biofuel production. The harvested biomass was separated into different anatomical fractions (leaves, stem and offshoots) to create multiple production streams from one feedstock (Figure P3-1). For 4.5 year old *A. tequilana* plants, 73% w/w of the above-ground biomass was attributable to leaves and 27% w/w to the stem (Table P3-2). By crushing the leaves, 68% w/w of the leaf mass could be extracted as a weakly acidic fermentable juice (Table P3-2).

The acidity of *Agave* juice (pH 4.6–5.0; Table P3-S1) is a characteristic of plants that use the Crassulacean acid metabolism (CAM) photosynthesis pathway, in which organic acids such as malic acid are stored in the vacuoles of cells during the night [10]. The two abundant organic acids measured by HPLC in the leaf juice were acetic and malic acid (Table PS-S2). High concentrations of weak acids such as acetic acid or low concentrations in the presence of other compounds (i.e furfural) have been shown to reduce biomass accumulation and production of ethanol, resulting in a need for superior strain selection [11]. A small percentage of the total mass of the juice was attributed to minerals at 1–2% w/w (Table P3-S1).

Total soluble solids (TSS) and °Brix can be used to estimate sugar content in a liquid solution (Table P3-2). The measured carbohydrate content for *A. tequilana* leaf (~9.6% w/v) and stem juice (~17.3% w/v) using the two methods, TSS and °Brix, were comparable. However, when juice was analysed using HPLC, it became clear that these methods overestimated the amount of carbohydrates in leaf and offshoot juice (Table P3-2).

Juice samples were treated with TFA or fructanase to cleave higher molecular weight carbohydrates, predominantly fructans, into monosaccharides for quantification. *A. tequilana* plants are considered to be high accumulators of branched fructans and neo-fructans [12]. However, only 10−15 g/L of the carbohydrates in *Agave* tequilana leaf and offshoot juice is attributed to fructans (Table P3-3). Whilst over 85% of the carbohydrates in stem juice are complex fructans, many microorganisms cannot ferment these in their native branched forms and thus a hydrolysis or cooking step is required [13]. Although such methods are considered to be efficient for the complete hydrolysis of fructans in both leaf and stem tissue, they can be costly and unfavourable for downstream processing [13, 14]. In addition, fructose is highly unstable relative to other monosaccharides such as glucose, even under moderate pre-treatment conditions, and is converted into compounds,

such as hydroxymethylfurfural, which are toxic to fermenting microorganisms [14]. As a result, acid and enzymatic pre-treatments were not further explored in this study.

Alternatively, autoclaving is considered a low-cost, low-input pre-treatment for *Agave* juice. In leaf juice samples, no detectable change in monosaccharides was observed in autoclaved juice when compared with raw juice (Table P3-3). Conversely, autoclaving increased measurable monosaccharides in stem juice by 50 g/L (Table P3-3). In addition, higher levels of the intermediate hydrolysis product sucrose were present; indicating that partial breakdown of the complex fructans was achieved where one sucrose moiety was liberated per molecule of inulin [14].

In the tequila industry, cooking the stem at elevated temperatures for an extended period is the standard method for fructan hydrolysis, for production of aromatic compounds and for softening of the recalcitrant tissue before milling [3]. Autoclaving can similarly be used to treat the juice prior to fermentation (Table P3-1), but there are inconsistent reports in the literature regarding the efficiency or necessity of the autoclaving step. Some studies indicate that autoclaving *Agave* juice partially hydrolyses fructans [15]. Other studies imply that autoclaving is employed strictly as a sterilization step and does not modify the structure or composition of carbohydrates in the juice [16], whilst others claim for complete hydrolysis (98%) of fructans to occur, the samples must be heated at about 80°C for more than 25 hrs [12]. Findings herein indicate that the efficiency of fructan hydrolysis by a simple thermal pre-treatment (autoclaving) is substrate-dependent, as there was no difference in the monosaccharide content between raw and autoclaved leaf juice but the amount of monosaccharides in the stem juice increased following autoclaving.

Although immature offshoots contributed a substantial amount of the total biomass (38% w/w; Table P3-2), they were at varying stages of development, potentially rendering biomass weights, carbohydrate content and ultimately ethanol yields unpredictable. As a

result, it may be more advantageous to harvest only the mother plant and to leave the offshoots to grow for future harvests, thus offshoots were not further analysed in this study.

Spontaneous fermentation of *Agave* juice is induced by endogenous species

In commercial tequila production, natural (spontaneous) fermentation is a common practice [17]. However, in a biofuel context natural fermentations are unlikely to be favourable and partial fermentation of the carbohydrates can occur prematurely during transport of feedstocks to processing facilities, thus reducing ethanol yields (Figure P3-S1). Here, the spontaneous fermentation of *A. tequilana* leaf and stem juice was tested by allowing raw leaf and stem juice to ferment for 120 h without the addition of any extra microorganisms. Both juice sources gave similar yields of ethanol: 9.0 g/L for leaf juice and 14.6 g/L for stem juice (Figure P3-2a). However, the fermentation profiles of these two substrates over 120 h were quite different (Figure P3-2a). The highest accumulation of ethanol in the stem juice was observed at 48 h and decreased over the next 24 h. This reduction in ethanol concentration may be due to native organisms metabolising ethanol in periods of stress or when monosaccharides are not immediately available. In contrast the concentration of ethanol in the spontaneous leaf juice fermentations increased consistently over 96 h and then plateaus.

In terms of the conversion of total carbohydrates to ethanol (Figure P3-2b), the performance efficiency of native organisms was higher in leaf samples. The higher conversion efficiency in leaf juice may be due to the majority of the carbohydrates being fermentable mono- or oligosaccharides, whereas in stem juice they are complex fructans (Table P3-3). The final ethanol yield for both spontaneous fermentations was similar (10−15 g/L), which is indicative of similarities between the membership of the microbial mixture both at the start and throughout the fermentation. The endogenous microorganisms found within these *Agave* samples may have a low tolerance to ethanol resulting in

incomplete fermentation (only 32% and 17% conversion of leaf and stem carbohydrates to ethanol, respectively), although other factors may be hindering the rate of conversion.

Spontaneous fermentations are heterogeneous microbiological and biochemical processes in which populations increase and decrease as selective pressures are induced, ultimately resulting in the dominance of species with superior catabolism rates and/or tolerance to the alcoholic end-products [2]. Microorganisms isolated from the early stages of *Agave* stem fermentations have been shown to contain a rich mixture of yeast species. For example, 192 yeasts were identified from alcoholic fermentation of *A. salmiana* juice when it was streaked on nutrient agar plates [18]. However, as fermentations progress, the number of species and their abundance can change. Some studies indicate that the diversity diminishes over the course of the fermentation [19], whereas in other studies, a more diverse mixture of microorganisms was observed due to the introduction of contaminants [18]. In both studies *Saccharomyces* was found to be the predominant strain at the onset of fermentation. A negative interaction between *S. cerevisiae* and non-*Saccharomyces* yeast strains has been proposed, which may be one of the factors contributing to the reduced efficiency of spontaneous fermentations [20].

In addition, active killer yeast (and bacterial) genera, such as *Kluyveromyces marxianus* var. *drosophilarum* and *Pichia membranaefaciens*, are frequently present in the spontaneous fermentation of plant biomass, including *Agave*, resulting in sluggish or stuck fermentations [21,22]. Strains that exhibit these killer properties tend to colonize fermentations early and produce zymocidal substances that can reduce beneficial yeast populations [23]. The rise and fall of populations during the course of the fermentation could explain the sporadic changes in ethanol content observed in the fermentation profiles of unmodified *Agave* leaf and stem juice. Prolonged spontaneous fermentations of *Agave* juice, if not monitored, could therefore inadvertently result in reduced ethanol yields. Such

information will be instructive when considering the processing, transport, handling and storage of *Agave* biomass and juice.

Screening and selection of microorganisms

As microorganisms exhibit a narrow range of tolerances to environmental conditions, identifying fermenting organisms that are particularly adapted to the substrate is crucial. In this work, five yeast strains were obtained from the ARS Culture (NRRL) Collection [7] for fermentation studies (Table P3-4). The selected yeast had been isolated directly from *Agave*, other succulents or carbohydrate-rich plant tissue such as grape or sugarcane molasses. The ability of three strains to ferment the primary carbohydrates in *Agave* juice (glucose, sucrose and inulin) as sole carbon sources had previously been tested (CBS-KNAW Fungal Biodiversity Centre, [24]; Table P3-4). For the other two yeasts, data specific to the strains were not available, but species level information regarding fermentation performance was considered (Table P3-4).

Of the yeast selected, three species had some capacity to ferment the primary carbohydrate in *Agave* tissue, inulin (fructans), *K. marxianus* (*Km*15918), *Candida akabanensis* (*Ca*7846) and *Saccharomyces cerevisiae* (*Sc*636 and *Sc*139) (CBS-KNAW Fungal Biodiversity Centre, [24]; Table P3-4). In addition, isolates of *Kluyveromyces marxianus* have been shown to produce fructan-hydrolysing enzymes [25]. The yeast *Pichia kluyveri* was also selected as it has been reported to display high fermentation efficiency in tequila making [26].

To investigate thermal tolerance and to determine the optimal growth temperature, each yeast was diluted to a standardized cell concentration and spotted on YPD plates placed at four different temperatures (28°C, 32°C, 37°C and 42°C). All yeast strains grew similarly at temperatures at or below 32°C (Table P3-S3). However, one yeast, *Kluyveromyces marxianus* (*Km*1598), tolerated temperatures above 37°C. This strain was

isolated from *Agave* that is able to thrive in arid regions and which can tolerate temperatures above 60°C [27]. Such information suggests that further analysis of the metagenome of *Agave* may identify novel, superior microorganisms for the production of bioethanol. For example, although less than 1% of microorganisms present in many natural environments can be cultured *in vitro* [28], endogenous microorganisms from *Agave* juice exhibited growth over a wide range of temperatures (28−42°C; data not shown). Yeast isolated from *Agave* also have the advantage of being tolerant to toxic compounds (i.e. furans) present in the juice and are less likely to become inactive during fermentation as they are more adapt to the substrate [29].

Comparison of microbial strains and treatment of *A. tequilana* leaf juice

In large-scale biofuel production, where rapid and reliable fermentations are essential, the use of robust and reliable pure yeast inocula of known performance is common practice, rather than spontaneous fermentation [2]. Here, five yeasts (Table P3-4) were selected for the fermentation of raw and autoclaved *Agave* leaf juice. Subsequently, yeast strains with the highest fermentation performance were cultured in autoclaved leaf juice after the re-introduction of endogenous microbes (Table P3-5).

S. cerevisiae is one of the most widely utilized yeasts for alcoholic fermentations such as wine making and brewing, yet the ethanol yields achieved from *Agave* leaf juice (raw or autoclaved) using two *Saccharomyces* isolates (*Sc*636 and *Sc*139) were lower compared with the yields achieved using the less commonly studied yeasts *Ca*7846, *Km*1598 and *Pk*17228 (Table P3-5). The poorer fermentation performance of the *Saccharomyces* isolates is likely to be attributable to the high levels of fructose present in the substrate, as *Saccharomyces* yeast species preferentially use glucose (although the level of preference varies) when in a heterogeneous culture with other monosaccharides such as fructose [30]. When glucose is transported across the plasma membrane in these mixed

cultures and ethanol is accumulated, the tautomeric equilibrium of fructose is shifted, converting it from fructopryranose to fructofuranose [31]. This shift in conformation has been suggested to lower the rate of fructose transport, further limiting its uptake by the yeast [30]. The accumulation of carbohydrates that are not metabolized by the yeast results in slowed or arrested fermentations, ultimately decreasing ethanol yields. Further reductions in ethanol yields have been reported for *Saccharomyces* inocula when used in *Agave* fermentations if the yeast were not isolated from *Agave* [32]. Therefore, for optimal fermentation microorganisms that are adapted to or can tolerate and metabolise fructose-containing polymers are required for the fermentation of *Agave*.

Higher ethanol yields were achieved using non-*Saccharomyces* yeasts than *Saccharomyces* yeasts. The increase in ethanol yields using these strains may be attributed to their superior ability to degrade fructose and fructan polymers, or possibly their tolerance to compounds inherent to the substrate (i.e. malic acid) or the production of ethanol and inhibitory by-products. For example, *K. marxianus* is known to produce fructan-specific enzymes, which enables the yeast to simultaneously hydrolyse fructans and ferment the liberated monosaccharides [25], resulting in higher ethanol yields (Table P3-5). Alcohol yields achieved using the non-*Saccharomyces* yeasts *P. kluyveri* and *K. marxianus* were similar, consistent with previous reports [26]. The most efficient isolate used was *Ca*7846 (78% conversion; Table P3-5), but this has been the first study to investigate its use for bioethanol production and little is known about its fermentation capabilities.

There is no benefit to autoclaving *Agave* leaf juice prior to fermentation. No significant differences between ethanol yields (g/L) or conversion rates (% of conversion of carbohydrates to ethanol) of *Agave* juice were observed between non-*Saccharomyces* strains when cultured into raw or autoclaved *Agave* leaf juice (Table P3-5). In addition, the accumulation and production of organic acids (acetic, citric, acetic and malic acid) and

glycerol in non-*Saccharomyces* fermentations of raw and autoclaved juice was similar (Table P3-S2), indicating that acid production and consumption patterns of the yeast are not influenced by autoclaving the juice. However, when endogenous microorganisms were re-introduced into autoclaved juice substrates, the ethanol yields were lower for all non-*Saccharomyces* yeasts tested than yields from fermentations where endogenous microorganisms were not re-introduced. The strain *Km*1598, which was originally isolated from *Agave sisalana* (Table P3-4), had the lowest rate of conversion in the presence of native microbes (raw and re-introduced) compared with *Ca*7846 and *Pk*17228.

Overall, the efficiency of the fermentations were influenced more by the yeast species present (Table P3-5) than by the treatment of the juice. There were no statistically significant differences observed between the ethanol yields achieved using raw or autoclaved leaf juice for any isolate. However, there was a clear and statistically significant distinction between fermentation performance of *Saccharomyces* and non-*Saccharomyces* yeast in both raw and autoclaved leaf juice (Table P3-5).

The current research has revealed that ethanol yields are likely to be compromised if *Agave* leaf juice is not autoclaved prior to fermentation and demonstrated the impact on ethanol yields when autoclaved juice becomes contaminated with native organisms following sterilization. The highest rate of conversion of carbohydrates to ethanol observed was 61%, using *Pk*17228 (Table P3-5). However, this value is lower than the rate observed when culturing raw leaf juice (69% conversion for *Pk*17228; Table P3-5). This reduction in ethanol yields is likely to be due to the compositional and chemical changes that occur as a result of autoclaving. One means by which autoclaving changes the composition of *Agave* juice is by degrading vitamins and thereby inducing a nutrient-limited environment [16]. Deficient vitamin levels can reduce cell growth rate, biomass production, decrease viability and ultimately effect fermentation performance [33]. In addition, it can result in the degradation of nitrogen containing compounds (i.e. ammonium), which is an essential

macronutrient required by microorganisms for the maintenance of stationary phase fermentation [33]. An unbalanced nitrogen level may result in stuck fermentations which may significantly reduce ethanol yields.

It should be noted that by culturing the endogenous microbes present in *Agave* juice on YPD plates, only a proportion of the microbial population was represented and therefore this inoculum was not identical to the breadth of microorganisms found in raw *Agave* juice. However, as in the tequila industry, even a small diversity of microbial contaminants can have a profound effect on the fermentation performance and end-products generated [19]. Contaminants from *Agave* will inevitably be present in *Agave* processing facilities. The current study provides founding data from which strategies to manage *Agave* storage and handling may be developed.

Fermentation of *Agave* stem juice and co-fermentation

Microbes that naturally deconstruct plant walls may provide the best enzymes for bioconversion of energy crops [34]. To explore this concept the fermentation of autoclaved *Agave* stem juice was investigated using *Km*1598, a strain which was originally isolated from *Agave*. A maximum conversion rate of 64% was achieved by 48 h (Figure P3-3a). This conversion rate was lower than previously reported for *K. marxianus* (94–96% efficiency after 72 h; Table P3-1), but the stem juice used for this study was not cooked for an extended period to hydrolyze the fructans [17].

When stem juice and leaf juice were combined, 85% conversion to ethanol was achieved (Figure P3-3b). The leaf and stem juice were combined in a volume leaf:stem ratio of 3:1, which was representative of a scenario where whole plants are crushed as one unit, without the leaves and stem being separated. This approach is advantageous as reduces the time and labour required for processing and harvesting of *Agave* plants, potentially reducing production costs.

Ethanol yield predictions

The theoretical ethanol yields for *A. tequilana* rival other currently studied lignocellulosic feedstocks [35]. Juice derived from *A. tequilana* stems had a higher proportion of carbohydrates than leaf juice (13.7% w/w or 17.1% w/v respectively; Table P3-2), but in total the juice from the leaves contributed more total carbohydrates, 5.3 kg per plant than stem, 3.5 kg per plant. Therefore, a higher ethanol yield was calculated for *A. tequilana* leaf juice, 2412 L/ha/yr relative to stem juice, 1602 L/ha/yr (Table P3-6). When combined the fermentation of juice collected from only the mother plants in an *Agave* plantation would yield up to 4014 L/ha/yr ethanol, leaving the offshoots to keep growing in the field for future harvests. However, the conversion of carbohydrates into ethanol is mediated via a complex chemical and enzymatic pathway and actual fermentation data is required to validate the estimates of the value of this biomass as a substrate for fermentation.

There was a range in the conversion of carbohydrates to ethanol in the fermentation of *Agave* leaf juice, 48–78% (Table P3-5). This correlates to yields of 1157–1881 L/ha/yr ethanol (Table P3-6). Therefore, the fermentation of *A. tequilana* leaves, which are currently discarded in the beverage industry, could add significant value to existing *Agave* industries. *Agave* production specifically for biofuel may also be profitable, particularly as *Agave* can be produced in agriculturally marginal regions. If grown specifically for biofuel production at a density of 4000 plants/ha, the juice derived from 4.5 y old *A. tequilana* plants (stem and leaf) could produce ethanol yields of 15 350 L/ha.

Conclusion

This data generated in this study challenges current practices in bioethanol production of supplementing and pre-treating *Agave* juice prior to fermentation. Leaf juice substrates do not benefit from autoclaving prior to inoculation, and the choice of fermenting organisms

is more important than pre-treatment. The best fermenters studied were isolated from succulents. A third species, *Candida akabanensis*, was successfully used for the first time in fermentation studies of *Agave* juice, suggesting that further exploration of non-traditional *Saccharomyces* species could improve bioethanol yields. Actual and extrapolated ethanol yields from *Agave* leaf juice confirm that this biomass has significant potential for bioethanol production.

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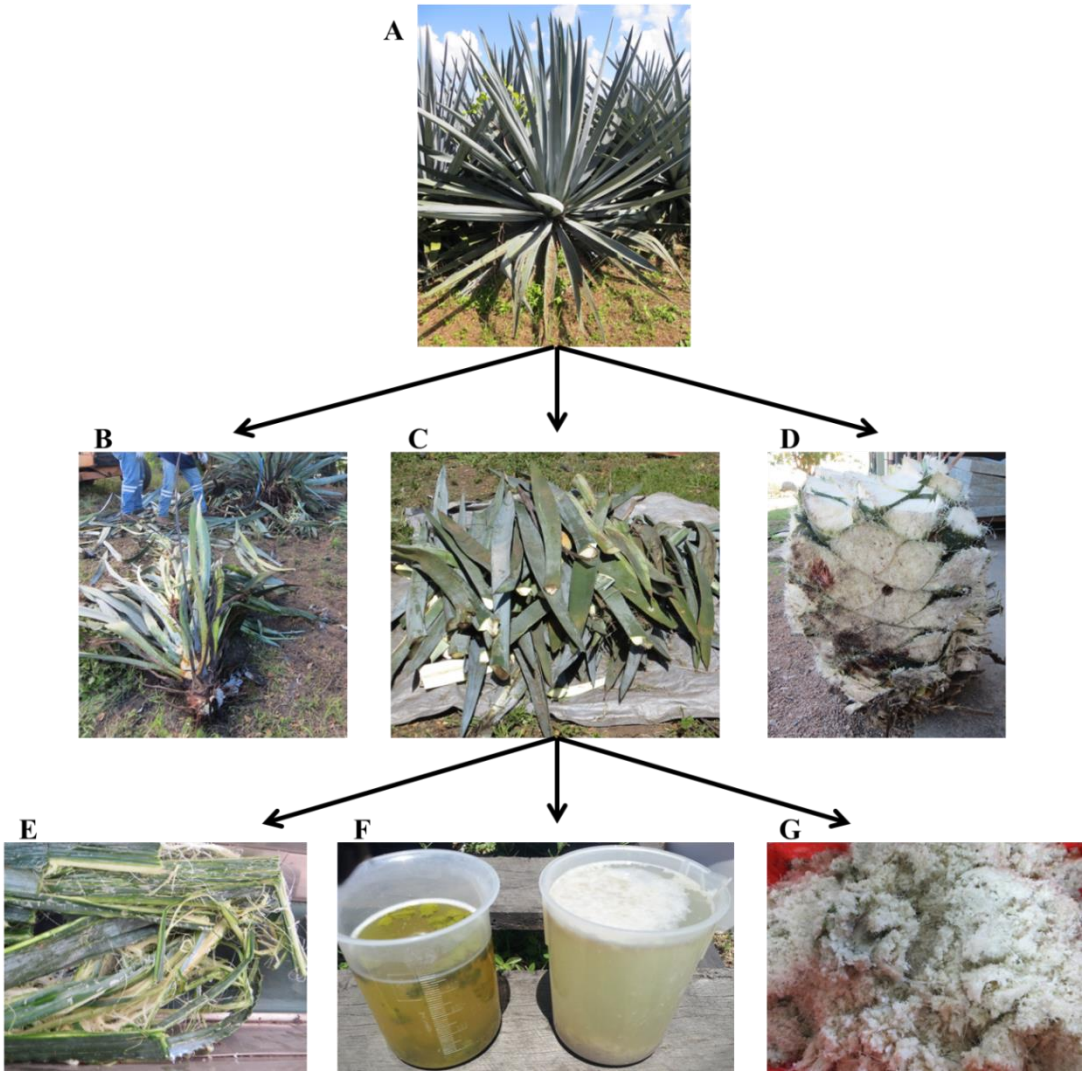

Figure P3-1 Diagram outlining the fractions that can be generated from processing of *Agave* plants

Whole units of *Agave* plants (A) were harvested, and divided into offshoots (B), leaves (C) and stem (D). Each of these fractions can be further processed to generate a cellulose-enriched fibrous bagasse (E), a fermentable juice (F) and residual biomass (G).

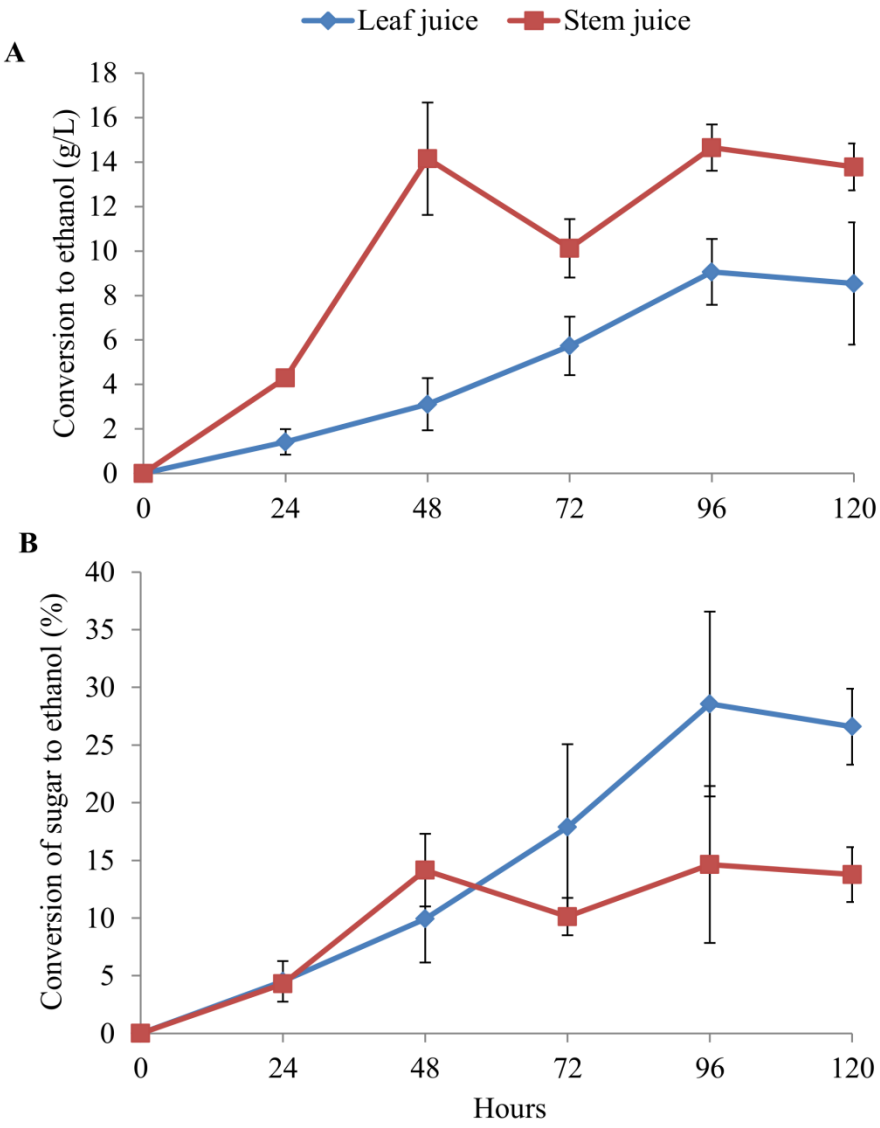

Figure P3-2 Spontaneous fermentation of *A. tequilana* juice

Raw *Agave* leaf and stem juice was fermented using only the native organisms present within the substrate; n=3. The production of ethanol (g/L) was higher in stem juice (A). However, when the conversion of carbohydrates to ethanol was considered the microflora of the leaf juice appeared to be more efficient (B). The total carbohydrate content in the leaf and stem juice was determined by HPLC (Table P3-3).

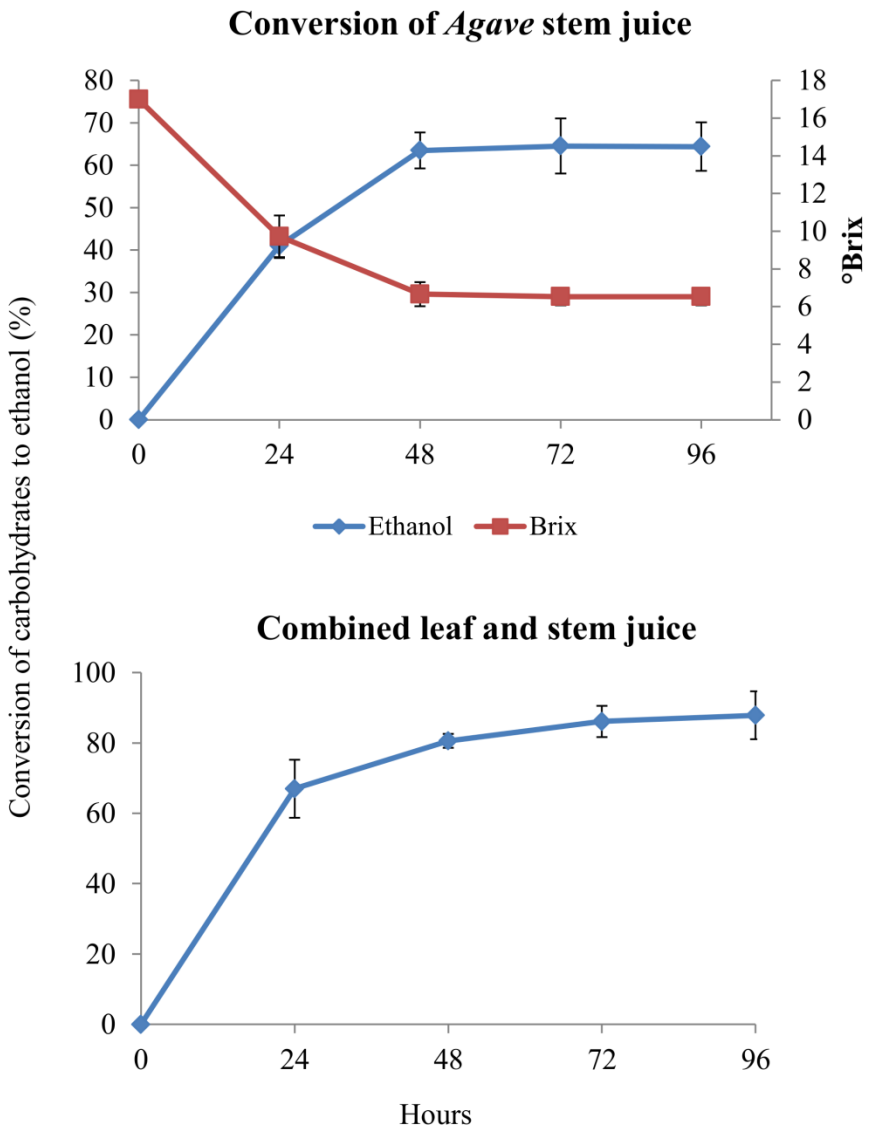

Figure P3-3 Conversion rates of total carbohydrates to ethanol using *Agave* stem juice

Kinetic profile of the fermentation of *Kluyveromyces marxianus* (*Km*1598) in autoclaved *A. tequilana* stem juice over time, 96 h, n=3 (A) and in combined leaf and stem juice (B). The autoclaved leaf and stem juice were combined in volumes equivalent to the mass distribution of a whole plant, namely 75% leaves and 25% stem, and fermented using *K. marxianus* (B). The fermentation of combined *Agave* juice from stem and leaf yielded 85% conversion of carbohydrates to ethanol.

Table P3-1 Selected fermenting strains and conditions using *Agave tequilana* as reported in literature

Substrate	Fermenting strain	Fermentation conditions	Sterilization	Sugar adjustments	Added nutrients	% conversion of carbohydrates to ethanol	Citation
Cooked *A. tequilana* stem	*Kluyveromyces marxianus* UMPe-1 *Saccharomyces cerevisiae* Pan1	20–28°C/72 h pH: 4–5	121°C for 15 min Filtered	YPD supplemented with high fructose *Agave* syrup Adjusted to 120 g/L fermentable sugars	Ammonium phosphate (60 mg/L) Ammonium salts (20 mg/L)	94–96 %; *K.marxianus* 70–76%; *S. cerevisiae*	[17]
A. tequilana stem juice source from tequila distillery	*Kloeckera africana* K1	30°C/80 h pH: n/a	121°C for 15 min Filtered	Adjusted to 100 g/L (reducing sugars)	Ammonium sulfate (220 mg N/l) or Mixture of amino acids, final concentration of 220 mg N/l	49%; supplemented with ammonium sulfate 94%; supplemented with Asparagine	[36]
Cooked *A. tequilana* stem (98°C for 12 h)	*Kloeckera africana* K1 *Saccharomyces cerevisiae* S1	30°C/24 h pH: 4.2	121°C for 15 min Filtered	Adjusted to 12° Brix with water (95 g/L reducing sugars)	Ammonium sulfate or Diammonium phosphate (1 g/L) or Yeast extract (2 g/L)	78%; *K. africana* 92–100%; *S. cerevisiae* Higher for fermentations supplemented with N	[37]
Cooked *A. tequilana* stem (100°C for 36 h)	*Saccharomyces cerevisiae* (S1or S2)	30°C/ length n/a pH: 2.5–4* *controlled using NaOH	121°C for 15 min	Adjusted to 100 g/L (reducing sugars)	Ammonium sulfate (1 g/L) Ammonium phosphate monobasic (4 g/L)	88%; no significant improvement due to pH, yields improved using aeration	[38]
Cooked *A. tequilana* stems (95–100°C for 4 h)	*Kloeckera africana* K1 *Kloeckera apiculate* K2 *Saccharomyces cerevisiae* (S1, S2 and S3)	35°C/72 h pH: 4.2	121°C for 15 min Filtered	Adjusted to 12° Brix (95 g/L reducing sugars)	Ammonium sulfate (1 g/L)	52%; *K. africana* 43%; *K. apiculate* 82–90%; *S. cerevisiae*	[16]

A. fourcroydes leaf juice combined with industrial molasses^	*Kluyveromyces marxianus* Cicy-Ki and/or *Saccharomyces cerevisiae*	35°C/48 h pH: 4.7	121°C for 1 h	Adjusted to 12° Brix* using varying ratios of juice + molasses *Sugar concentration in g/L not given	Ammonium sulfate (1.5 g/L)	5.2% v/v ethanol; 25/75 mix of *K. marxianus*/ *S. cerevisiae*	[15]

The definition of stem is used interchangeably with the terms pine, head, must and piña. Data was sourced from the following publications: [15–17, 36–38]. ^No information was provided regarding the source of molasses.

Table P3-2 Mass distribution and carbohydrate content of *Agave tequilana* juice

	Average mass per plant (kg)	% juice collected per fraction (w/w)	Total soluble carbohydrates in juice (% w/v)*	°Brix (w/v)	TSS (% w/v)
Mother plant					
Leaf	163 ± 24	68	6.0	9.6 ± 2.2	9.3 ± 2.1
Stem	60 ± 15	43	17.1	17.3 ± 3.8	17.4 ± 3.7
Offshoots	137 ± 50	27	4.0	7.9 ± 0.3	7.5 ± 0.5

The weight of biomass derived from *Agave tequilana* plants was recorded at the time of harvest (fresh wt; kg). The mother plant (4.5 y old) was separated into leaves, stem and root fractions and the younger plants (offshoots) removed. The majority of the above-ground mass of mother plants is attributed to the leaves at 73% w/w. Three different methods were used to determine the carbohydrate content in *Agave* juice; *HPLC (Table P3-3), Brix and total soluble solids (TSS). The density for all *Agave* juice samples is assumed to be 1.25.

Table P3-3 Analysis of *Agave* juice to quantify total carbohydrate content

Tissue	Treatment of juice	Fructose (g/L)	Glucose (g/L)	Sucrose (g/L)	Total (g/L)
Leaves	Raw	12.7 ± 2.7	25.6 ± 4.9	6.0 ± 4.5	**44.3**
	TFA	23.9 ± 6.0	35.9 ± 7.8	–	**59.8**
	Fructanase	22.4 ± 6.8	33.2 ± 10.0	–	**55.6**
	Autoclaved	15.2 ± 4.4	25.6 ± 4.5	4.6 ± 1.0	**45.4**
Stem	Raw	6.2 ± 1.2	11.3 ± 3.2	8.0 ± 5.6	**25.5**
	TFA	129.8 ± 32.5	39.8 ± 9.4	–	**169.6**
	Fructanase	132.0 ± 33.9	38.7 ± 8.1	–	**170.8**
	Autoclaved	34.3 ± 2.9	15.7 ± 1.1	25.2 ± 7.2	**75.2**
Offshoots	Raw	10.4 ± 3.4	15.1 ± 4.6	2.1 ± 0.2	**27.6**
	TFA	20.9 ± 7.3	17.3 ± 3.6	–	**38.2**
	Fructanase	22.5 ± 8.1	17.7 ± 3.4	–	**40.2**
	Autoclaved	12.2 ± 0.6	14.4 ± 4.5	2.7 ± 1.3	**29.4**

The amount of detectable soluble carbohydrates (glucose, fructose and sucrose) in *Agave* juice (leaf, stem and offshoots) is dependent on the treatment method employed: raw, TFA-treated, fructanase-treated and autoclaved juice samples. Data are presented as g/L; leaves and stem n=3; offshoots n=2.

Table P3-4 Selected microorganisms for fermentation of *Agave* juice

			Fermentation			Tolerance
Source	**Accession number**	**Organism**	**Glucose**	**Sucrose**	**Inulin**	**Ethanol**
Rotting *Agave sisalana* leaves	**1598* (CBS: 745)**	***Kluyveromyces marxianus***	**+**	**+**	**+**	**Growth**
Rotting *Opunita stricta*	**17228 (CBS: 7274)**	***Pichia kluyveri***	**+**	**n/a**	**n/a**	**Growth**
Jamaica sugarcane	7846	*Candida akabanensis*	+	+	+/-	Weak/delayed
Molasses	636	*Saccharomyces cerevisiae*	+	+/-	weak	Variable
Fermenting grapes	**139 (CBS: 1539)**	***Saccharomyces cerevisiae***	**+**	**+**	**n/a**	**Growth**

Five yeast were used for the fermentation of *Agave* juice. Organisms were sourced from the ARS Culture Collection (NRRL) [7]. Accession numbers for strains in NRRL database are listed first, followed by the corresponding number in the CBS database. Fermentation and tolerance data for microorganisms was obtained from CBS-KNAW Fungal Biodiversity Centre, [24]; bold text indicates data for the specific strain was available in the database. **Km*1598 has been reported to secrete fructan hydrolysing enzymes [25].

Table P3-5 Comparison of ethanol yields achieved from fermentation of *A. tequilana* leaf juice

		Ethanol yield(72 hr)	
Treatment	Microbial strain	Yield (g/L)	Conversion (% w/w of total sugars converted to ethanol)
Non-autoclaved + Inoculation	*Km*1598	23.0 ± 3.4	66.7 ± 0.4
	*Pk*17228	23.4 ± 0.1	68.7 ± 10.2
	*Ca*7846	24.8 ± 6.0	71.3 ± 6.5
	*Sc*636	13.7 ± 0.3	48.1 ± 1.5
	*Sc*139	14.6 ± 2.4	48.9 ± 3.7
Autoclaved + Inoculation	*Km*1598	26.1 ± 6.4	75.1 ± 7.4
	*Pk*17228	25.7 ± 4.2	74.9 ± 4.8
	*Ca*7846	26.9 ± 1.9	78.4 ± 6.3
	*Sc*636	19.5 ± 0.4	57.0 ± 7.6
	*Sc*139	16.8 ± 5.1	48.2 ± 7.2
Autoclaved + Indigenous microbes	*Km*1598*	16.8 ± 1.7	54.6 ± 4.9
	*Pk*17228*	18.9 ± 2.0	61.4 ± 6.1
	*Ca*7846*	18.3 ± 2.0	59.2 ± 6.7

Comparison of ethanol yields (g/L) and conversion efficiency (%) of five different yeast strains when cultured in raw and autoclaved *A. tequilana* leaf juice; n=2. In addition indigenous microbes were re-introduced into autoclaved leaf juice and fermented using non-*Saccharomyces* yeast; *n=3. Ethanol conversion are based on the carbohydrate content of TFA-treated leaf juice quantified by HPLC (Table P3-3).

Table P3-6 Predicted ethanol yields for *Agave tequilana*

Leaf	78820	4729	2412	48−78	1158−1881
Stem	18347	3142	1602	64	1025
Whole Mother plant			*4014*	85	3411

Calculations are based on 4000 *Agave* plants ha^{-1} using a juice density of 1.25. The productivity and carbohydrate content of *Agave tequilana* is calculated using real-harvest data (Table P3-2) and HPLC data (Table P3-3). ^ Maximum ethanol yields assume 100% efficiency of carbohydrate conversion to ethanol at 0.511 g ethanol/g sugar [9]. The total ethanol yield is divided by the age of plant (4.5 y old) and expressed as the value per year. Actual conversion rates were determined in this study to be 48−78% using leaf juice (Table P3-5) and 64% using stem juice (Figure P3-3a) but 85% when juice from the whole plant was used (Figure P3-3b). Italics indicate values derived from an additive calculation rather than direct measurement. *The predicted ethanol yields are based on the maximum ethanol yields that could be generated from each fraction and the conversion rates achieved in this study.

Supporting Information

Table P3-S1 pH and minerals in *Agave tequilana* juice

Tissue	pH	Minerals % (w/w)
***A. tequilana* leaves**[1]	4.6 ± 0.1	1.5 ± 0.8
***A. tequilana* stems**[1]	4.9 ± 0.1	0.8 ± 0.1
Offshoots[2]	5.0 ± 0.1	2.0 ± 0.1

Variation in the pH and mineral content was observed between the different juice samples collected from *Agave tequilana*.

Table P3-S2 Metabolite concentrations in raw, autoclaved and fermented (72 h) *A. tequilana* leaf juice

Treatment of juice	Strain	Acid compounds (g/L)				Glycerol
		Acetic	Citric	Lactic	Malic	
Raw leaf juice[1]	–	14.5 ± 9.5	3.9 ± 1.8	3.2 ± 1.1	15.8 ± 4.9	–
Fermented: non-autoclaved leaf juice + inoculation[2]	7846	17.5 ± 10.4	6.4 ± 4.2	17.4 ± 2.8	18.4 ± 8.0	1.2 ± 0.1
	1598	16.0 ± 9.1	5.5 ± 3.2	12.5 ± 0.8	16.7 ± 6.4	2.1 ± 0.6
	17228	16.8 ± 9.1	6.7 ± 2.1	21.0 ± 11.7	16.7 ± 5.6	1.6 ± 0.2
Autoclaved leaf juice[1]	–	5.6 ± 2.0	3.7 ± 1.4	9.2 ± 5.1	15.6 ± 5.9	–
Fermented: autoclaved leaf juice + inoculation[2]	7846	17.7 ± 11.8	6.5 ± 3.8	30.1 ± 2.6	21.3 ± 5.9	1.0 ± 0.5
	1598	18.3 ± 10.1	6.6 ± 4.0	18.1 ± 1.8	20.0 ± 8.7	2.4 ± 0.7
	17228	18.3 ± 11.4	6.6 ± 1.6	21.7 ± 5.7	21.5 ± 10.7	1.2 ± 0.9

Metabolite concentrations are presented as a mean ± SD of [1]three replicates; [2]two repl

Figure P3-S1 *Agave* juice naturally ferments

Fermentation of *Agave* stem juice was observed 6 h after the plant was harvested. Left beaker contains the starting juice and the right beaker contains juice left at room temperature for 6 h.

Table P3-S3 Thermo-tolerance of selected commercial yeast

Source	Strain	Organism	28°C	32°C	37°C	42°C
Agave or other succulent	**1598**	***Kluyveromyces marxianus***	+	+	+	+
	17228	***Pichia kluyveri***	+	+	+	–
High sugar content (sugarcane)	**7846**	***Candida akabanensis***	+	+	–	–
	636	***Saccharomyces cerevisiae***	+	+	+	–
High glucose/fructose content (grapes)	**139**	***Saccharomyces cerevisiae***	+	+	+	–

Relative thermo-tolerance of five yeast strains obtained from the ARS (NRRL) Culture Collection (2015) assessed by spotting a standardized cell concentration on YPD plates with incubation at 28, 32, 37 and 42°C.

12.0 Thesis Conclusions and Key Findings

The research described in this thesis explores the potential of utilizing agro-industrial residues derived from *Agave* and *Vitis vinifera* for biofuel production. Overall, *Agave* plants exhibit characteristics that are favorable for the production of hydrocarbon fuels: they have high biomass yields, are adapted to growing in sub-optimal regions under challenging environmental conditions, have low cost production systems and are not used for animal or human food. The current research demonstrates that *Agave* plants have compositional features that further increase their suitability, such as a low lignin content, high accumulation rates of soluble carbohydrates, easily hydrolyzed cellulose polymers and high ethanol yields (theoretical and actual) relative to other commonly studied lignocellulosic feedstocks (paper I and III). In contrast, grape marc carbohydrates are complex and the homogenized material is rich in the phenolic polymer lignin, making marc an unfavorable feedstock for bioethanol production in its raw form (paper II). However, because grape marc is generated as a waste product from a well-established industry, it could still generate a value-adding stream of income for grape growers and the wine industry, as currently the disposal of grape marc waste is incurred at a cost to vineyards.

The optimal conversion methods for *Agave* leaves and grape marc were determined by biochemical analyses, deconstructive processing and anaerobic fermentations. There were four focus areas: refining the techniques used for biomass processing; increasing monosaccharide availability; identifying efficient low cost pre-treatments; and ensuring complete utilization of sugars during fermentation.

12.1 Refining the techniques used for biomass processing

The impact of processing and fermenting *Agave* plants in different fractions or as a whole was investigated in this thesis. The data indicate that harvesting techniques will influence final biofuel yields. For the two selected feedstocks separation of the more recalcitrant tissue from fermentable sugars during harvesting could increase the efficiency of converting the biomass to ethanol. For example, the compositional data (paper I) indicated that *Agave* juice is rich in fermentable sugars that can be converted directly to ethanol with no additional inputs of nutrients or carbohydrates, using standard *S. cerevisiae* strains. Mechanically crushing the leaves to collect these fermentable sugars leaves a residual cellulosic bagasse fraction that could be utilized in an alternative processing stream for bioethanol or biochemical production.

In paper I and III, the processing of *Agave* biomass and juice is explored to assess ethanol yields under different harvesting scenarios. Predicted ethanol yields using extracted leaf juice from 3 yr old plants, 2350 L/ha/yr (paper I), is similar to the values predicted from 4.5 yr old plant, 2412 L/ha/yr (paper III). Allowing *Agave* plants to grow for another 1.5 yr did not significantly change the amount of extractable carbohydrates (48 g/L compared to 56 g/L); however the amount of biomass per hectare was increased. As a result, the total ethanol (predicted) that can be achieved using 3 yr old *Agave* leaf juice was lower (7 050 L/ha; paper I) than that of 4.5 yr old plants (10 854 L/ha; paper III). Ethanol yields can be further increased if *Agave* plants are grown specifically for biofuel production at a density of 4000 plants/ha, in which the juice derived from whole plants (stem and leaf; 4.5 yr) could generate yields of 15 350 L/ha.

Alcoholic fermentations involve complex chemical and enzymatic reactions either step-wise or simultaneously until conversion is complete, slows or is inhibited. As a result, conversion of carbohydrates to ethanol (1 g sugar converted to 0.51 g ethanol) is unlikely to be achieved at maximum efficiency and real-production and fermentation data is required. For the fermentation of *Agave* juice the production of ethanol was influenced more by the choice of fermenting organisms than the juice pre-treatment (paper III). This study demonstrated that ethanol yields are reduced if fermentation is reliant solely on endogenous microorganisms or if pre-treated juice becomes contaminated with a mixture of microbes that originated from the biomass itself. In paper I, the fermentation performance of two *Saccharomyces* yeast was investigated when cultured in autoclaved *Agave* leaf juice from 3 yr old plants. When the same yeast (*Sc*139 and *Sc*636) were cultured in juice from 4.5 yr old plants (paper III) the ethanol yields produced were lower, (66% of the predicted yield paper I compared to 57%, paper III). This variation in the fermentation performance is likely substrate induced and may be attributed to an increase in fructan complexity as the plants matures or a lack of nutrients. Further investigations showed that non-*Saccharomyces* yeast including *Kluyveromyces marxianus* and *Candida akabanensis*, are more robust than standard *Saccharomyces*, resulting in higher yields of ethanol.

It should be noted that the conversion efficiency (carbohydrate to ethanol) was greater, 85%, when leaf and stem juice were combined (paper III). Therefore, direct fermentation of the whole plant may produce higher yields than separating juice from different fractions and limit the time and cost incurred at the time of harvest or crushing. On-site crushing of *Agave* biomass may reduce the costs associated with transporting this moisture dense (up to 90% water) plant material from the field to the processing facility. However, proper storage and

handling of the juice is critical to preventing the occurrence of spontaneous or natural fermentations, which compromise ethanol yields (paper III).

12.2 Increasing monosaccharide availability and identifying efficient pre-treatments

A valuable source of carbohydrate is retained in the solid mass that remains after the crushing of *Agave* leaves or the extraction of soluble carbohydrates from grape marc. The compositional data reported in paper I indicated that the residual fibrous bagasse from *Agave* is a recalcitrant, complex matrix of cellulose and non-cellulosic polysaccharides. Complete hydrolysis of the cell wall components for both biomass sources was achieved using concentrated sulphuric acid. However, this methodology is not suitable for large-scale production. An alternative methodology was tested using unpurified cellulase preparations to hydrolyze cellulose polymers to glucose monomers. Liberation of glucose was achieved at nearly 40% efficiency using non-pretreated *Agave* leaf tissue, but this method could be further optimized if enzymatic hydrolysis were combined with a pre-treatment step (such as a dilute acid treatment.

Fructose is highly unstable relative to other monosaccharides such as glucose, even under moderate pre-treatment conditions, and is degraded into compounds which are toxic to many fermenting microorganisms [107, 108]. As a result, autoclaving was tested as a low-cost, low-input pre-treatment for *Agave* juice. In leaf juice samples no detectable change in monosaccharide extraction or composition is observed in autoclaved juice when compared with the raw juice (Table P3-3; paper III), and thus this standard practice of autoclaving *Agave* juice (paper III) offers no benefit unless a sterilization step is specifically required. In contrast to the data for *Agave* leaf juice, autoclaving was found to increase the amount of

monosaccharides in stem juice by 50 g/L (Table P3-3; paper III), and this may be related to loosening of the bonds within and between fructans by this moderate thermal pre-treatment.

Grape marc was found to be predominantly composed of non-cellulosic polysaccharides (homogalacturonan, xyloglucan and arabinoxylan) and the non-fermentable polymer lignin (paper II). To circumvent the need for costly processing of grape marc, soluble carbohydrates may be extracted using aqueous solutions (paper II). By extracting the soluble carbohydrates, one third of the dry weight of white marc (Sauvignon Blanc) could be directly fermented to ethanol. Fermenting these easily extracted carbohydrates could yield up to 270 L/tonne ethanol. After extraction of the soluble carbohydrates the recalcitrant biomass that remains may be pre-treated. The use of dilute acid pre-treatments increased the liberation of monosaccharides trapped in the cell wall remnants, relative to raw non-pretreated grape marc (paper II). Subsequent rates of hydrolysis were higher for grape marc samples that had been acid-treated. The thermal pre-treatments tested were ineffective at facilitating the solubilization of non-cellulosic polysaccharides and had no significant impact on the saccharification rate of grape marc. Another value adding prospect compatible with this process may be extracting the phenolic components (i.e. anythocyanins) from the grape marc (section 14.3).

12.3 Ensuring complete utilization of carbohydrates during fermentation

Historically, bioethanol studies have focused on the optimization of converting homogenous polymers such as starch and cellulose to hydrocarbon fuels using glucophilic yeast. The conversion of heterogeneous polymers and the identification of microorganisms

suitable for these substrates is an area of research still in its infancy. As a result, small-scale production of ethanol from *Agave* juice was investigated by exploring different processing methods and microorganisms (paper I and III). Raw *Agave* leaf juice was converted to ethanol at rates similar to those of autoclaved juice, indicating that this pre-treatment step had no benefit. The yeast strains isolated directly from the substrate (i.e. *Kluyveromyces marxianus*) were superior, in regards to their efficiency of fermentation of *Agave* juice, relative to the commonly used strain *S. cerevisiae.* However, spontaneous fermentation of the juice by endogenous microbes resulted in unreliable ethanol yields.

13.0 Future Directions

13.1 Trialing the scalability of bioethanol production from lignocellulosic residues

The scalability of using *Agave* or grape marc biomass to make bioethanol, that is the conversion potential of small amounts (grams) to large quantities (tonnes) of feedstock, was not investigated in this study. Further studies should combine data generated herein with other published literature to develop a technoeconomical model of *Agave* and grape marc processing for biofuel and biochemical production. Such a study would allow a more direct comparison to other lignocellulosic biomasses and help to identify "superior" biomass sources. In addition, it would highlight areas of production requiring further research and advancement. Technoeconomical modeling could be expanded to include a life cycle analysis (LCA) based on industry scale data from harvest, composition, conversion, and fermentation information. An LCA is needed to provide information about the environmental impact of using these feedstocks for bioethanol production from "cradle to grave" [109]. Field and industrial scale

production data need to be combined to identify bottlenecks in the production process. This information could underpin the development of large-scale bioethanol production that is energetically and financially profitable.

13.2 Investigating alternative conversion methods

It is unlikely that all cell wall components will be converted to ethanol in any particular process. However, there is scope for maximizing yields by optimizing fermentation and tailoring microorganisms to suit the substrate. For example, if *Agave* leaves are crushed at the time of harvest to extract the juice, a bagasse fraction remains that is rich in recalcitrant polymers such as cellulose. As a result, the carbohydrates in these polymers are inaccessible to fermenting microorganisms unless additional pre-treatment steps or enzymes are added. Alternatively, if whole leaves are fermented the selected microorganisms would need to be able to hydrolyze and ferment a complex cocktail of polymers such as fructans, non-cellulosic polysaccharides, cellulose and soluble carbohydrates simultaneously. In both scenarios a portion of the biomass and thus carbohydrates is likely to be unused. It has been suggested that for lignocellulosic biomass to play an important role in the future of chemical energy production, all cell wall components must be converted at maximum efficiency [14]. Considering this, future studies could test the conversion efficiency of grape marc and *Agave* using alternative methods that may be more robust, such as hydrothermal liquefaction (HTL).

Hydrothermal liquefaction is a thermochemical process, in which all the macromolecules of the biomass are reduced and/or degraded into smaller molecules, ultimately producing bio-oil [110]. The chemical properties of the bio-oil are dependent on the compositions of the starting biomass because each compound is converted into a distinct range

of hydrocarbon compounds [110]. However, pre-treatments are not required to remove the lignin or to make the carbohydrates more accessible, as is standard for fermentation assays. In HTL, lignin remains in the residue fraction and non-cellulosic polysaccharides are readily decomposed [111]. HTL may be beneficial for conversion of feedstocks with complex non-cellulosic polysaccharides such as grape marc that would alternatively require costly processing to obtain competitive ethanol yields. Unlike other processing methods, such as pyrolysis, HTL is suitable for biomasses with high moisture contents, such as *Agave* and grape marc, without the need for costly drying steps [111]. In addition, the use of moisture dense feedstocks is beneficial as the exogenous water required for thermochemical processing is reduced.

Further studies should also investigate the composition and complexity of each individual component in grape marc: seed, stem and skin. This study considered grape marc as a homogenized material, but depending on the harvest, storage, processing, and fermentation methods used both in the vineyard and winery the composition of this material may differ greatly. For instance, lignin or other non-fermentable compounds may be prevalent in a specific component (i.e. stems) and depending if the grapes were hand harvested or mechanically harvested the proportion of stems present would vary. Differences in pressing and crushing regimes are also likely to affect juice release and therefore the residual sugar in the marc. Further studies should investigate and compare the composition of grape marc and residual sugar content under various processing conditions. In addition, further studies should investigate how the length of fermentation (of red grape marc) affects the amount of ethanol that can be produced from this waste material. Such information would highlight areas of processing that require optimization and provide insight into the potential value of grape marc as a biofuel feedstock.

13.3 Mining the microbiome of lignocellulosic feedstocks

Plant biomass contains a trove of microorganisms that have symbiotic, commensal, competitive or parasitic interactions with each other, with the vegetative tissue, with root systems and/or with the soil. The study of microbial populations is hampered by the fact that less than 1% of microorganisms present in many natural environments can be cultured *in vitro* [112] and more advanced methodologies, such as Next-Generation Sequencing (NGS) are required to analyze the total prokaryotic and eukaryotic diversity within plant microbe populations. In brief, microbiome metagenomic analysis has two main levels of resolution, namely taxonomic profiling of microbial populations and microbiome shotgun sequencing of the genome of an entire microbial population.

Further studies should take into consideration the microbiome of *Agave* and grape marc with regards to employing appropriate harvesting, storage, transport and fermentation methods. It has been purposed that fungi that naturally deconstruct plant cell walls may provide the best enzymes for bioconversion of energy crops. For example, a recent study demonstrated that fungi isolated from decaying leaves of energy grasses were superior to the most widely used industrial bioconversion fungus *Trichoderma reesei* when applied to *Miscanthus* [113]. Thus, mining the natural microflora of *Agave* and grape marc could result in the isolation of microbes that outperform commercially available strains, where directly fermenting the biomass in its native form could obviate the need for inoculation.

Microbes from fructose-rich biomasses such as grape marc or *Agave* may be beneficial in alternative industries. For example, slow and incomplete consumption of sugars by yeast during grape fermentation is a prominent problem in wine making [114]. It has been suggested that a high concentration of fructose relative to glucose and depletion in nutrients result in

yeast having difficulty maintaining metabolic activity, and this results in sluggish, stuck or incomplete ferments [89]. Fructose competitively inhibits glucose uptake [89], so identifying microbes that can efficiently metabolize fructose may be beneficial in preventing stuck grape fermentations.

14.0 Appendices

14.1 Appendix A: Unpublished data paper I

Comparison of water-extraction methods

Four water-extraction methods were compared using leaves harvested from *A. tequilana*, as outlined in the Materials and Methods section (Table A-1). The extraction of glucose, fructose and sucrose was quantified by HILIC (paper I). The sums of WSC extracted under the selected conditions were similar and ranged from 10.3–11.0% w/w. Although variation was observed between the different treatments, this is negligible (Table A-1). The conditions (15 min, 80°C) used for Treatment 1 are the same as those used for fructan extraction according to Megazyme (paper I), and were thus chosen as the standard WSC (monosaccharides, disaccharides and fructans) method employed for *Agave* compositional analyses.

Table A-1 Comparison of four WSC extraction methods using *A. tequilana* leaves

Treatment	Glucose (% w/w)	Fructose (% w/w)	Sucrose (% w/w)	Sum WSC (% w/w)
1	4.6 ± 0.9	2.8 ± 0.5	3.0 ± 1.0	10.3 ± 1.0
2	5.0 ± 1.0	3.0 ± 0.8	2.6 ± 1.2	10.7 ± 0.9
3	4.3 ± 0.7	3.5 ± 0.5	3.0 ± 1.2	10.9 ± 1.4
4	4.8 ± 0.9	3.0 ± 0.6	3.2 ± 1.1	11.0 ± 1.2

The WSC content in *A. tequilana* leaves was extracted using four different methods and the carbohydrate content (glucose, fructose and sucrose) was quantified by HILIC; n=6.

Quantification of monolignols: S/G ratio

Agave leaves are considered to be low in lignin content (paper I) and highly pectinaceous (Figure A-1; Method described in section 10.2) when compared to other lignocellulosic feedstocks. Lignin is composed of *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) monomers which are methoxylated to varying degrees [115]. The composition of lignin varies between species, cell types, and individual cell wall layers and is influenced by developmental and environmental cues [116]. However, the monolignol pathway is highly conserved across the evolution of vascular plants [117]. In this study the quantification of monolignols was conducted by thioacidolysis and gas chromatography/mass spectrometry as described in section 10.3 (Materials and Methods).

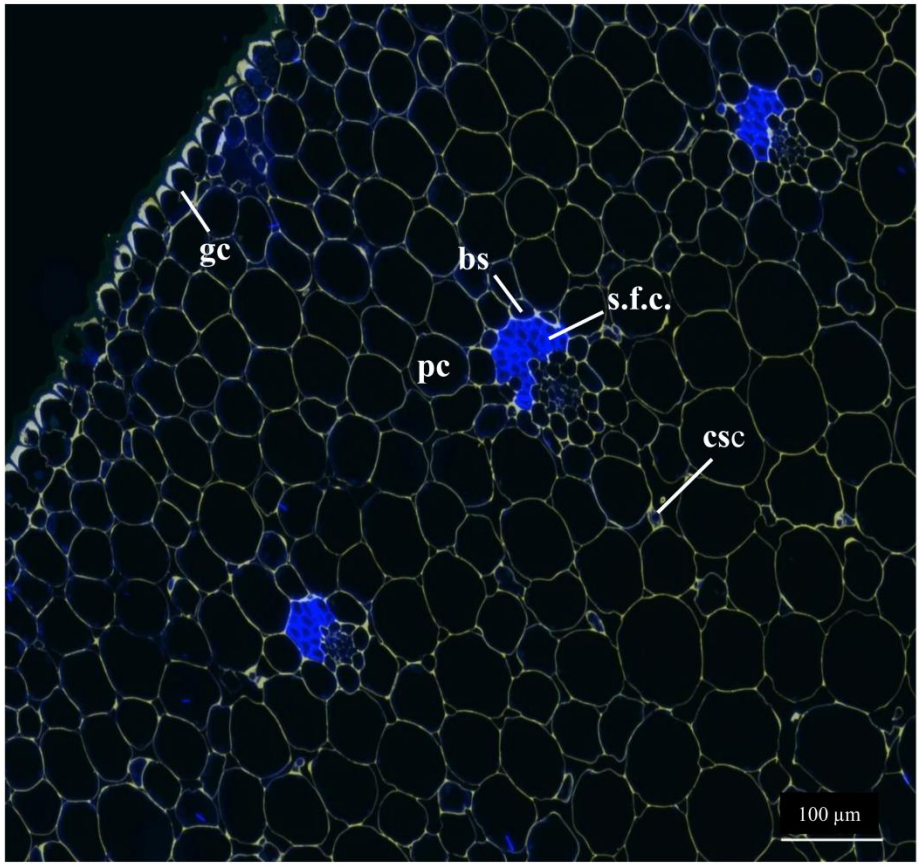

Figure A-1 Distribution of pectin labelling in an *A. tequilana* leaf section

Section labelled with LM19 against partially methyl-esterified homogalacturonan (pectin). Pectic polysaccharides were detected in most cell types in the leaf with the heaviest labelling observed in the sclerenchymatous fiber caps (s.f.c). Bundle sheath (bs); parenchyma cells (pc); guard cells (gc); cube shaped crystals (csc). Scale bar =100µm.

The S/G ratio for monolignols is important as it can be used as an indicator of how strong the carbon-carbon linkages are within and between lignin and other polysaccharides. For example, G units form highly condensed lignin that requires extensive processing and the use of expensive chemicals to remove [118]. Conversely, S units are linked via more labile ether bonds and are more easily degradable compared to linkages between G subunits [118]. Variability in the S/G ratio was observed between leaf and stem tissue of *A. tequilana* (Table A-2). The S/G ratio for *A tequilana* leaves and leaf bagasse was similar (4.7 and 4.9, respectively) and contained a higher proportion of S units (83%) when compared with the monolignol profile of stem bagasse (2.6; 72% S monolignols). Previously it has been shown that the negative influence lignin has on the conversion of plant biomass is less pronounced if the lignin is composed of a higher S/G ratio [119]. As a result, it can be concluded that the liberation of carbohydrates entrapped in the cell wall of *Agave* leaves may be more easily hydrolyzed than stem biomass.

Table A-2 Comparison of monolignol ratios (S/G) in *A. tequilana* biomass

Sample	S/G ratio	% S
Dried leaves	4.7 ± 0.2	82
Leaf bagasse	4.9 ± 0.1	83
Stem bagasse	2.6 ± 0.1	72

The monolignols sinapyl alcohol (S) and coniferyl alcohols (G) were quantified in *Agave* leaves and bagasse (leaf and stem) by thioacidolysis and gas chromatography/mass spectrometry, n=2.

Compositional analysis of leaf and stem bagasse

In paper I, the compositions of *Agave* juice and isolated fibers were characterized, but whole bagasse tissue, which includes fibers, residual juice, pith tissue and the waxy skin, was not considered. The composition of bagasse tissue collected from both *A. tequilana* leaf pressings and stem a pressing was investigated (Table A-3). Characterization of the soluble and insoluble components of the bagasse tissue was completed as outlined in paper I. For both samples there was a similar ratio of cell wall carbohydrates (about 37% w/w), extractables (about 20% w/w), and non-fermentable components (i.e. lignin, acetyl and minerals; about 25% w/w). However, within these categories, a higher percentage of glucan (25% w/w) and lignin (13% w/w) was measured in the leaf bagasse. Alternatively the stem bagasse was richer in xylan (14% w/w) and minerals (15% w/w) (Table A-3).

Table A-3 Mass balance of *A. tequilana* leaf and stem bagasse

	Leaf bagasse (% w/w)	**Stem bagasse (% w/w)**
***Extractable components**	**21.0 ± 3.1**	**24.3 ± 1.6**
***Insoluble components**	**63.3 ± 5.1**	**59.2 ± 1.7**
Carbohydrates	37.3 ± 3.6	33.2 ± 0.4
Glucan	25.3 ± 2.5	17.5 ± 0.4
Xylan	10.6 ± 1.1	13.6 ± 0.2
Arabinan	1.4 ± 0.1	2.1 ± 0.1
Acetyl	2.1 ± 0.3	2.4 ± 0.1
Lignin	12.7 ± 1.2	8.8 ± 1.3
Minerals (structural inorganics)	11.2 ± 0.2	14.8 ± 0.3
Biomass accounted for (%)	**84**	**84**

Data are presented as percentage dry weight (% w/w), n=2. Extractable components were not quantified in this study but include soluble carbohydrates, minerals, lipids, and proteins. The alcohol-insoluble residue was composed of carbohydrates, acetyl, lignin and minerals. * indicates values used to calculate the total biomass accounted for. Unaccounted- for mass is likely to include volatile compounds, lipids, waxes and unhydrolyzed polysaccharides.

14.2 Appendix B: Compositional analysis of *Agave sisalana* leaves

The species *A. sisalana* was also considered in this study, but these plants were not at the same stage of maturity as *A. americana* and *A. tequilana* and thus the data were not included in paper I. Historically, the agronomical practices and harvesting of the leaves from this feedstock were specialized for sisal (fiber) industries. However, approximately 0.6 M ha of land previously dedicated to plant-based fiber industries has fallen out of production, creating an opportunity for alternative markets for this underutilized biomass source [12]. Current predictions indicate that 6.1 billion L/yr of ethanol could be produced if areas previously sown with *Agave* were reestablished as a dedicated bioenergy feedstock [12]. To determine the practicality of using this feedstock for biofuel production, compositional characterization of the biomass is necessary.

Similar to the findings for *A. americana* and *A. tequilana* the majority of the mass of dry *A. sisalana* leaves was attributed to components that are soluble in aqueous solutions (Figure B-1). The most abundant monosaccharide detected in the alcohol insoluble residue (AIR) following acid hydrolysis was glucose (9% w/w). The non-fermentable components lignin (9.0 % w/w), acetyl (1.0 % w/w), minerals (7.1% w/w) and protein (7.0% w/w) also contributed to the total composition of this feedstock.

Linkage analysis, as described in paper I, was employed to distinguish the structure and relative percentage of cell wall polysaccharides present in the biomass. Cellulose was the most predominant polymer detected contributing 52 mol % (Table B-1). The non-cellulosic polysaccharides arabinoxylan and xyloglucan likewise contributed significantly, at 14 mol % and 11 mol %, respectively. One of the major differences observed between the compositions of *A. sisalana* compared to other species studied in paper I was a lower percentage of

homogalacturonan. However, methyl-esterified homogalacturonans were detected using antibody labelling (LM20) and TEM on the surface and the outer sheath of oxalate crystals that accumulated in the parenchyma tissue (Figure B-2). The distribution of arabinoxylan (LM11), (1,4)-β-mannan) and pectic polysaccharides (LM19 and LM20) were also identified in *A. sisalana* leaf sections by immuno-labelling and transmission electron microscopy (Figure B-3), and appeared similar to those of *A. americana* and *A. tequilana*. .

In addition, lower levels of soluble carbohydrates were detected in juice collected from *A. sisalana* leaves compared to the species examined in paper I. The carbohydrates present in *A. sisalana* juice were predominantly glucose, fructose and sucrose (Figure B-4). An enzyme specific for the hydrolysis of fructans (fructanase) was used to cleave higher molecular weight oligomers into its constituent fructose and glucose. Following enzymatic hydrolysis the total amount of detectable monosaccharides using HPLC was 28 g/L. However, only about 2 g/L of carbohydrate was attributed to these higher molecular weight polymers, rendering the extra processing step unnecessary. The lower levels of fructans detected in *A. sisalana* juice may be a characteristic inherit to this *Agave* species, which historically has not been selected for its fructans, but for its cellulose enriched-fibers (Table B-2). Alternatively, the low abundance of carbohydrates present in the juice may be attributed to the age of the plants, as fructans accumulate and become more complex as the plants age [120]. The method used for the quantification of the carbohydrates present in the juice and from isolated fibers is described in paper I.

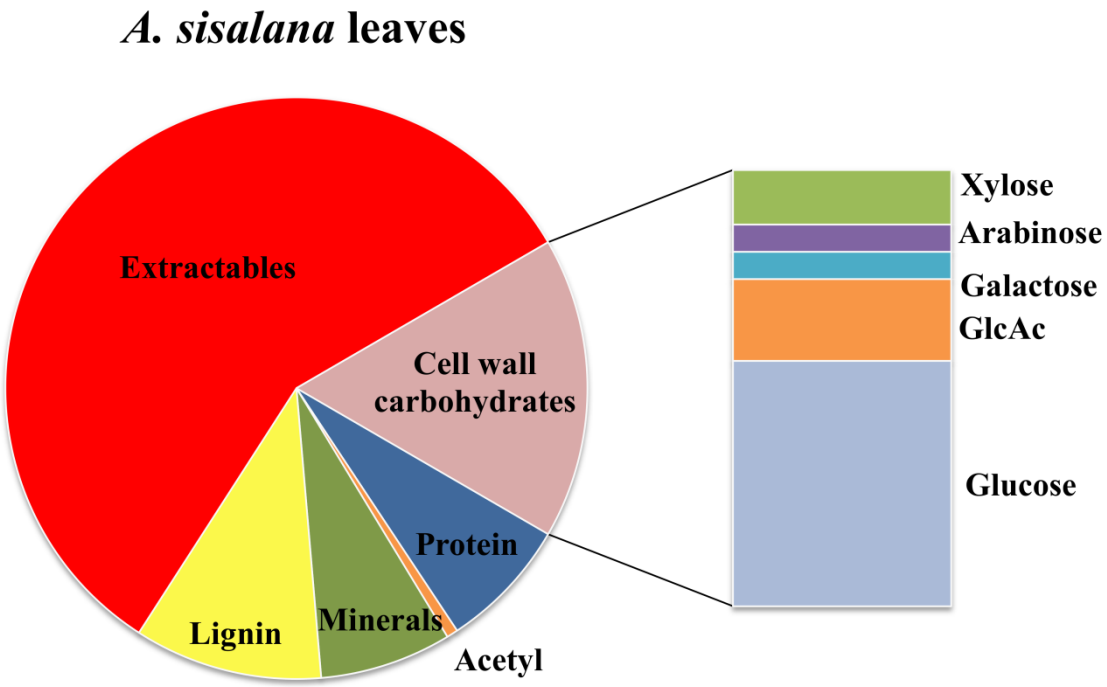

Figure B-1 Mass balance of *A. sisalana* leaves: soluble and insoluble components

Data are presented as percentage of dry weight (% w/w), n=5.

Table B-1 Linkage analysis of *A. sisalana* leaves

Polysaccharide	Mol % of polysaccharide	Derivative linkage	Mol % per linkage
Arabinan	5.3 ± 0.9	*1,5-Ara (f)* *1,2,5-Ara (f)* *t-Ara*	4.9 ± 0.8 0.3 ± 0.1 0.1 ± 0.1
Type I arabinogalactan	0.7 ± 0.6	*1,4-Gal (p)*	0.7 ± 0.6
Type II arabinogalactan	1.1 ± 0.1	*1,6-Gal (p)* *1,3,6-Gal (p)* *t-Gal*	0.5 ± 0.1 0.1 ± 0.1 0.5 ± 0.1
Arabinoxylan	14.0 ± 3.2	*1,4-Xyl (p)* *1,2,4-Xyl (p)* *1,3,4-Xyl (p)* *1,2,3,4-Xyl (p)* *t-Ara*	9.4 ± 1.4 0.8 ± 0.3 0.6 ± 0.4 1.4 ± 1.4 1.8 ± 0.5
Cellulose	52.4 ± 2.0	*1,4-Glc(p)*	31.9 ± 2.1
Heteromannan	7.0 ± 0.4	*1,4-Man (p)* *1,4-Glc (p)*	3.5 ± 0.3 3.5 ± 0.3
Homogalacturonan	4.5 ± 0.5	*1,4-Gal A (p)* *t-Gal A (p)*	4.0 ± 0.6 0.6 ± 0.1
Rhamnogalactan I/II	0.3 ± 0.1	*1,2,4-Rha (p)*	0.3 ± 0.1
Xyloglucan	10.7 ± 3.5	*1,4,6-Glc (p)* *1,4-Glc (p)* *1,2-Xyl (p* *1,2-Gal (p)* *t-Fuc (p)* *t-Xyl (p)*	3.9 ± 0.2 1.3 ± 2.4 1.3 ± 0.1 0.6 ± 0.2 1.2 ± 0.5 2.4 ± 0.4
Unassigned	3.9 ± 0.6		

Linkage analysis was used to quantify the cell wall polysaccharides in *A. sisalana* leaves (n=3). Data are presented as relative percent molarity (mol %). Linkages are assigned according to [121]. Unassigned linkages include the linkages measured where the polysaccharide of origin was not clear.

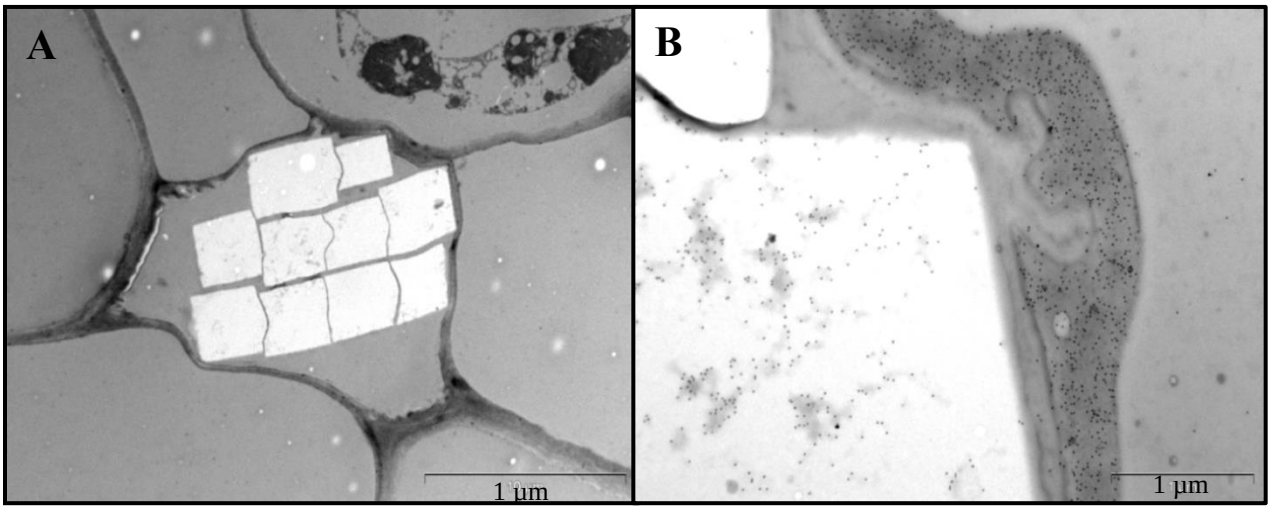

Figure B-2 *A. sisalana* leaves are enriched with calcium oxalate crystals

Transmission electron microscopy (TEM) image of oxalate crystal clusters located in the parenchyma cells (A). Labelling of methyl-esterified homogalacturonan (pectin) with LM20, was identified on the surface and the outer sheath of the crystals (B). Scale bars = 1µm.

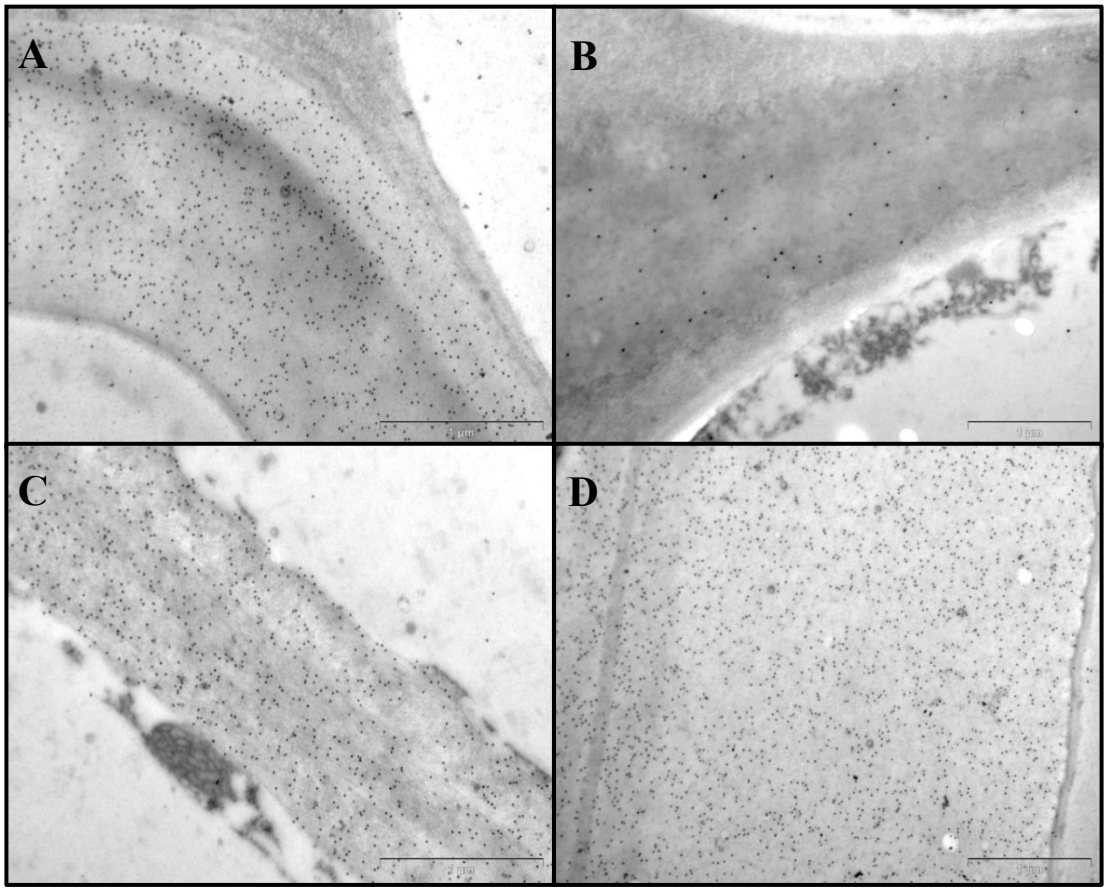

Figure B-3 Cell wall polysaccharides in *A. sisalana* leaves

Cell wall polysaccharides were detected by immuno-labelling and transmission electron microscopy. Phloem tissue labelled with LM11, an antibody specific to arabinoxylan (A). Inner epidermis tissue labelled with an antibody for (1→4)-β-mannan (B). Parenchyma cells labelled with LM20, an antibody for methyl-esterified homogalacturonans (C). Phloem tissue labelled with LM19, an antibody recognizing partially methyl-esterified homogalacturonan (D). Scale bars = 1µm.

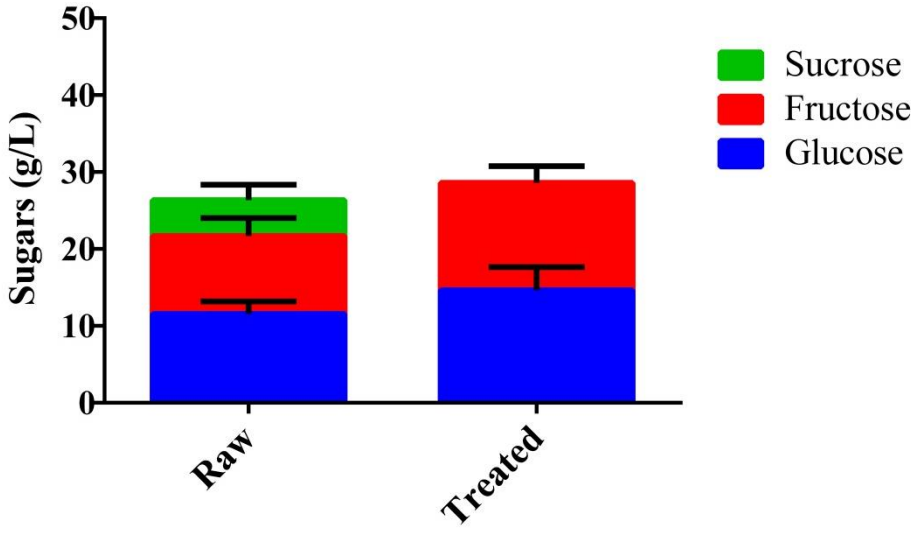

Figure B-4 Quantification of mono- and di-saccharides in *A. sisalana* leaf juice

The amount of glucose, fructose and sucrose present in both raw juice and fructanase treated juice samples. Data are presented as g/L, n=5.

Table B-2 Composition of fibers isolated from *A. sisalana* leaves

Crystalline cellulose	Glucose	Xylose	Arabinose	Other[1]
54.4 ± 2.3	3.0 ± 0.6	12.5 ± 0.7	0.4 ± 0.2	2.5

Values are presented as an average of three replicates.[1] Other cell wall components include: mannose, rhamnose, glucuronic acid, galacturonic acid and galactose.

14.3 Appendix C: Unpublished data paper II

Grapes contained significant amounts of polyphenolic compounds (Figure C-1) and pectic polysaccharides (Figure C-2). During the processing of grapes for wine making small amounts of polyphenolics are liberated from the skin, pulp and seeds [122]. However, it has been shown that approximately 60–65% of phenolic compounds remain in the grape pomace after red wine production [123]. Polyphenolic compounds include phenolic acids, flavonoids, anthocyanins and proanthocyanins [124]. The total phenolic content was measured using a modified Folin-Ciocalteau method (described in the section 10.4 Materials and Methods; [103]) and quantified as ferulic acid equivalents (FAE) (Figure C-1). The same extraction method was used for the quantification of anthocyanins in the grape marc using HPLC (Figure C-1).

Cabernet Sauvignon grape marc had a total phenolic content of 45.5 mg/g (FAE), which is about 30 mg/g lower than the previously reported value of 74.8 mg/g [125] but significantly higher than the value obtained in other studies, which were ~20 mg/g GAE [123]. The difference may be attributed to variation in growing conditions and/or methodology used (mg/g gallic acid equivalent vs. mg/g ferulic acid equivalents). This variation observed between studies (varieties of grape marc, extraction methods, and quantification) has previously been reported [126]. The average phenolic content for the two vintages of Sauvignon Blanc was lower at 27.0 mg/g (FAE), similar to findings elsewhere [123]. These compounds may be used as a precursor in phytochemical synthesis, an additive or food supplement (they have anti-oxidizing properties), a colorant in food products, or prescribed for disease prevention and suppression of existing medical conditions in humans (postprandial hyperglycemia), further increasing the value of this raw material [127-132].

The spatial distribution of pectic polysaccharides was observed by immuno-electron microscopy in white marc (Figure C-2). Sections of seed, skin and stem were labeled with antibodies specific to partially- (LM19) and fully-methyl esterified (LM20) pectic polysaccharides. The distribution of labeling varied between tissue types, but labeling of both types of pectins was observed in all fractions (Figure P2-2), with a higher concentration of labeling detected in berry skin. Previous studies have shown that the galacturonan content in the mesocarp of grape berries increases from 26–46% of cell wall polysaccharides and becomes more soluble as ripening progresses [21]. Souble pectins were quantified in the water extracts of the grape marc and contributed 4% and 9% w/w of the mass of Sauvignon Blanc and Cabernet Sauvignon, respectively (Table P2-1). Pectic polysaccharides were also detected in seed, skin and stem pieces of red marc (data not shown). The distribution of pectic polysaccharides in grape marc was investigated following the method described in section 10.5.

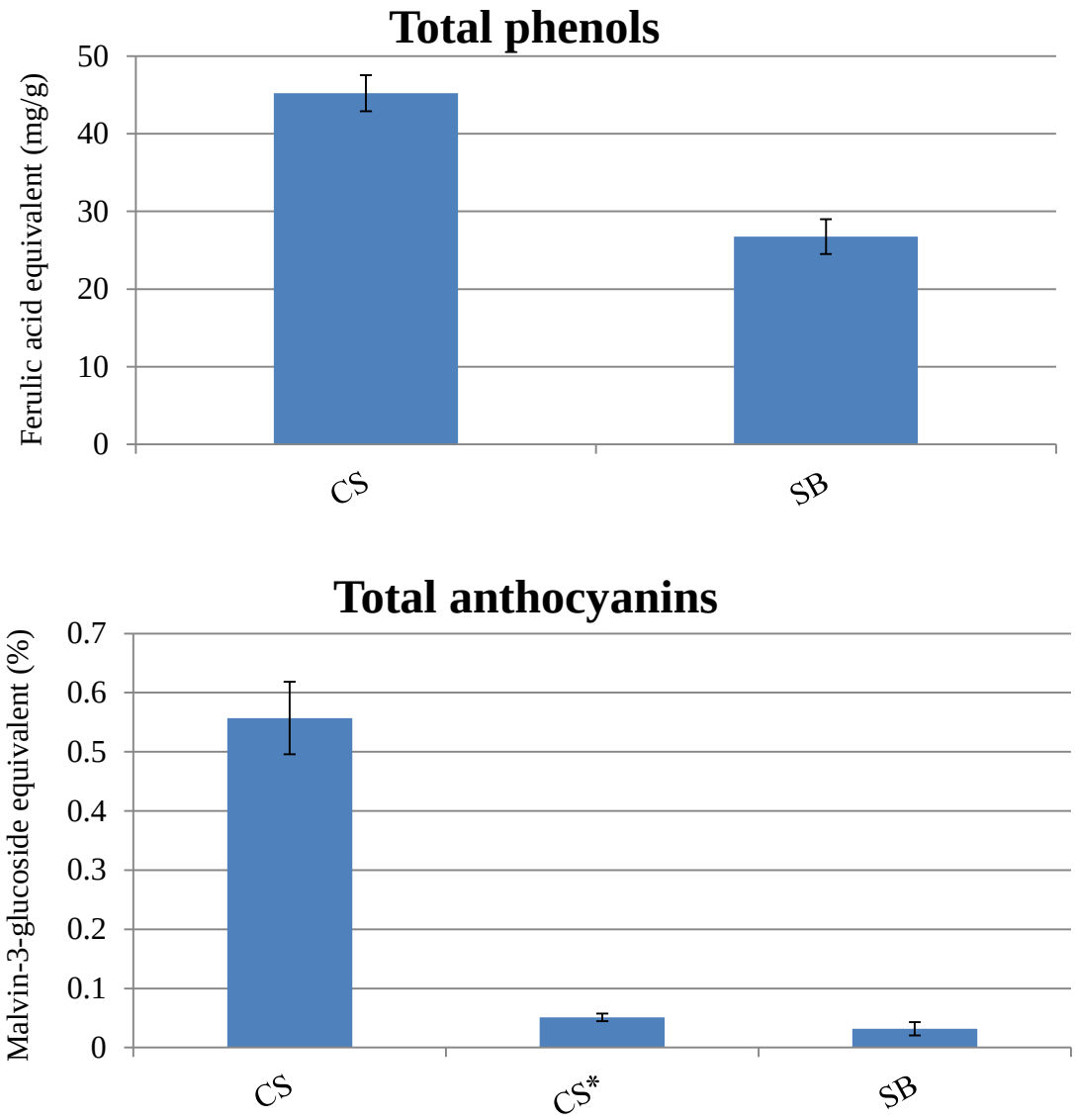

Figure C-1 Total phenolic and anthocyanin content in grape marc

The total phenolic content for Cabernet Sauvignon (CS) and Sauvignon Blanc (SB) grape marc expressed as ferulic acid equivalents (mg/g) n=2. The total anthocyanin content was determined using HPLC and is expressed as malvin-3-glucoside equivalent (%). Cabernet Sauvignon was extracted twice for quantification of anthocyanins, CS*.

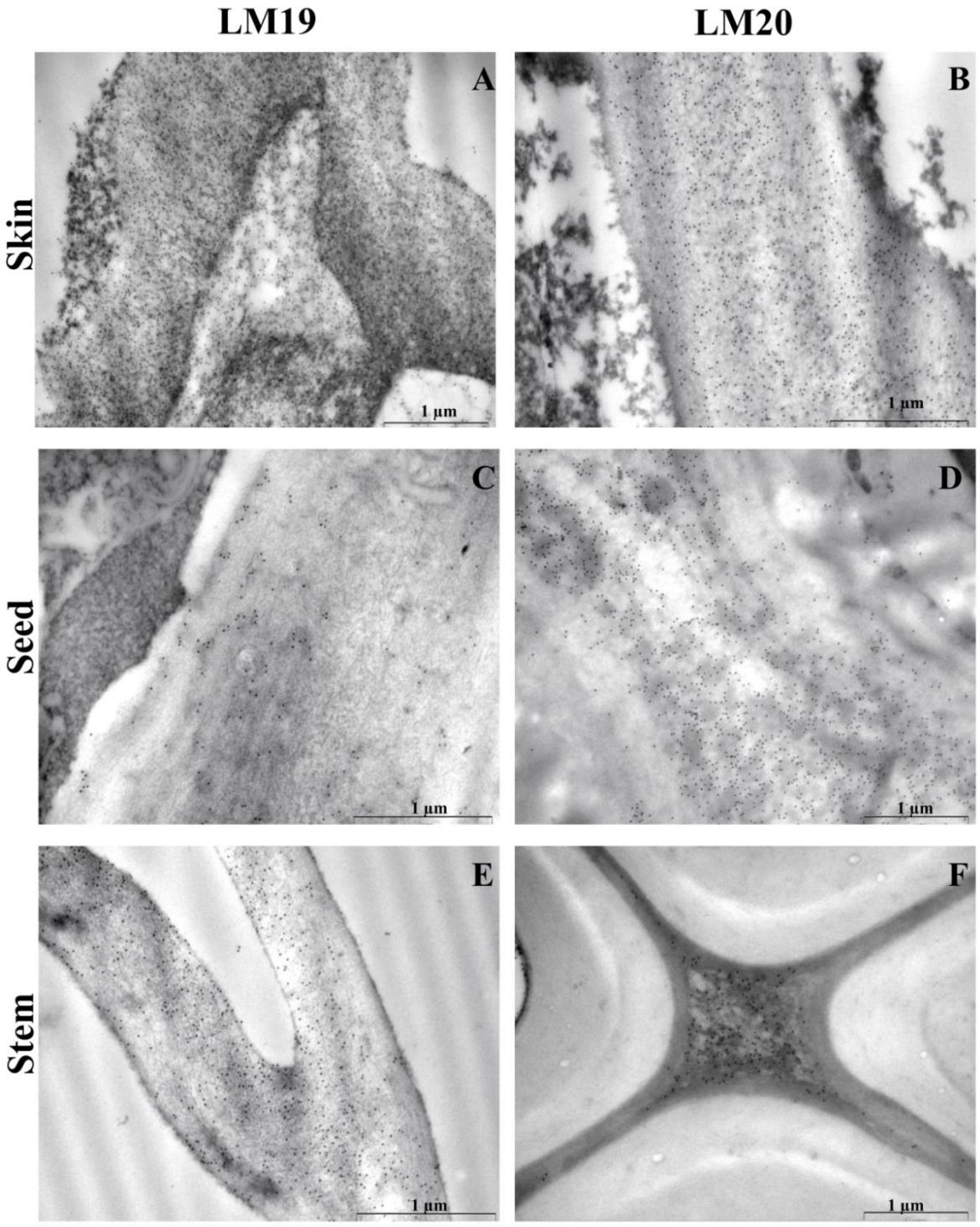

Figure C-2 Distribution of pectin labeling in Sauvignon Blanc grape marc

Partially (A) and fully (B) methyl-esterified homogalacturonan (pectins) were detected in the internal walls and epidermis of transverse sections of grape skin, respectively. Heavy labeling of pectic polysaccharides was detected using LM19 in the middle layer of Sauvignon Blanc seed sections (C) and the inner wall of the stem (E). Methyl-esterified homogalacturonans were labeled with LM20 in seed endosperm (D) and the internal schlerenchyma of stem sections (F). Scale bars = 1µm.

14.4 Appendix D: Additional data paper III

At the time of harvest, the masses of the *A. tequilana* plants (4.5 y old) were recorded (as described in paper III). The total biomass per plant was subsequently split into different anatomical fractions, namely leaves, roots and stems. In addition, the mass of the attached offshoots was recorded. Although all plants analysed in this study were cultivated in the same environment and were at the same stage of maturity, variability in the accumulation of biomass was observed between plants (Table D-1). In addition, the carbohydrate content in the juice extracted from each plant (paper III) and the composition of the mineral content (ash) was variable between the tissues (Figure D-1; method described in paper III).

Table D-1 Mass distribution is variable between plants of *A. tequilana* (4.5 y old)

	Plant 1 (kg)	**Plant 2 (kg)**	**Plant 3 (kg)**
Mother plant	***237.5***	***277.0***	***210.8***
Leaves	173.2	184.0	137.0
Stem	45.0	73.6	62.4
Roots	19.3	19.4	11.4
Offshoots	***189.1***	***107.0***	***110.2***
Total	**426.6**	**384.0**	**321.0**

Weights were taken of each plant at the time of harvest (fresh wt; kg). The total mass ranged from 321−430 kg per plant the majority of which was attributed to the leaves. The offshoots, which were attached to the mother plant, contributed a substantial amount of biomass (107−190 kg/plant); however the offshoots were all at varying stages of development making ethanol yields more unpredictable.

Figure D-1 Variation in the mineral content of extracted *Agave* juice

The composition of the minerals present in *Agave* juice appears variable between different tissue types; leaf juice (A), stem juice (B) and juice extracted from offshoots (C).

For the conversion of carbohydrates derived from *A. tequilana* to ethanol, different processing conditions and selected microorganisms were trialed under anaerobic fermentation conditions. Microorganisms were sourced from the ARS (NRRL) Culture Collection for fermentation studies [133]. The ten selected yeast (Table D-2) had been isolated either from *Agave* or substrates with high fructose content such as molasses and grapes. However the fermentative performance of yeast is substrate dependent, because yeast are unable to function effectively outside a narrow range of parameters and thus the fermentation performance of the yeast on pure carbon sources found in *Agave* juice was considered (Table D-2). In addition, ethanol can exert inhibitory effects on the yeast, such as increasing plasma membrane disruption, disrupting passive proton flux, damaging intracellular enzymes and causing cell death [87,91,92]. As a result, to obtain maximum ethanol yields careful selection of microorganisms is essential for the fermentation of *Agave* juice. A subset of five yeast was selected for the fermentation studies (Table D-2).

Table D-2 Microorganisms sourced from ARS Culture Collection for fermentation of *Agave* juice

			Fermentation			Tolerance
Source	**Strain**	**Organism**	**Glucose**	**Sucrose**	**Inulin**	**Ethanol**
Agave or other succulent	**1598**	***Kluyveromyces marxianus***	**+**	**+**	**+/-**	**Weak/delayed**
	17228	***Pichia kluyveri***	**+**	**+/-**	**−**	**Growth**
	10965	*Pichia cactophila*	weak	−	−	Growth
	12918	*Pichia deserticola*	−	−	−	Growth
High sugar content (sugarcane)	**7846**	***Candida akabanensis***	**+**	**+**	**+/-**	**Weak/delayed**
	636	***Saccharomyces cerevisiae***	**+**	**+/-**	**weak**	**Variable**
High glucose/fructose content (grapes)	**139**	***Saccharomyces cerevisiae***	**+**	**+/-**	**weak**	**Variable**
	6682	*Kluyveromyces lactis*	+/-	+/-	−	Variable
	1774	*Pichia kluyveri*	+	**+/-**	**−**	Growth
	17672	*Pichia mexicana*	weak	weak	−	Growth

Ten microorganisms were selected for the fermentation of *Agave* juice. Organisms were sourced from the ARS (NRRL) Culture Collection [133]. Fermentation and tolerance for microorganisms was previously studied CBS-KNAW Fungal Biodiversity Centre [134]. Bold text indicates the five strains that were selected for further fermentation studies.

The ten selected yeasts were screened to determine their thermo-tolerance as described in paper III (Figure D-2) and spotted on pure carbohydrates to test ability to utilize selectec carbohydrates (Figure D-3). Another indicator for fermentation performance (and rate) is the accumulation of yeast biomass under fermentation conditions [135]. When cultured in glucose the non-*Saccharomyces* yeasts (*Km*1598, *Pk*17228 and *Ca*7846) were found to accumulate biomass more rapidly than *Saccharomyces* yeast (*Sc*636 and *Sc*139) (Figure D-4). The method used to investigate biomass accumulation of yeast is outlined in section 10.6 Materials and Methods. It was noted that when the selected strains were cultured in autoclaved *Agave* juice (method described in paper III) the fermentation performance (conversion of carbohydrates to ethanol) of the non-*Saccharomyces* strains was superior to *Saccharomyces* yeast at all time points measured (24−72 h) (Figure D-5).

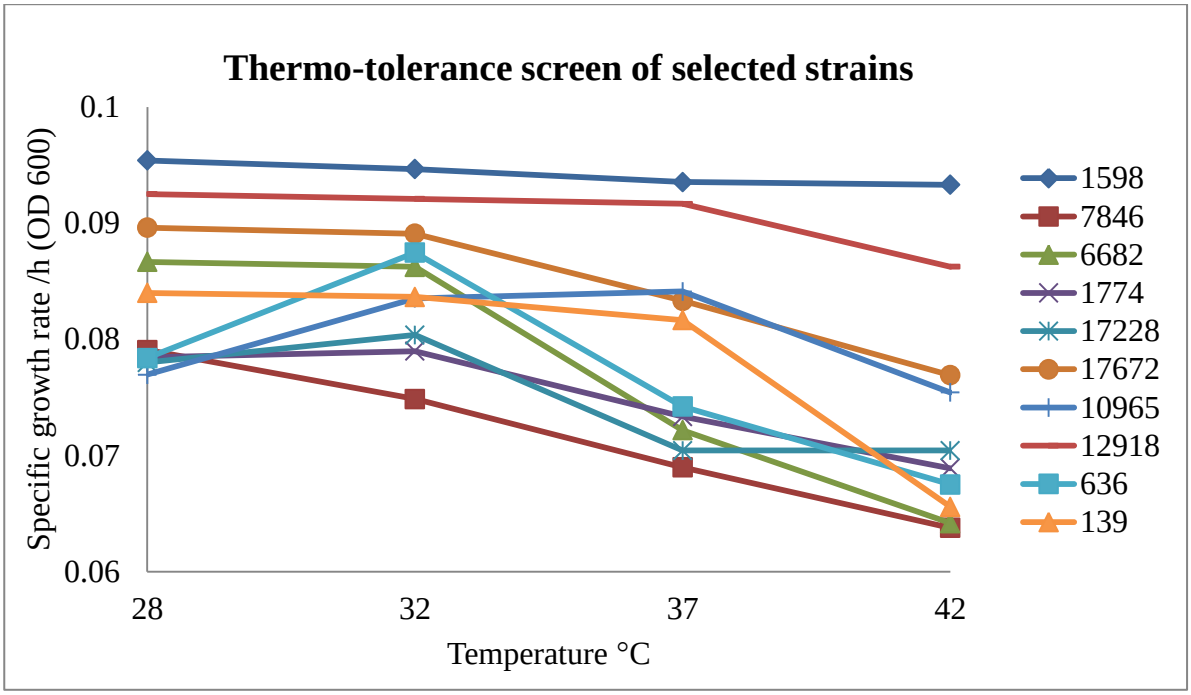

Figure D-2 Identifying the optimal growth temperature for selected microorganisms

A standardized cell count for each strain was inoculated into liquid culture (YPD) and grown at four different temperatures (28, 32, 37 and 42°C) to investigate mesophilic tolerance and to identify optimal growth conditions. An increase in cell density (OD reading) was observed for all strains grown at ambient temperature up to 28ºC, and growth held stable or moderately increased when grown at 32ºC. However, above 32ºC there was a significant decrease in the turbidity of cultures. Only one strain was identified to have a stable growth pattern over the range of temperatures investigated (28−42ºC), *Km*1598, which was originally isolated from rotting *Agave sisalana* [133].

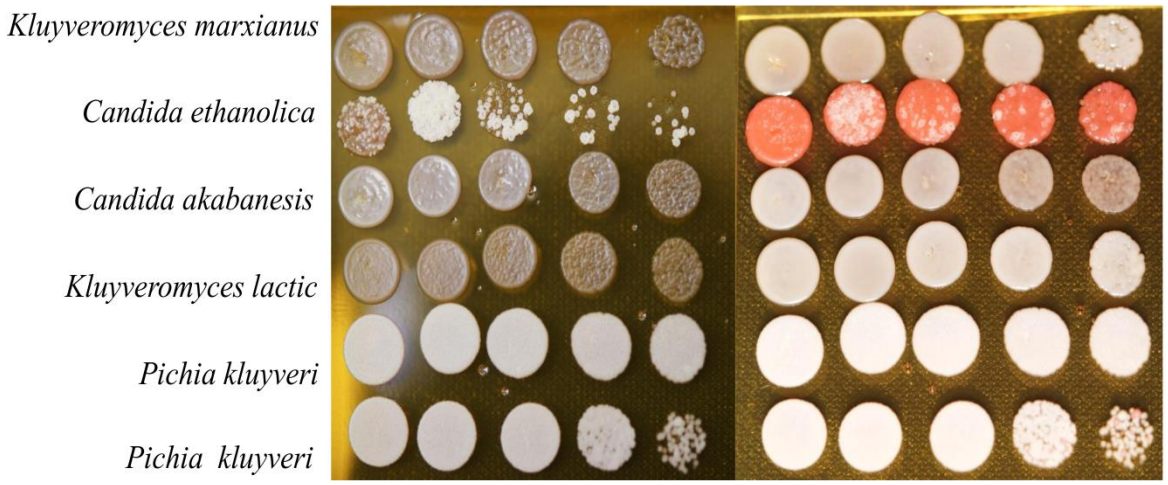

Figure D-3 Example of carbon source utilization by selected yeast strains

Selected microorganisms were spotted by serial dilution on induction medium supplemented with a sole carbon source. Growth was observed for all strains on glucose at 28°C (A). However, when plates were stored at 4°C contamination (pink colour) was visible in the spots of *Candida ethanolica* (B). Contamination is likely to be due to the yeast *Rhodotorula.*

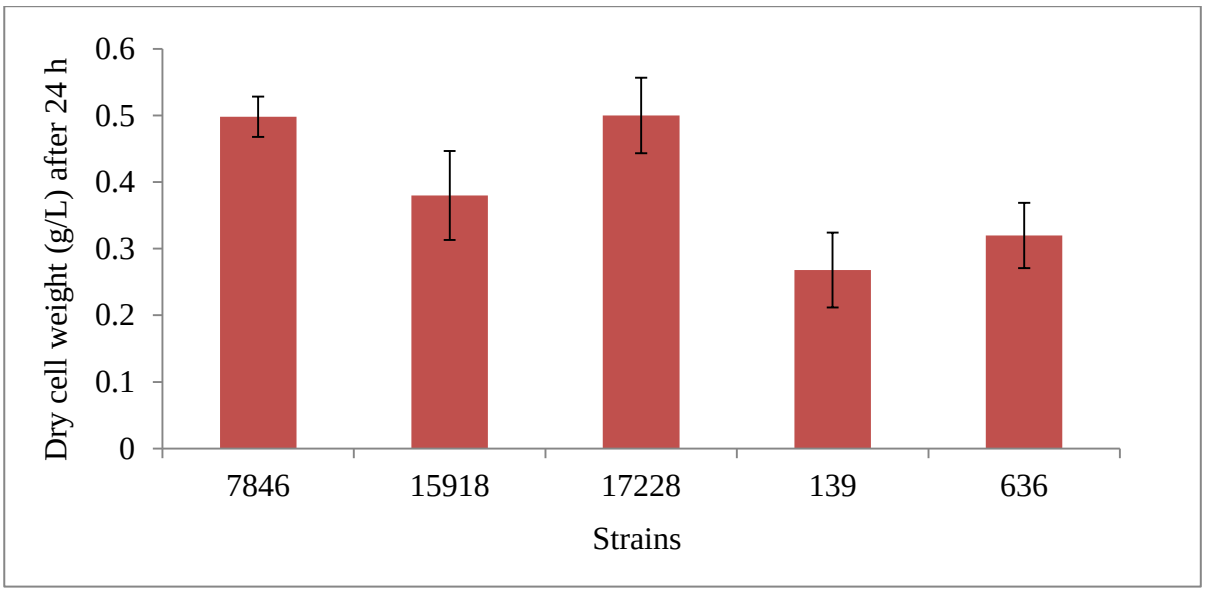

Figure D-4 Dry weight of cell pellets of selected strains after 24 h growth

The accumulation of biomass after growth at 28°C and in the presence of a sole carbohydrate (glucose) was measured for selected strains; n= 2.

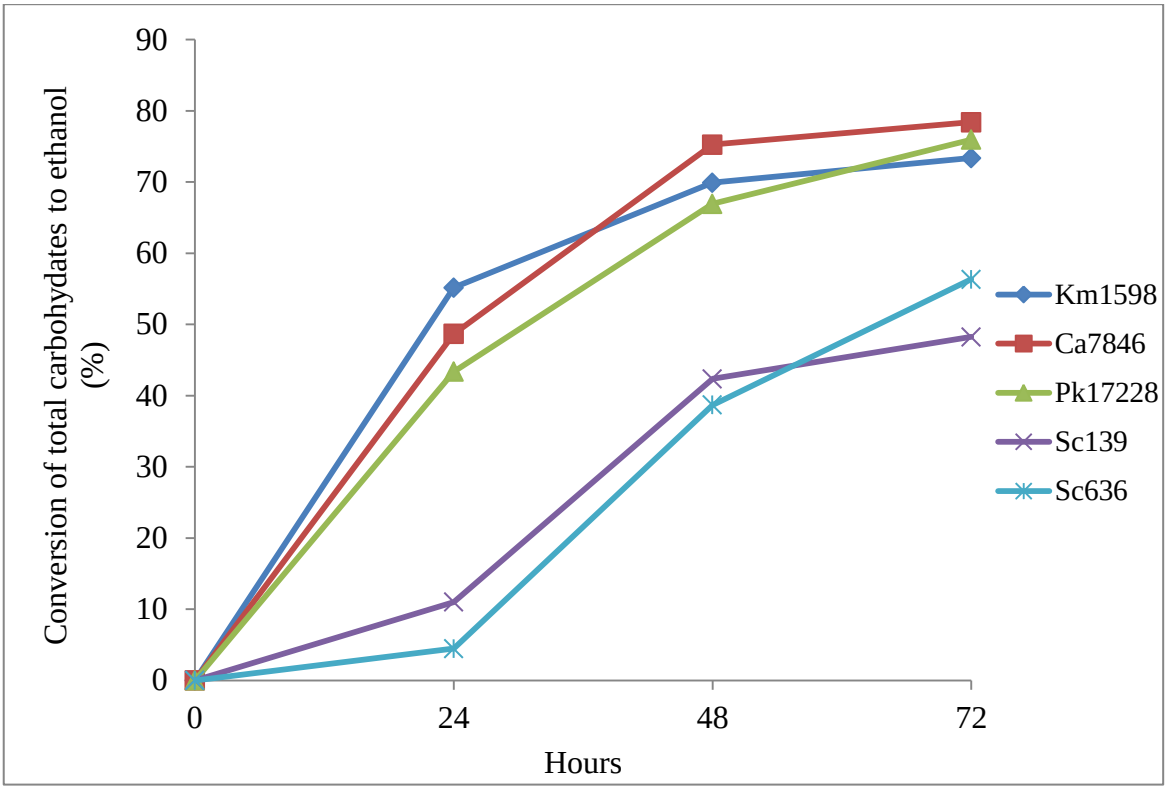

Figure D-5 Fermentation profiles of selected yeast in autoclaved *A. tequilana* leaf juice

At all the time points assayed (24, 48 and 72 hrs) the rate of ethanol production was greater in fermentations using non-*Saccharomyces* yeast (*Km*1598, *Pk*17228 and *Ca*7846) compared to *Saccharomyces* yeast (*Sc*636 and *Sc*139).

14.5 Appendix E: Published Paper I

PLOS | ONE

RESEARCH ARTICLE

Prospecting for Energy-Rich Renewable Raw Materials: *Agave* Leaf Case Study

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Abstract

Plant biomass from different species is heterogeneous, and this diversity in composition can be mined to identify materials of value to fuel and chemical industries. *Agave* produces high yields of energy-rich biomass, and the sugar-rich stem tissue has traditionally been used to make alcoholic beverages. Here, the compositions of *Agave americana* and *Agave tequilana* leaves are determined, particularly in the context of bioethanol production. *Agave* leaf cell wall polysaccharide content was characterized by linkage analysis, non-cellulosic polysaccharides such as pectins were observed by immuno-microscopy, and leaf juice composition was determined by liquid chromatography. *Agave* leaves are fruit-like—rich in moisture, soluble sugars and pectin. The dry leaf fiber was composed of crystalline cellulose (47–50% w/w) and non-cellulosic polysaccharides (16–22% w/w), and whole leaves were low in lignin (9–13% w/w). Of the dry mass of whole *Agave* leaves, 85–95% consisted of soluble sugars, cellulose, non-cellulosic polysaccharides, lignin, acetate, protein and minerals. Juice pressed from the *Agave* leaves accounted for 69% of the fresh weight and was rich in glucose and fructose. Hydrolysis of the fructan oligosaccharides doubled the amount of fermentable fructose in *A. tequilana* leaf juice samples and the concentration of fermentable hexose sugars was 41–48 g/L. In agricultural production systems such as the tequila making, *Agave* leaves are discarded as waste. Theoretically, up to 4000 L/ha/yr of bioethanol could be produced from juice extracted from waste *Agave* leaves. Using standard *Saccharomyces cerevisiae* strains to ferment *Agave* juice, we observed ethanol yields that were 66% of the theoretical yields. These data indicate that *Agave* could rival currently used bioethanol feedstocks, particularly if the fermentation organisms and conditions were adapted to suit *Agave* leaf composition.

Introduction

Plant biomass is a source of chemical energy that can be converted to combustible transport fuels and biochemicals by fermentation or chemical conversion of plant-derived sugars [1]. Currently, plant materials from farming-intensive food production systems, such as corn, wheat grain or cane sugar, are being used to make bioethanol and biochemicals. In the future, alternative sources of energy-rich plant material from low-input systems that are independent from the food chain will be needed [2,3].

Plant biomass contains soluble and structural sugars: for example the vacuoles of storage cells in the stem of sugarcane contain high concentrations of sucrose, a soluble disaccharide and the cell walls in the trunks of willow trees contain a large amount of cellulose, a structural sugar composed of glucose [4]. The composition of historical agriculture plant species have been reported (Table 1; [5]); however, the relative importance of plant species is likely to change as agricultural industries adapt to new markets and climate change. Research into novel plants may reveal non-food sources of valuable raw materials. One example of a plant species that is likely to gain importance is *Agave*. Historically *Agave* has been used for production of alcoholic beverages, fibers, chemicals and sugar additives [6] and there is growing interest in using *Agave* for biofuel production.

Alcoholic beverages such as tequila and mescal are made from the stem tissue of *A. tequilana* plants that are 8–12 years old. Fructans in mature stem tissue are degraded by heat to release fermentable fructose [7] and the leaves, which account for up to 66% dry weight of the biomass, are discarded [8]. *Agave* is a productive water-use efficient plant that grows in regions with extreme environments [9–11] and recent literature has considered the potential for using *Agave* as a feedstock for bioethanol production [12–18]. However, the composition of *Agave* leaf tissues from plants at an earlier stage in development has not been well characterized and may represent an energy-rich raw material that can be produced rapidly in a low-input system [19,20].

There are standard protocols for determining the composition of plant biomass, such as the analytical procedures published by the United States Government National Renewable Energy Laboratory (NREL) [21–26]. Biomass composition analyses may include determination of moisture content, total solids, acid-soluble and insoluble residues and the amount of water soluble carbohydrates (WSC), starch, mineral, lignin, protein, crystalline cellulose and non-cellulosic polysaccharides. In the context of using biomass to make biofuels and biochemicals, it is of interest to determine not only the amount of fermentable sugars that can be

Table 1. Comparison of potential biofuel feedstocks.

Species	Common name	Tissue	Cellulose (% w/w)	Non-cellulosic polysaccharides (% w/w)	Lignin (% w/w)
Zea mays	Corn	Stover without cobs	31–38	19–25	17–21
Triticum aestivum	Wheat	Whole plant	33	23	17
***Saccharum* spp.**	Sugarcane	Bagasse	32–43	12–26	23–28
Sorghum bicolor	Sorghum	Whole plant	23	14	11
Panicum virgatum	Switchgrass	Whole plant	30–35	24–28	17–20
***Populus* spp.**	Hybrid poplar	Whole tree without leaves	41–43	17–20	24–28
***Agave* spp.**	Agave	Whole residue from tequila brewing	31	17	17

Cellulose is the major source of glucose in feedstocks. Non-cellulosic polysaccharides contribute some fermentable hexose (glucose and galactose) and pentose (xylose and arabinose) sugars. Lignin is a non-sugar polymer that inhibits cell wall degradation and subsequent fermentation. Data are presented as percentage of dry weight (% w/w). Data may be accessed through the United States Department of Energy, Energy Efficiency & Renewable Energy, Biomass Feedstock Composition and Property Database, 2013 [5].

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extracted from plant biomass, but also the amount of inhibitory compounds that are formed during processing which may interfere with conversion of the biomass to bioethanol [27]. For example, acetic acid is generated from the hydrolysis of acetyl groups associated with non-cellulosic polysaccharides. Weak acids like acetic can reduce yeast growth and ethanol yields by prohibiting monosaccharide metabolism and causing intracellular anion accumulation [27]. In addition, the compositions and proportions of sugar present in soluble forms and structural forms, and the recalcitrance of these structural sugars are important as they influence the processing methods and costs. These data are also used to estimate the bioethanol yields for a feedstock of interest.

Here, the composition of *Agave* leaves is determined, including a detailed analysis of the fermentable and non-fermentable compounds in *A*. *americana* and *A*. *tequilana*. The efficiency of enzymatic hydrolysis of *Agave* leaf cellulose and hydrolysis of fructans in juice samples is quantified. Compositional data is then extrapolated to calculate theoretical ethanol yields and *A*. *tequilana* leaf juice is fermented using two *Saccharomyces cerevisiae* strains. These compositional and fermentation data can be used to inform the development of biotechnology to exploit this energy-rich raw material.

Material and Methods

Plant material

A. *tequilana* and *A*. *americana* plants were approximately 2–3 y old at the time of harvest and had begun to reproduce asexually. The heights of the plants from the base to the tip of the tallest leaf were at least 2 m. Six plants of *A*. *tequilana* were harvested from Ayr (Queensland, Australia) and six plants of *A*. *americana* were harvested from the Adelaide Hills (South Australia, Australia). From each individual plant stem tissue and at least three leaves were collected. Permission for the described field studies were granted by either the crop manager or land owner.

The stem and leaves were separated at the time of harvest and fresh weights recorded. Juice from the stem tissue of each *A*. *tequilana* plant was collected after shredding (Cutter-Grinder CG03, Jeffco) and three leaves per plant (*A*. *americana* and *A*. *tequilana*) were collected for compositional analysis. A subset of the remaining leaves was pooled and two experimental shredders were used to extract juice (Cutter-Grinder CG03, Jeffco and Food processor, Abode). Wet bagasse was dried at 60°C to a constant moisture content. Juice and whole leaves were transported to the University of Adelaide on dry ice and stored at –80°C. Prior to analysis, samples were cut into 200–400 mm^2 pieces, weighed, lyophilized (Labconco-Freezone, Missouri, United States) and moisture loss was calculated. Dried leaf material was ground in a 25 mL stainless steel grinding jar with one 7 mm steel ball. The grinding jars were shaken at 30 Hz for 3 min (Retsch mill MM400, Retsch GmbH; Haan, Germany). A flowchart of methods employed for compositional analysis is included in Fig 1.

Fiber extraction. Whole leaves were frozen at –80°C and subsequently thawed at room temperature. Fibers were pulled from three plants of each species and separated from the vegetative tissue manually. The fibers were further cleaned using forceps to remove any attached pith tissue. Fibers (1–2 mm) were dried overnight at 60°C. Dried fibers were hydrolyzed using 1M sulfuric acid (H_2SO_4) for 3 h at 100°C [28], cooled and centrifuged at 28 000 g for 5 min. The monosaccharides in the supernatant were analyzed using high-performance liquid chromatography (HPLC). Derivatisation and quantification of monosaccharides was completed according to [29] with modifications to the gradient conditions. Elution was performed with 10% acetonitrile, 40mM ammonium acetate (A) and 70% acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient for solvent B is as follows: 0–9.5 min, 8% B; 9.5–10 min, 17% B; 10–11.5 min, 100% B; 11.5–14.5 min, 8% B.

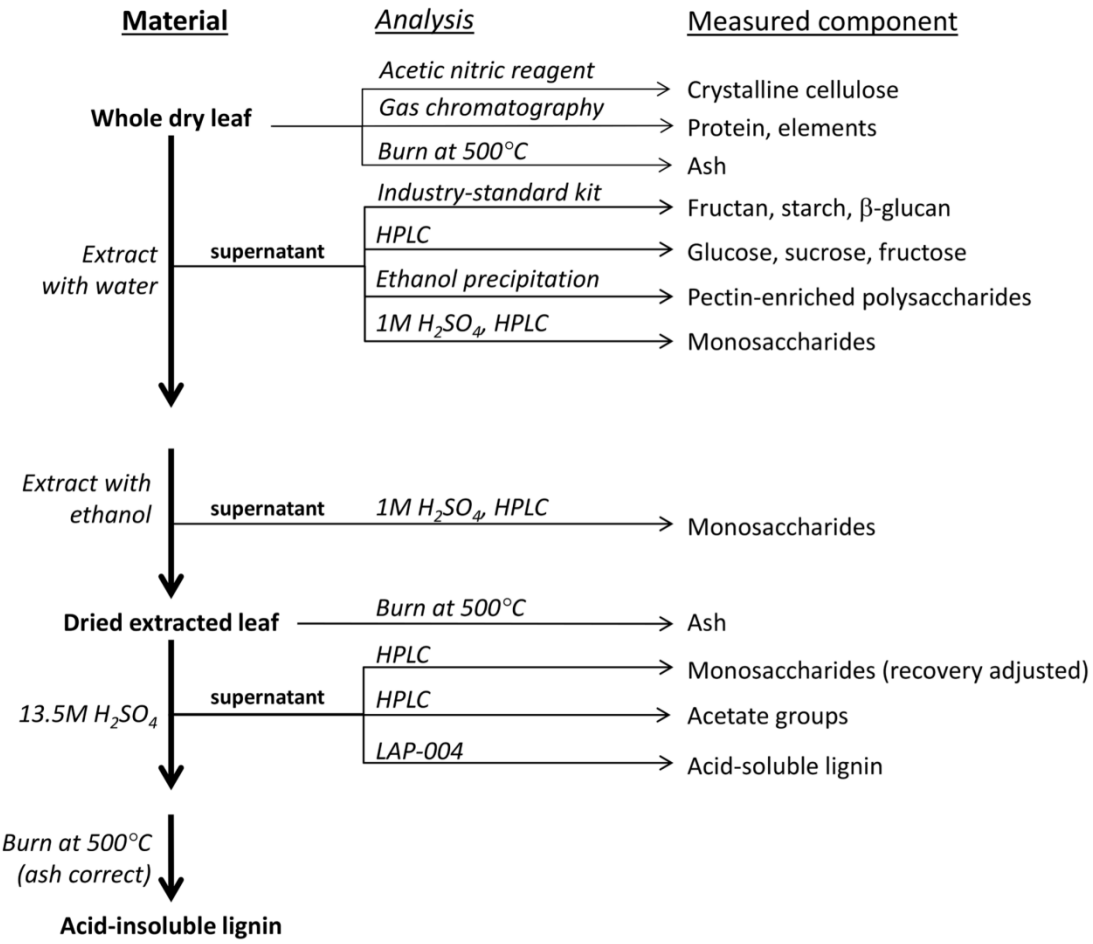

Fig 1. Flowchart outlining the steps taken to process and analyze *Agave* leaves.

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Measurement of leaf composition

Total soluble solids (TSS) in *Agave* juice. Aluminum pans (Fisher Scientific, Australia) were dried at 60°C and their initial weight recorded. Juice samples were centrifuged at 10 000 g for 10 min and 2 mL aliquots of supernatant were added to the pans and heated at 60°C for 48 h, leaving a solid residue in the pan. The final weight of the pan and solid residue was subtracted from the initial weight to calculate the total soluble solids (TSS).

Crystalline cellulose. Crystalline cellulose in leaf tissue and fiber-enriched samples was determined using a modified Updegraff method according to [30].

Elemental analysis and protein and mineral (ash) quantification. Samples for the elemental analysis included 300 mg of dry, ball milled, whole leaf tissue or 1 mL of juice. Elements

(Al, Ca, Fe, Mg, P, K, Na, S and Zn) were measured using a closed tube nitric acid/hydrogen peroxide digest and radial view inductively couple plasma-optical emission spectrometry [31].

The total nitrogen content was measured by the Waite Analytical Services, University of Adelaide using complete combustion gas chromatography (Carlo Erba Instrument) and 100 mg of biomass or 1 mL of juice. The nitrogen value was converted to an estimate of the protein content using the nitrogen factor (NF) 6.25 [21]. Mineral content of extracted and non-extracted material was calculated by heating samples to 500°C for 3 h [22].

Water- and ethanol-soluble carbohydrates in *Agave* leaves. Leaf samples were dried at 60°C and extracted sequentially in water, 95% v/v ethanol and 70% v/v ethanol at 80°C for 15 min using a 1:5 ratio of biomass to extraction liquid. The residual biomass was dried at 60°C.

The total fructan and (1,3;1,4)-β-glucan content in water extracts was measured using commercial assay kits (Fructan HK-Megazyme: AOAC Method 999.03 and AACC Method 32.32 and AACC Method 76.13, Mixed-Linkage Beta-Glucan-Megazyme: AACC Method 32–23, AOAC Method 995.16, EBC Methods 3.11.1, 4.16.1, 8.11.1 and ICC Standard Method No. 166; International Ireland Ltd., Wicklow, Ireland), respectively.

Glucose, fructose and sucrose in water extracts were measured by hydrophilic interaction chromatography (HILIC), using a Prevail Carbohydrate ES column (150 × 4.6 mm) (Alltech; Illinois, United States) on an Agilent 1200 series liquid chromatography instrument equipped with an evaporative light scattering detector (Alltech ELSD 800). The mobile phase consisted of water (A) and 90% acetonitrile (B) at a flow rate of 1.0 mL/min at 20°C. The gradient for solvent B is as follows: 0–18 min, 94.5% B; 18–19 min, 64.5% B; 19–20 min, 0% B; 20–30 min; 94.5% B. The pectin-enriched polysaccharide content in water extracts was determined using an ethanol precipitation method according to [32].

Solvent was removed from water and ethanol extracts separately by centrifugal evaporation (Savant SC110 Speed Vac, Thermofisher; Massachusetts, United States). The concentrated material was hydrolyzed using 1M sulfuric acid (H_2SO_4) for monosaccharide analysis using HPLC, as previously described [29].

Measurement of structural carbohydrates, lignin and acetyl content. For compositional analysis, samples were extracted using an Automated Extraction System (ASE) following [23]. *Agave* leaves (cut to 2–4 mm in size); aluminum pans and Whatman GF/C 55 mm glass microfiber filters (Sigma-Aldrich, United States) were dried at 105°C. Extraction cells (11 mL) were fitted with pre-weighed filter paper and 1 g of dried material added. Material was extracted with three water cycles followed by three 190 proof ethanol cycles at 100°C (ASE300, Dionex). Extraction settings were modified to 60 s nitrogen purges following extraction, 5 min static time and 120% rinse volume. Following extraction the remaining alcohol insoluble residue (AIR) and filter paper were placed in pre-weighed aluminum pans and dried at 105°C. Dried, extracted biomass was ground using a Retsch mill MM400, as previously described. The percentage of extractables was calculated based on the difference between the initial weight (before water and ethanol extraction) and final weight (after extraction).

Following extraction the alcohol insoluble residue was analyzed following [24]. Briefly, a 30 mg sample of dried ground material was treated with 13.5M sulfuric acid at room temperature for 1 h. The samples were diluted to 0.75M acid and autoclaved at 121°C for 15 minand centrifuged for 10 min at 10 000 g. The supernatant was collected for monosaccharide, acid-soluble lignin and acetate analyses. A sugar recovery standard for monosaccharides was carried through the acid hydrolysis as outlined in [25]. Monosaccharides were measured following derivatisation as previously described using HPLC. The acid-soluble lignin content was measured using a spectrophotometer (Thermo Fischer, Waltham, MA, USA) set at a wavelength of 205nm and calculated following LAP-004 using the extinction coefficient value 110 L/g-cm [26]. The acetyl content in the supernatant was analyzed at 60°C using an Aminex HPX-87H

column (300 x 7.8 mm) (Bio Rad; California, United States) on a 1100 series liquid chromatography instrument. Elution was performed isocratically with 2.5mM H_2SO_4 at a rate of 0.5 mL/min [33]. Starch was measured in extracted samples following a commercial assay (Total Starch-Megazyme: AOAC Method 996.11; International Ireland Ltd., Wicklow, Ireland).

The residual biomass was washed to a neutral pH and filtered through pre-dried and pre-weighed Whatman GF/C 55 mm glass microfiber filters (Sigma-Aldrich, United States). The filter paper and collected sample residue was heated to 105°C overnight and weighed (*M1*). The material was ash corrected by heating at 500°C for 3 h and weighed (*M2*). The lignin content was calculated based on the difference between *M2 –M1* divided by the initial weight.

Linkage analysis of cell wall residue in whole leaf. Lyophilized leaf material was ground in a 25 mL stainless steel grinding jar with one 7 mm steel ball. The grinding jars were shaken at 30 Hz for 3 min (Retsch mill MM400, Retsch GmbH; Haan, Germany) until all cells were ruptured. Samples were extracted sequentially with 80% v/v ethanol on ice, and acetone and methanol at room temperature. Samples were digested with α-amylase (*B. licheniformis*; EC 3.2.1.1) to remove starch. Linkage analysis and carboxyl reduction of the material followed [34].

Enzymatic saccharification

For saccharification, Celluclast 1.5 L (cellulase preparation from *Trichoderma reesei*) and Novozyme 188 (cellobiase preparation from *Aspergillus niger*) (Sigma-Aldrich; St Louis, MO, USA) were mixed in equal volumes. Enzymatic activity of the cellulase cocktail was measured according to the National Renewable Energy Laboratory (NREL) analytical procedure, Measurement of Cellulase Activities (LAP 006) [35]. The saccharifications used an enzyme concentration of 60 filter paper units (FPU). Alcohol insoluble cell walls were prepared according to [36]. Modifications to the micro scale saccharification were made using equivalent amounts of 0.02 g cellulose for all samples (NREL; LAP 009) and the total reaction volume reduced to 1.5 mL [37,38]. The glucose concentration was measured using a Yellow Springs Instrument (YSI) glucose analyzer (Yellow Springs, OH, USA) over 48 h, n = 3.

Analysis of hydrolyzed juice fraction

Samples of diluted, centrifuged, juice were treated with trifluoroacetic acid (TFA) to a final concentration of 0.2M TFA or fructanase (Fructan HK-Megazyme: AOAC Method 999.03; International Ireland Ltd., Wicklow, Ireland). For the TFA hydrolysis, juice and acid were mixed in equal proportions and samples were heated at 80°C for 1 h. For enzymatic hydrolysis, juice and enzyme mix were combined in equal proportions and samples incubated at room temperature for 30 min, then heated to 100°C for 15 min to deactivate the enzyme. Carbohydrates in the raw and treated juice samples were measured by HILIC, using a Prevail Carbohydrate ES column (150 × 4.6 mm) as previously described.

Microscopy

Fresh tissue was fixed in a solution of 0.25% glutaraldehyde, 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS) for 24 h at 20°C. Samples were washed twice with PBS, dehydrated in an ethanol series, infiltrated in LR White resin (ProSciTech Pty Ltd, Australia), and polymerized in a gelatin capsules at 58°C for 48 h [39,40].

Light microscopy. Embedded *Agave* leaf tissue was sectioned at 1 μm using a diamond knife on a Leica Ultracut R microtome. Sections were collected and dried onto poly-L-Lysine-coated microscope slides and stained with either toluidine blue (Sigma-Aldrich, United States) or methylene blue/basic fuchsin (ProSciTech Pty Ltd, Australia). Sections were viewed using a

Leica light microscope (Version 4.3) and images captured with a Zeiss M2 Axio Imager fitted with an MRm Rev. 3 AxioCam.

Immuno-electron microscopy. Ultrathin sections of 70–90 nm were collected on collodion-coated nickel grids and labeled following Aurion Immunogold Specific Localisation Methods [41] using the primary antibodies LM19 (diluted 1/20), LM11 (diluted 1/500), LM20 (diluted 1/20) (Plant Probes, UK), or (1→4)-β-Mannan (diluted 1/50; Biosupplies, AU) [42–44]. Diluted (1/30) secondary antibodies goat-anti-rat IgM (LM19, LM11 and LM20; Jackson ImmunoResearch Labs Inc., USA) and goat-anti-mouse IgG (Mannan; ProSciTech, Australia) were used. Labeled sections were examined and imaged using a Philips CM100 Transmission Electron Microscope.

Preparation of inoculums, fermentation conditions and analysis

Two *Saccharomyces cerevisiae* strains (Y-139 and Y-636) were kindly provided by the ARS Culture (NRRL) Collection, National Center for Agricultural Utilization Research (Peoria, IL, USA). Strains were streaked on 1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar (YPD) plates. Plates were grown overnight at 28°C and a single colony picked. The single colony was grown in YPD liquid broth (28°C) in a shaker incubator (120 rpm). The YPD cultures were used to inoculate autoclaved *Agave* leaf juice at a cell density of 5 x 10^6 cells/mL. Juice samples were autoclaved (121°C, 15 min) and centrifuged at 5000 rpm for 10 min to remove excess leaf tissue. The fermentations were completed in Erlenmeyer flasks with side arm sampling ports and sealed with water-filled airlocks. The fermentation flasks were placed in a shaker (150 rpm) set at 28°C for 96 h. The cells were removed from the fermentation broth by centrifugation (1m / 10 000 g) and the supernatant stored at -20°C until analysis. Ethanol concentration was determined using an Aminex HPX-87H column (300 x 7.8 mm) (Bio Rad; California, United States) as described above, following [33].

Results and Discussion

Processing of *Agave* biomass: leaf and stem fractions

One feature of *Agave* plants that differs from traditional biofuel feedstocks is its high moisture content and inversely, its low water requirements. The seasonal water requirement of *Agave* (300–800 mm/yr) is minor compared with other biomass sources such as sugarcane (*Saccharum* spp., 1500–2500 mm/yr) [18]. The lower water requirement for *Agave* is attributed to its ability to store large volumes of water in its leaves (>83% w/w) (Fig 2). This water storage is common for crassulacean acid metabolism (CAM) plant assimilatory organs and aids in buffering the plant against periods of extended drought [45]. Such physiological characteristics make *Agave* a favorable biofuel feedstock for dry, marginal regions. However, moisture content directly contributes to biomass weight, which affects transport and processing costs. Separating *Agave* juice from the biomass at the time of harvest may result in higher yields and lower input costs such as transportation.

The above-ground portion of *Agave* plants can be separated into leaves and stems (Fig 3a). For 3 year old *Agave* plants, the ratio of leaf: stem dry weight is 4:1, but becomes more variable with age [8]. Whole leaf and stem tissue may be dried and ground to remove excess moisture and to reduce particle size (Fig 3b). Alternatively, crushing the leaves by mechanical force releases 69% of the wet weight (Fig 2a) as a sugar-rich juice (Fig 3c). The biomass that remains after crushing is a fibrous bagasse, which may be further dried to remove excess moisture (Fig 3d).

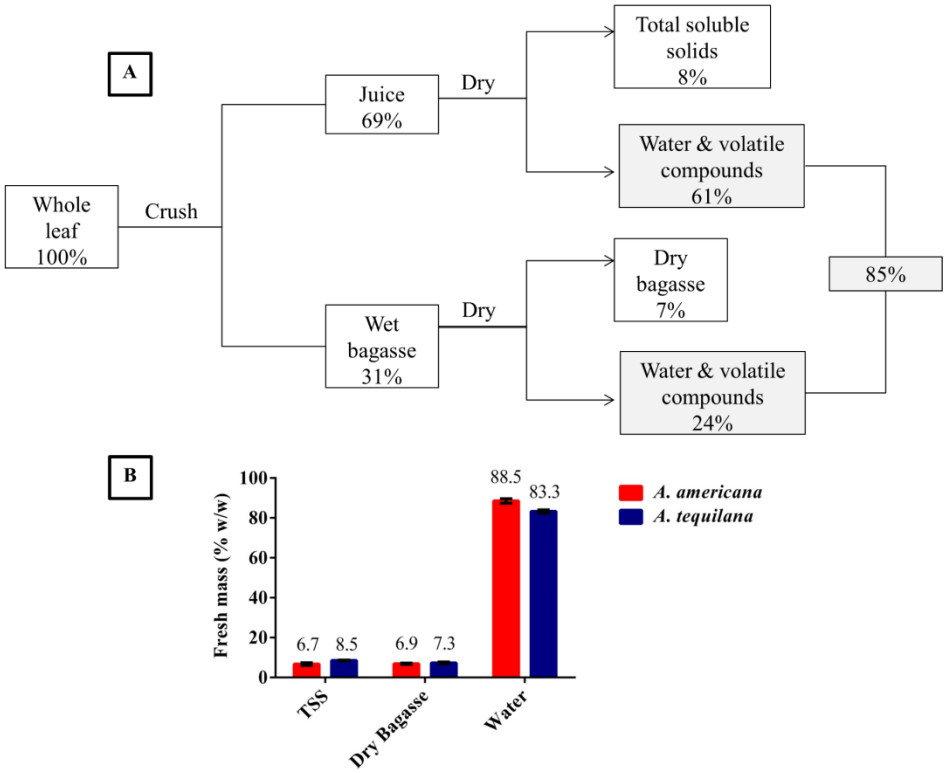

Fig 2. *Agave* processing and moisture content. Whole leaves were crushed, producing juice and wet bagasse fractions (a). These fractions were dried separately to calculate moisture content. Data is presented as percentage of fresh (wet) starting mass (% w/w). The values shown in gray are used to calculate total moisture content. The distribution of leaf fresh mass (% w/w) in *A. americana* and *A. tequilana* (b).

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Analysis of the whole leaf fraction

Pectic polysaccharides occur in crystal sheaths. The morphology of *Agave* cells and the spatial localization of polysaccharides in the leaf tissue was investigated. Transverse sections of *A. tequiliana* leaf were stained with toluidine blue to observe the morphology of the tissues (Fig 4a). Toluidine blue recognizes carboxyl groups on polysaccharides and proteins, and shows the distribution, but not amount or structure, of polysaccharides. Staining was observed in and around the parenchyma cells, with sclerenchymatous fiber cap cells staining very brightly. Further examination revealed that the sclerenchymatous fiber caps around the vascular bundles in *A. tequilana* (Fig 4b) had thicker cell walls than in *A. americana* (Fig 4c). These fiber caps surrounding the xylem and phloem cells are the main structural support for the leaves [46], and the thicker cell walls explain the more erect leaf morphology of *A. tequilana* plants.

Crystal clusters were identified at the junction between cells in *Agave* leaf tissue (Fig 5a). Crystals have been identified in a range of photosynthetic organisms but the abundance, distribution and crystal structure varies between organisms and within tissue types [47]. The accumulation of crystals is correlated with oxalic acid production in plant tissue during normal

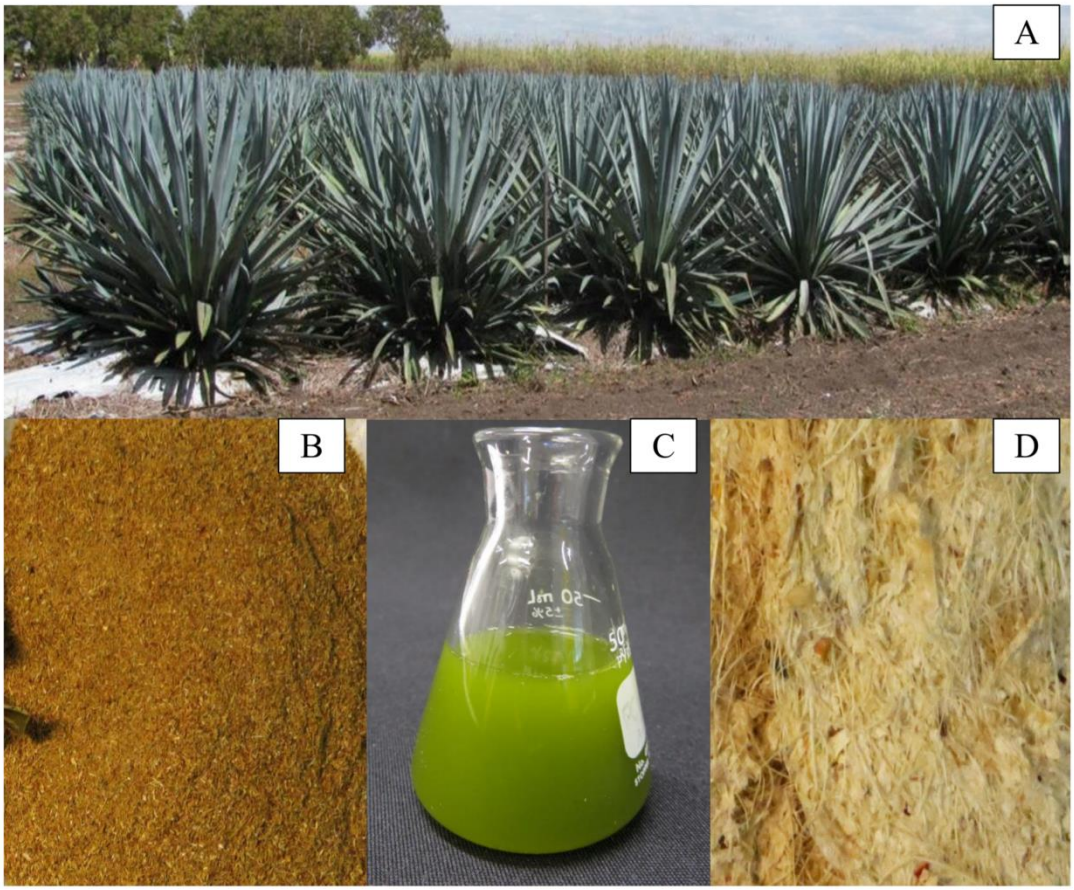

Fig 3. Different fractions of *Agave* material. Two year old *A. tequilana* plants in Australia (a). Partially dried leaves reduced to smaller particle sizes using a ball mill (b). Juice extracted from leaves using an experimental shredder (c). Dried fibers after extraction from wet bagasse (d).

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development and in fungal-plant symbiosis [48]. A pectin-specific antibody that detects methyl-esterified homogalacturonan (LM20) [44] revealed the presence of pectic polysaccharides in the sheath surrounding the crystals (Fig 5b). There is conflicting information about the sheath surrounding the crystals in *Agave* plants; our results support a finding that polysaccharides are present [49], but this is not consistent with another report indicating that no polysaccharides are present in this sheath [50].

Labeling of partially (LM19; [44]) and fully (LM20; [44]) methyl-esterified homogalacturonan was also observed in xylem parenchyma cell walls in both species (Fig 6a–6d)]. Both linkage analysis and results from the water soluble fraction confirm that high levels of pectins are present in *Agave* leaves. However, the amount of pectin-enriched polysaccharides in water

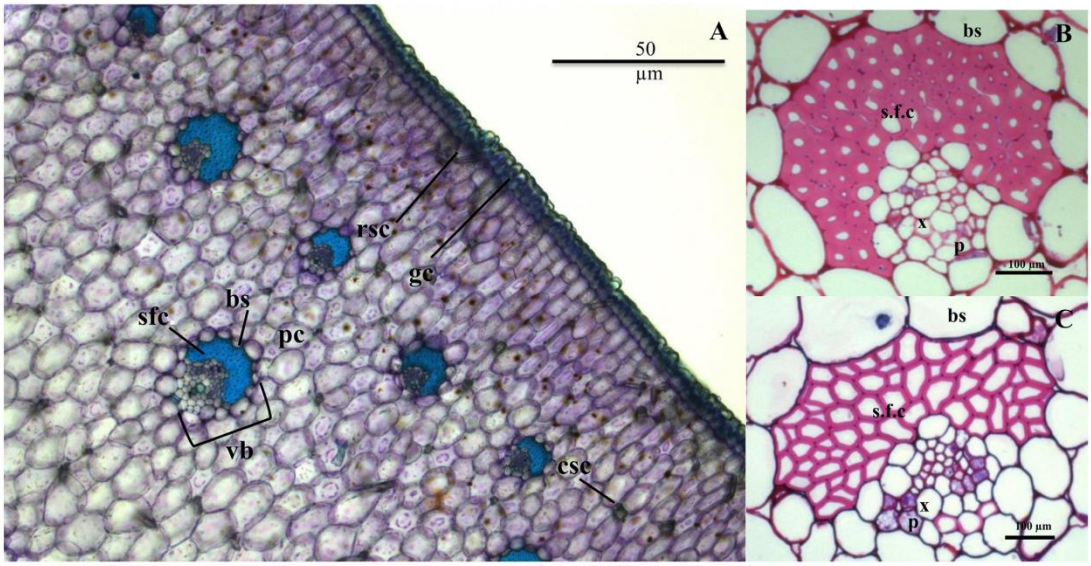

Fig 4. *Agave* leaf morphology. Transverse section of *A. tequilana* leaf stained with toluidine blue (a). Crystals are situated at the junction between some parenchyma cells within the tissue and at the site of stomata at the epidermis. Vascular bundles and fibers in *A. tequilana* (b) and *A. americana* leaf (c) stained with basic fuchsin. Sclerenchymatous fiber cap (sfc); bundle sheath (bs); parenchyma cells (pc); guard cells (gc); cubic shaped crystals (csc); rod shaped crystals (rsc); vascular bundle (vb)

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extracts of *A. tequilana* was five times higher than in *A. americana* (Table 2); whereas linkage analysis indicated that homogalacturonan levels were considerably higher in *A. americana* (17.6 mol%) than in *A. tequilana* (6.5 mol%; Table 3). These data indicate that pectins in *A. tequilana* leaves may be more soluble than those in *A. americana*.

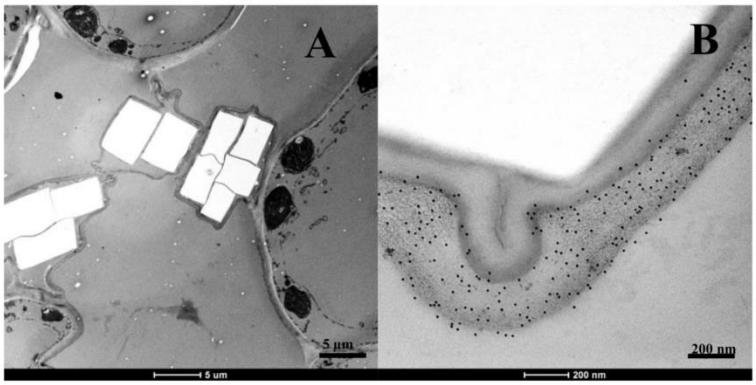

Fig 5. *Agave* tissue has pectinaceous crystal clusters localized at cell junctions. Transmission electron microscopy (TEM) image of crystals between junctions of cells (a) in *A. tequilana*. Labeling of methyl-esterified homogalacturonan (pectin) with LM20, was identified in the outer sheath of the crystals (b).

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The distribution of other cell wall polysaccharides was investigated using antibodies specific to xylan (LM11) [42] and (1→4)-β-mannan [43]. Xylan labeling was observed in the phloem walls (Fig 6e and 6f), consistent with linkage data (Table 3) indicating that heteroxylan is present in *Agave* cell walls. Mannan was detected to a similar extent in cell walls of parenchyma and inner epidermal tissue in both species (Fig 6g and 6h), again consistent with the linkage data (Table 3) that indicated heteromannan in both species.

The soluble fraction contains high levels of fermentable sugars. Sections of whole *Agave* leaves were dried, milled into fine particles, and sequentially extracted with water and ethanol to generate soluble and insoluble fractions. The water soluble carbohydrates (WSC), comprising glucose, fructose, fructans and sucrose, ranged from 15–29% dry weight. In mature *Agave* plants, fructans are the main storage carbohydrate in the stems [10]. Fructans were also the predominant WSC found in *A. tequilana* leaves, but *A. americana* leaves were richer in glucose, fructose and sucrose (Table 2). Total leaf WSC content was lower than the 36–64% w/w found in 6 year old *Agave* stems [10], which have been traditionally selected and used for tequila production, but was much higher than the 5% and 11% w/w found in the biofuel feedstock switchgrass (*Panicum virgatum*) [51] and fructan-rich chicory (*Cichorium intybus*) [52], respectively.

Other soluble sugars were analyzed by hydrolyzing acid-labile polysaccharides into monosaccharides, which were subsequently identified by HPLC. For both species, these monosaccharides comprised a very small proportion of the total mass (Table 2), which is not surprising as the higher molecular weight polymers usually have limited solubility in aqueous solutions [53]. Unhydrolyzed polysaccharides were precipitated with ethanol to create a pectin-enriched fraction [32], which, in *A. tequilana*, comprised over 10% of the dry weight of the leaves (Table 2). From a biofuel perspective, pectins play mixed roles: soluble pectins can be hydrolyzed into monosaccharides for fermentation [54], however acetate substituents on pectins can hinder hydrolysis by blocking cleavage sites for lytic enzymes [55] and once liberated from the polymer these compounds can be toxic to susceptible fermenting microorganisms such as *Pichia stipitis* [56]. Alternatively, when thermochemical conversion processes such as catalytic pyrolysis are used instead of fermentation to produce a hydrocarbon based biofuel the amount of non-carbohydrate cell wall components (i.e. acetyl) in the biomass is less important [57].

The insoluble fraction is predominantly cellulose with low levels of lignin. The remaining insoluble residue, largely cell wall material, was dried, milled, and hydrolyzed with concentrated sulfuric acid. The resulting monosaccharide profiles of *A. americana* and *A. tequilana* leaves were similar, with 12–16% w/w glucose, 3–4% w/w xylose, 3–4% w/w galacturonic acid, 1–3% w/w galactose and less than 1% w/w arabinose (Table 2). However, acid hydrolysis does not permit identification of cell wall polysaccharides, so linkage analysis was used to obtain structural information. Linkages were assigned to polysaccharides according to Pettolino *et al.*, 2012 [34] (S1 Table).

For both species, the majority of the material was composed of hexose (C6) sugars. Cellulose was the most abundant polysaccharide, comprising 32–45 mol% of the cell walls (Table 3). *A. americana* leaf cell walls had higher amounts of pectin-associated polysaccharides such as Type I arabinogalactan and homogalacturonan. There was more heteroxylan in *A. tequilana* than in *A. americana* but the heteroxylan in *A. americana* was less substituted than the heteroxylan in *A. tequilana* (S1 Table). Xylans with low degrees of substitution are reported to bind more strongly to cellulose [58]. The amounts of other cell wall polysaccharides were similar between the two species (Table 3).

Starch, a (1,4)-α-glucan, was removed from the biomass samples prior to linkage analysis to reduce interference with cellulose quantification. Starch was measured separately using a commercial assay at 1–6% w/w (Table 2). The polysaccharide (1,3;1,4)-β-glucan was not detected by enzymatic assays or by linkage analysis.

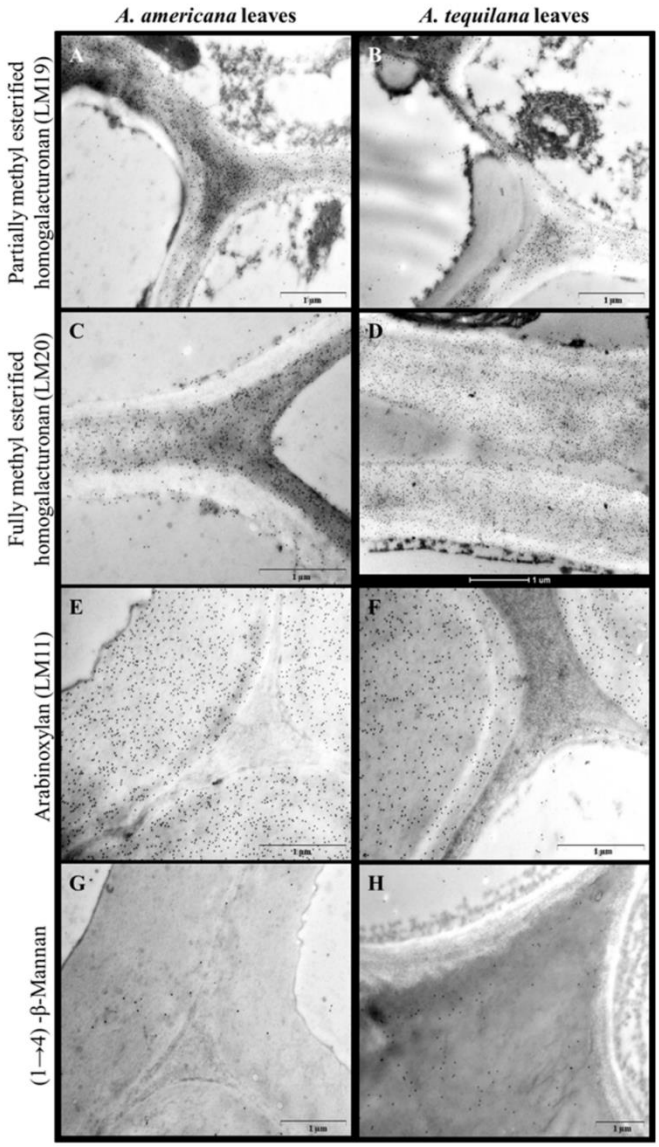

Fig 6. Cell wall polysaccharides detected by immunolabeling and transmission electron microscopy. Xylem tissue labeled with LM19, an antibody for partially methyl-esterified homogalacturonan (a-b) (pectin, [44]). Parenchyma cells labeled with LM20, an antibody for methyl-esterified homogalacturonans (c-d) [44]. Phloem tissue labeled with LM11 indicating the presence of arabinoxylan [42] (e-f). Leaf inner epidermal cells labeled with an antibody for (1→4)-β-mannan indicating the presence of mannan (g-h) [43]. Scale bars = 1μm.

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Table 2. Composition of *A. americana* and *A. tequilana* leaves.

	A. americana (% w/w)	*A. tequilana* (% w/w)
Soluble extracts	**55.5 ± 2.9**	**45.8 ± 2.5**
*WSC	9.1 ± 5.9	15.3 ± 3.0
Glucose	13.5 ± 3.6	4.6 ± 0.8
Fructose	7.8 ± 1.4	2.8 ± 0.6
Fructan	3.4 ± 2.5	4.9 ± 2.5
Sucrose	4.4 ± 0.5	3.0 ± 1.1
*Polysaccharides	4.0 ± 0.2	12.6 ± 1.1
Hydrolyzed monosaccharides	2.2 ± 0.3	2.4 ± 0.2
Ethanol-insoluble (pectin-enriched)	1.8 ± 0.4	10.2 ± 1.1
*Ethanol-soluble monosaccharides	6.0 ± 1.6	1.3 ± 0.2
Ash (non-structural inorganics)	*6.4 ± 1.4*	*15.1 ± 1.6*
Other	*10.0*	*1.5*
Insoluble components	**44.5 ± 2.9**	**54.1 ± 2.5**
*Monosaccharides	21.3 ± 1.7	26.1 ± 3.6
Glucose	12.0 ± 1.8	16.4 ± 2.3
^Starch	5.7 ± 1.4	1.4 ± 0.3
Xylose	2.9 ± 0.7	4.4 ± 0.7
Galacturonic acid	2.8 ± 0.2	3.1 ± 0.7
Galactose	2.7 ± 0.6	1.4 ± 0.1
Arabinose	0.9 ± 0.1	0.8 ± 0.1
Lignin	9.3 ± 0.9	12.7 ± 1.1
Acid-insoluble	5.3 ± 1.0	9.1 ± 1.4
Acid-soluble	4.0 ± 0.7	3.6 ± 0.3
Protein	6.2 ± 2.0	5.8 ± 0.7
Acetate groups	1.0 ± 0.2	0.7 ± 0.2
Ash (structural inorganics)	2.1 ± 1.0	5.5 ± 1.1
Other	*4.6*	*3.3*

The soluble extracts and insoluble residue, comprising structural carbohydrates and other cell wall components, were quantified (n = 6). Data are presented as percentage of dry weight (% w/w).

* indicates the values used to calculate total sugar content: 60.4% w/w for *A. americana* and 55.3% w/w for *A. tequilana*. Italics indicate values derived from calculation rather than direct measurement.

^Indicates values (starch) which were not included in the mass balance. Components of 'Other' (otherwise unaccounted for mass) are likely to be lipids and waxes in the soluble fraction or unhydrolyzed crystalline cellulose and pectin in the insoluble fraction.

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The total lignin content of the leaves was 9.3–12.7% w/w (Table 2). Compared with other biofuel feedstock crops such as corn, sugarcane and poplar, which all have lignin contents >17% w/w (Table 2), *Agave* is considered a low lignin feedstock. Lignin is a non-sugar aromatic polymer that binds strongly to cell wall polysaccharides via covalent and non-covalent linkages. This barrier limits enzyme binding sites on the polymers and reduces the rate and efficiency of hydrolysis [59]. Alternatively, lignin can be acid-soluble. High levels of soluble lignin in the hydrolyzate can be an inhibitor to both yeast and bacteria, reducing the yield of ethanol produced [60]. In *Agave*, 28–43% of the total lignin was acid-soluble (Table 2). Acid-soluble lignin has been shown to be predominantly composed of syringyl lignin and, to a lesser degree, secondary hydrophilic compounds [61].

Cellulose undergoes 40% saccharification without pre-treatments. The predominant polysaccharide identified in both species of *Agave* using linkage analysis was cellulose

Table 3. Polysaccharides detected by linkage analysis in *Agave* leaf.

Polysaccharide	*A. americana* (mol%)	*A. tequilana* (mol%)
Arabinan	5.5	4.7
Type I arabinogalactan	7.4	2.3
Type II arabinogalactan	2.4	1.5
Arabinoxylan	13.4	16.4
Cellulose	31.9	45.3
Heteromannan	6.6	6.0
Homogalacturonan	17.6	6.5
Rhamnogalactan I/II	0.7	0.3
Xyloglucan	10.6	12.7
Unassigned	3.9	4.3
Total	**100.0**	**100.0**

Polysaccharides detected in alcohol-insoluble residues (AIR) of *A. americana* and *A. tequilana* leaves (n = 3). Data are presented as relative percent molarity (mol%). Individual linkages were classified as described in S1 Table. Unassigned linkages include the linkages measured where the polysaccharide of origin was not clear.

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(Table 3). Due to its recalcitrance, cellulose quantification after hydrolysis with sulphuric acid can be an underestimate [62]. As a result, a method optimized for the isolation and measurement of cellulose was employed [30]. The amount of cellulose in whole tissue was slightly lower in *A. americana* (15.7% w/w) than in *A. tequilana* (16.5% w/w).

Cellulose is embedded *in muro* within a complex matrix of non-cellulosic polysaccharides, lignin and proteins. Saccharification tests were thus performed on the heterogeneous alcohol insoluble residue (removing all free glucose from the matrix) on identical cellulose loadings rather than on purified cellulose. The liberation of glucose was monitored over 48 h of enzymatic digestion using a cellulase cocktail. The extent of saccharification was similar for both species (40–35%) but slightly higher for *A. americana* (Fig 7). The efficiency of cellulose breakdown

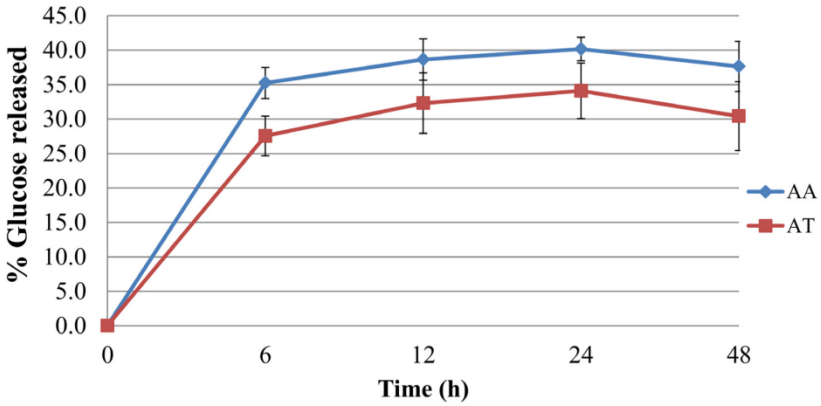

Fig 7. Cellulose, the most predominant polymer in *Agave* leaf tissue is degraded by cellulases. Liberation of the monomer glucose from the alcohol insoluble residue of *A. americana* (AA) and *A. tequilana* (AT) was measured over 48 h. The rate of saccharification is expressed as a percentage of cellulose converted into glucose (n = 3).

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and therefore the total ethanol yield from *Agave* may be increased if the biomass is further processed using pre-treatments, thus loosening the bonds within and between cellulose chains.

Analysis of leaf juice and fiber fractions

Agave leaf juice is rich in fructans. The total moisture content of whole *Agave* leaves is upwards of 89% (Fig 2). Pressing released 69% of the fresh weight as a sugar-rich juice that was analyzed for glucose, fructose and sucrose content. The amounts of these directly fermentable sugars were also measured in *A. tequilana* stem juice, which is commonly used for tequila production. *A. americana* leaves and *A. tequilana* stems had similar amounts of free sugars in the juice (38–39 g/L), with a lower level detected in *A. tequilana* leaves (Fig 8a). Glucose was the most abundant sugar in all three samples although stem juice had a similar amount of sucrose. Additional, unidentified oligosaccharides were also detected in the raw juice samples (Fig 8b), indicating that these monosaccharide values were likely to be an underrepresentation of the total sugar content.

Two methods were used to hydrolyze the unidentified oligosaccharides into monosaccharides: 1) a non-specific acid hydrolysis using trifluoroacetic acid (TFA); and 2) specific enzymatic cleavage of fructans by a broad specificity fructanase. This fructanase exhibits both *exo*-inulinase activity, which degrades sucrose and kestose (glucose-fructose-fructose), and *endo*-inulinase activity, which liberates fructose from the non-reducing ends of long-chain fructans. Both TFA (Fig 8c) and fructanase (Fig 8d) cleaved the unidentified oligosaccharides completely into glucose and fructose, confirming that these oligosaccharides were fructans.

The total concentration of fermentable hexose sugars after hydrolysis in leaf samples was 41–48 g/L and increased to 104 g/L in *A. tequilana* stem juice. Fructose accounted for 68% of the stem monosaccharides, comparable to previous studies that found 60% of the total soluble sugars in *A. tequilana* stem to be fructans [10]. Galactose and galacturonic acid were detected in hydrolyzed juice samples at less than 0.5 g/L.

Inorganic elements in leaf juice that may affect fermentation were measured and compared with the inorganic content of whole leaf (S2 Table). The concentration of inorganic elements in *A. tequilana* juice was twice as high as in *A. americana* juice, although whole *A. americana* leaves had 20% more inorganic elements than *A. tequilana* leaves. High levels of calcium were observed in both species, particularly *A. americana* whole leaves, which may be attributed to inorganic calcium oxalate crystals detected in the tissue (Fig 5). Calcium levels in *A. tequilana* juice and whole leaves were similar to each other, but much higher than *A. americana* juice and much lower than *A. americana* whole leaf. It is possible that the difference in calcium detected between the two *Agave* species is an artefact of the shredding processes or different growing conditions for the two species.

***Agave* fibers are predominantly crystalline cellulose.** With increasing reliance on synthetic fibers to meet consumer demands, production and markets for *Agave* fibers has been on the decline [14]. In recent years research has begun to investigate *Agave* fibers for emerging markets such as use in thermoplastics [63,64]. However, limited information is available regarding the composition of this waste material.

Crystalline cellulose comprised just under half (47–50% w/w) of the dry weight of fiber-enriched leaf fractions (Table 4), lower than the 68.4% w/w previously reported for crystalline cellulose in *A. americana* fibers [65]. The total cellulose in fibers of *A. lechuguilla* and *A. fourcroydes*, species specifically grown for their fibers, accounted for ~80% w/w of dry fiber weight, with the remainder composed mainly of lignin [66].

Non-cellulosic polysaccharides accounted for 22.4% and 15.8% of the dry weight of *A. americana* and *A. tequilana* leaves, respectively. These values are consistent with the values

A

Tissue	Type of juice	Fructose (g/L)	Glucose (g/L)	Sucrose (g/L)	Total (g/L)
A. americana leaves	Raw	13.0 ± 1.3	22.7 ± 3.3	3.3 ± 0.7	**39.0**
	Treated	20.8 ± 4.0	26.9 ± 4.7	–	**47.7**
A. tequilana leaves	Raw	10.0 ± 0.4	17.7 ± 0.2	2.3 ± 0.1	**30.0**
	Treated	20.2 ± 1.6	20.9 ± 2.9	–	**41.1**
A. tequilana stem	Raw	9.4 ± 0.6	15.1 ± 0.4	14.2 ± 0.1	**38.7**
	Treated	71.2 ± 5.6	32.8 ± 2.1	–	**104**

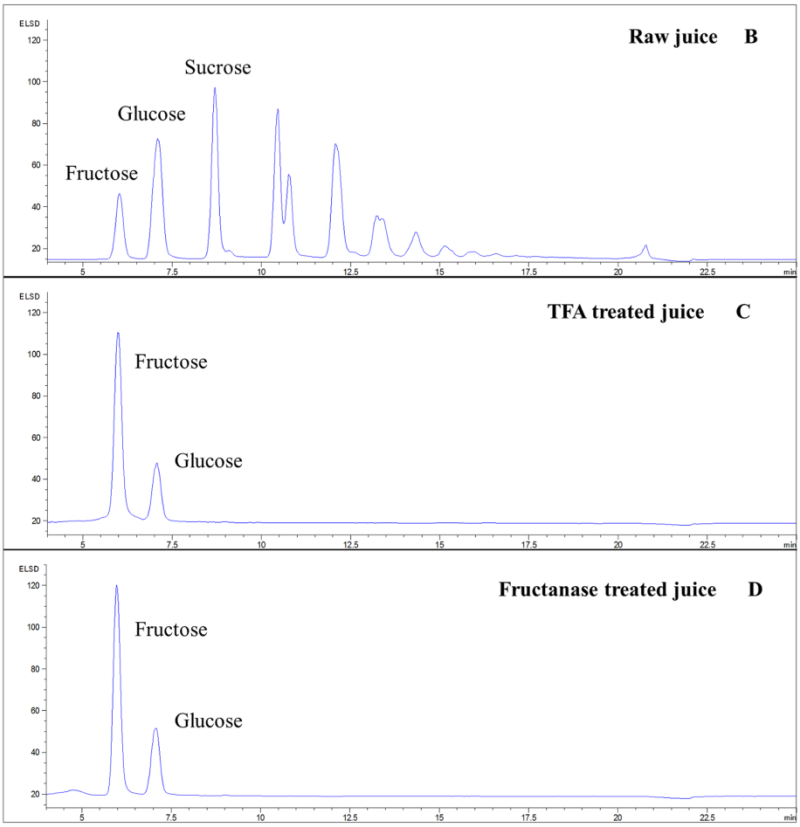

Fig 8. Quantification of juice sugars from *A. americana* leaves and *A. tequilana* leaves and stem. The amount of glucose, fructose and sucrose present in both raw and TFA-treated juice samples (a). Data are presented as g/L. Additional peaks for which there are no known standards were detected in the chromatograms of raw juice (b). *A. tequilana* stem juice is used as a representative of all three, very similar, chromatograms for the raw and treated samples. Chromatogram of TFA-treated *A. tequilana* stem juice (c). Chromatogram of fructanase-treated *A. tequilana* stem juice (d).

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Table 4. Carbohydrates in fiber-enriched fractions from *Agave* leaves.

Component	*A. americana* (% w/w)	*A. tequilana* (% w/w)
Crystalline cellulose	47.2 ± 2.3	49.5 ± 1.9
Non-cellulosic polysaccharides	22.4 ± 0.8	15.8 ± 1.3
Arabinose	*0.6 ± 0.1*	*0.3 ± 0.1*
Glucose	*8.6 ± 0.3*	*2.7 ± 0.6*
Xylose	*9.4 ± 0.9*	*11.4 ± 1.0*
*Other monosaccharides**	*3.8 ± 0.1*	*1.4 ± 0.1*

Data are presented as a percentage of dry weight (% w/w).

*Includes mannose, rhamnose, glucuronic acid, galacturonic acid and galactose

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reported in the literature suggesting that *A. tequilana* fibers contain 17% w/w non-cellulosic polysaccharides [67]. Xylose and glucose were the most abundant monosaccharides detected in the fibers after hydrolysis in 1M sulfuric acid, agreeing with linkage analysis that detected heteroxylans and xyloglucan in insoluble leaf fractions. In addition, similar to other studies [67] about ~30% of the fiber mass for both species was unaccounted for which may be attributed to unidentified or unhydrolyzed carbohydrates, lignin, inorganic compounds and protein.

Fermentation of Agave juice

A. tequilana leaf juice was used as a substrate to investigate fermentation efficiency using two different strains of *Saccharomyces cerevisiae*. *A. tequilana* juice was autoclaved to minimize microbial contamination from native organisms and inoculated with one yeast strain. Sugar content of the starting juice was 41.4 g/L of total sugars and 30.0 g/L of readily fermentable WSC. After 96 h, both strains produced ethanol concentrations of 11–14 g/L (Table 5). Up to 90% of the monomers were fermented, which represent only 54–66% of the total sugars. Sugars in the *Agave* leaf juice, predominantly the fructans, are therefore being underutilized by these yeast strains.

Historically, *Saccharomyces cerevisiae* is the most readily studied and utilized yeast for alcoholic fermentation assays [68] and can efficiently convert sucrose, glucose and fructose [69]; the main sugars in *Agave* leaf juice. However, alternative microorganisms may be more efficient at fermenting *Agave* juice sugars. For example, microorganisms such *Kluyveromyces marxianus* and *Torulaspora delbrueckii*, isolated from fermenting mezcal (a distilled alcohol made from *Agave*), express enzymes that hydrolyze fructooligosaccharides [70]. Activation of fructanase enzymes was induced by Ca^{2+}, which is present in significant amounts in the leaves and juice of both *A. americana* and *A. tequilana* (S2 Table) [71]. In addition, using organisms such as *Escherichia coli* that can catabolise galacturonic acid may be a sensible choice for *Agave*

Table 5. Fermentation of *Agave tequilana* leaf juice using *Saccharomyces cerevisiae*.

	Ethanol yield (96 hr)		
***S. cerevisiae* strain**	**Yield (g/L)**	**Conversion (% of total sugars)**	**Conversion (% of monomers)**
139	11.4 ± 0.6	54%	74%
636	13.8 ± 0.5	66%	90%

Two strains of *S. cerevisiae* were used to ferment untreated *A. tequilana* leaf juice with a starting sugar concentration of 41.4 g/ L and WSC concentration of 30.0 g/L. Conversion efficiencies are based on a maximum conversion rate of sugar to ethanol of 51.1% w/w.

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if the pectic sugars in leaf tissue are to be fermented [72]. The use of readily studied *S. cerevisae* strains should thus be considered a benchmark by which to judge other organisms since it may be not be optimal for *Agave*. Careful selection of fermenting organisms may obviate the need for expensive pre-treatment processes or use of additional enzymes, which would increase the return on investment of using *Agave* spp. for biofuel production.

Agave ethanol yields rival current biofuel feedstocks

Ethanol yields from three different *Agave* substrates were modelled: 1) the dry mass of the entire *Agave* plant based on leaf sugar composition, thereby underestimating sugar content because the additional sugar in the stem is not accounted for; 2) waste *A. tequilana* leaves from tequila production, and 3) juice from *A. tequilana* and *A. americana* leaves (Table 6). Theoretical ethanol yields were calculated using standard conversion assumptions [73].

The theoretical ethanol yield values for the whole leaf sugars of *A. americana* and *A. tequilana* were 437 L/t and 401 L/t, respectively. These values are comparable to estimates for other lignocellulosic biofuel feedstocks such as corn stover, sugarcane and switchgrass (Table 6). However, *Agave* plants may out-perform current biofuel feedstock crops in terms of productivity per hectare. Whole *A. tequilana* plants were predicted to yield 4000–13600 L/ha/yr and *A. americana* plants were predicted to yield 4400–14800 L/ha/yr. At the low end, these values exceed theoretical yields from first-generation feedstocks such as corn, wheat (*Triticum aestivum)* and sugarcane and at the high end, they double the yields of more recently investigated second generation feedstocks such as poplar, sorghum and switchgrass. The current values are consistent with those reported previously in the literature, which estimated that ethanol yields for *Agave* spp. may range from 3000–12000 L/ha/yr [18,20].

Table 6. Theoretical ethanol yields for lignocellulosic feedstocks.

Biomass	Source of sugars	Ethanol yield (L/t)	Productivity (t/ha/yr)	Ethanol yield (L/ha/yr)
Corn	Stover without cobs	362–456*	3[18]	1086–1369
Wheat	Straw	406*	2.6[74]	1055
Sugarcane	Bagasse	318–500*	10[18]	3179–4996
Sorghum	Whole plant	268*	24–32.5[75,76]	6430–8708
Switchgrass	Whole plant	392–457*	5.2–23[77,78]	2036–10508
Poplar	Whole tree, no leaves	419–456*	5–11[18]	2096–5011
Agave	Whole residue	347*	10–34[18]	3474–11811
A. americana	Whole plant, extrapolated from leaf sugar content	437^	10–34[18]	4368–14851
A. tequilana	Whole plant, extrapolated from leaf sugar content	401^	10–34[18]	4009–13636
A. tequilana leaves	Whole leaf	401^	5.7–19#	2273–7728
A. americana leaves	Juice†	34^,‡	34–115.7‡	1165–3961
A. tequilana leaves	Juice†	30^,‡	23.4–79.7‡	691–2350

*Calculations were based on the compositional values listed in Table 1 [5].

^Calculations based on data obtained in this study.

#Assumes that 56.7% dry w/w of the whole 3 year old plants is leaf material [8].

†Assumes that juice accounted for 69% of plant wet weight; *A. americana* leaf was 88.5% w/w water; and *A. tequilana* leaf was 83.3% w/w water.

‡ Tonnes of wet weight rather than dry weight. Units for data are given in table headings. Constants for ethanol calculations are consistent with the National Renewable Energy Laboratory Theoretical Ethanol Yield Calculator [73]: 1.111 kg monomeric C6 sugar per 1 kg polymeric C6 polymer (glucan, fructan); 1.1363 kg monomeric C5 sugar per 1 kg polymeric C5 polymer (xylan, arabinan); 0.51 kg of ethanol produced from 1 kg of sugar. Productivity per hectare is based on previous studies [18,74–78].

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Waste *A. tequilana* leaves could generate 2300–7900 L/ha/yr and increase the value of existing *Agave* industries. However, since the majority of the mass of *Agave* plants is water, it may be more economically viable to directly separate and ferment the sugar-rich juice, which could yield 690–4000 L/ha/yr (Table 6). Even using a generic *S. cerevisiae* strain unadapted to *Agave* substrates, yields of up to 1500 L/ha/yr from *A. tequilana* leaf juice and 2600 L/ha/yr from *A. americana* leaf juice could be obtained (assuming a fermentation conversion of 66% for both substrates; Table 5). More efficient fermenting organisms may increase the value of using *Agave* juice as a biofuel feedstock in terms of yield and revenue returns.

It is worth noting that *Agave* cultivation systems have not yet been optimized to produce sugar for biofuel and biochemical industries. Information about agronomical practices, such as planting density or the optimal age to harvest the plants, is limited. If the plants are harvested at 2–3 years of age rather than the traditional 8–12 years of age, plant spacing could be reduced further, increasing density per hectare. In addition, further information about microorganisms that are naturally found within *Agave* may be beneficial for the industries that grow and commercialize these plants. In a biofuel context, it may be useful to isolate and characterise organisms that naturally grow on *Agave*, as they presumably utilize sugars such as fructans efficiently and are tolerant to a range of environmental conditions. The isolation and use of microorganisms found on or within biomass for the conversion of carbohydrates to biofuel is not novel; grape marc, an agro-industrial waste material, has been found to be a rich source of robust organisms that are economically and productively favourable for second generation bioethanol conversion [79]. Further research is required to identify the microorganisms associated with the *Agave* microbiome.

Conclusion

The leaf tissues of *A. americana* and *A. tequilana* species contain 56–60% (dry weight) of potentially fermentable sugars, over half of which are present in a soluble fraction. These same tissues also contain relatively low amounts of lignin. Ethanol yields (ha/yr) that could be generated from *Agave* leaves and whole plants rival those of the most successful biofuel feedstock crops such as switchgrass and poplar. *Agave* differs from most common feedstocks in its high moisture content, but nearly 70% of plant mass can be extracted with simple mechanical pressing to release a sugar-rich juice. Crushing and fermenting the juice on site without any pretreatment can produce competitive ethanol yields, with room for improvement by judicious selection of fermenting organisms, and by-products may be produced from the crystalline cellulose enriched bagasse waste. The comprehensive compositional data for *Agave* leaves and fermentation trials reported herein will be instrumental in the development of agronomic, saccharification and fermentation methods for converting *Agave* raw material into biofuel or biochemical products.

Supporting Information

S1 Table. Monosaccharide linkage analysis data for *Agave* leaves (mol%). Analysis completed on alcohol insoluble residues (AIR). Data are presented as relative percent molarity (mol %).
(DOCX)

S2 Table. Elemental analysis of *Agave* juice and whole leaf. Data are presented as mg/kg of material. [1]Average of two biological replicates. [2]Average of three biological replicates.
(DOCX)

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Author Contributions

Conceived and designed the experiments: GF RB DC. Performed the experiments: KC MH GK CTB CSB SB. Analyzed the data: KC NB CSB SB SD CTB AB JL. Contributed reagents/materials/analysis tools: DC JH SD SB JL. Wrote the paper: KC NB CSB RB GF. Method development: JL KC.

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14.6 Appendix F: Published Paper II

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Grape marc as a source of carbohydrates for bioethanol: Chemical composition, pre-treatment and saccharification

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HIGHLIGHTS

- The chemical composition of white and red grape marc was determined.
- Marc carbohydrates were characterized using enzyme digests, HPLC and MALDI-TOF-MS.
- Dilute acid pre-treatments liberated glucose more efficiently than thermal treatments.
- White marc contains 40% fermentable water-soluble carbohydrates.
- Theoretical ethanol yields were calculated based on chemical composition.

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ABSTRACT

Global grape production could generate up to 13 Mt/yr of wasted biomass. The compositions of Cabernet Sauvignon (red marc) and Sauvignon Blanc (white marc) were analyzed with a view to using marc as raw material for biofuel production. On a dry weight basis, 31–54% w/w of the grape marc consisted of carbohydrate, of which 47–80% was soluble in aqueous media. Ethanol insoluble residues consisted mainly of polyphenols, pectic polysaccharides, heteroxylans and cellulose. Acid and thermal pre-treatments were investigated for their effects on subsequent cellulose saccharification. A 0.5 M sulfuric acid pre-treatment yielded a 10% increase in the amount of liberated glucose after enzymatic saccharification. The theoretical amount of bioethanol that could be produced by fermentation of grape marc was up to 400 L/t. However, bioethanol from only soluble carbohydrates could yield 270 L/t, leaving a polyphenol enriched fraction that may be used in animal feed or as fertilizer.

1. Introduction

The carbohydrates in plant biomass can be used as a raw material for producing liquid biofuels and valuable biochemicals. However, plant material is heterogeneous and recalcitrant to degradation. Further, the carbohydrates may take a variety of chemical forms, including polysaccharides, oligosaccharides, monosaccharides, or form glyco-conjugates like glycoproteins and glycolipids. To overcome the structural complexity of plant biomass, research has focused on modifying plants to make the carbohydrates more available (Abramson et al., 2010). Alternatively, physical or chemical pre-treatments combined with enzymatic hydrolysis have been used to facilitate the liberation of fermentable sugars (Mosier et al., 2005). However, these additional processing steps are energetically and financially costly, and potentially create bottlenecks in large scale production (Klein-Marcuschamer et al., 2012). Thus the need to identify dedicated biomass sources that are structurally favorable in their

Abbreviations: A:X, arabinose:xylose ratio; AIR, alcohol-insoluble residue; Ara, arabinose; ASE, accelerated solvent extractor; CDTA, cyclohexane-1,2-diamine tetraacetate; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; HILIC, hydrophilic interaction chromatography; HMF, 5-(hydroxymethyl)furfural; HPLC, high-performance liquid chromatography; Man, mannose; mol%, relative percent molarity; MS, mass spectrometry; Na_2CO_3, sodium carbonate; NCPs, noncellulosic polysaccharides; Rha, rhamnose; WSC, water soluble carbohydrates; Xyl, xylose.

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native form, require negligible processing, and are socially and environmentally advantageous is essential to the progression and prosperity of the biofuels industry.

One way to circumvent the issues imposed by using classic lignocellulosic feedstocks is to identify waste materials that are produced in abundance, have been accumulated for other uses, can be sourced cheaply and have carbohydrates in a form amenable to fermenting microorganisms. One such potential source of raw material is grape marc. Grape marc (or pomace) is the waste that remains after the juice is collected from the pressing of grapes for wine production; it includes grape skin, pulp, seeds, stems and residual juice. The composition of grape marc is related to the grape variety, the method of processing, environmental conditions and the ratio of skin:seeds:stem.

The global production of grapes is 67.1 Mt/yr with China, the United States of America and Italy being the leading producers (FAO, 2015). It has been estimated that 18–20% of the grapes used for wine making remains as waste marc, which could generate up to ~13.4 Mt (fresh weight) of waste biomass (Spanghero et al., 2009). Currently, the global accumulation of grape marc is lower at 4.8 Mt/yr (average of last 10 years; FAO, 2015) and is considered to have limited economic value. Grape marc is generally disposed of at a cost to the winery but may be utilized as filler in livestock feed, fermented to make the alcoholic beverage grappa, reapplied as fertilizer or used as a source of phenolic compounds.

In recent years, grape marc has been proposed as a raw material for bioenergy production (Toscano et al., 2013) and has been used to generate butanol and biogas (Cáceres et al., 2012; Law and Gutierrez, 2013). Grape marc may also be used to produce biofuel at the winery site and the evolution of local biofuel processing plants will obviate costs associated with transport of the biomass. However, further information about the composition of grape marc is required to allow estimates to be made of biofuel yields and hence facilitate cost-benefit analyses of different types of marc for biofuel production.

Here the composition of grape marc derived from two grape varieties, Cabernet Sauvignon and Sauvignon Blanc was investigated. Various pre-treatments were analyzed for their effect on depolymerisation of non-cellulosic polysaccharides, and the residual cellulosic biomass was subsequently hydrolyzed using crude cellulase preparations. Finally, the chemical composition of grape marc was compared with other known agro-industrial waste materials, and these data were extrapolated to estimate theoretical ethanol yields.

2. Methods

2.1. Collection and preparation of plant material

Material was collected after the pressing of Cabernet Sauvignon and Sauvignon Blanc grapes in both April 2012 and March 2013 at the University of Adelaide Waite Campus vineyards (Adelaide, Australia), $n = 2$ for each variety. Amounts of 30 g (three technical replicates) were separated into seed, skin and stem fractions. The weight of each fraction was recorded and the mass distribution calculated.

Whole grape marc was lyophilized (Labconco-Freezone, Kansas City, MO, USA) to determine moisture composition. Lyophilized grape marc was ground in a Retsch mill MM400 (Retsch GmbH; Haan, Germany) at 30 Hz for 3 min for compositional analyses.

2.2. Extraction and analysis of soluble and insoluble components

Grape marc samples were extracted with three water cycles followed by three 95% v/v ethanol cycles at 100 °C using an Accelerated Solvent Extractor (ASE300, Dionex) (Sluiter et al., 2005). Briefly, ASE (accelerated solvent extractor) stainless steel extraction cells (11 mL) were fitted with dried (105 °C) and pre-weighed Whatman GF/C 55 mm glass microfiber filters (Sigma–Aldrich, United States) and 1 g dried marc was added. Extraction settings were modified to 60 s nitrogen purges following extraction, 5 min static time and 120% rinse volume. Following extraction, the biomass was dried overnight at 105 °C. The percentage of material extracted was calculated based on the difference between the initial weight (before the water and ethanol extractions) and final weight (after extraction).

The carbohydrates and lignin content in the ASE extracted biomass was quantified according to Sluiter et al. (2008). Briefly, the material (30 mg) was incubated with 13.5 M H_2SO_4 at room temperature for 1 h, followed by dilution to 0.75 M H_2SO_4 and autoclaving at 121 °C (Tuttnauer 3850 ELC Benchtop Sterilizer). The solubilized material and the residual biomass were separated by centrifugation for 10 min at 10,000g. The supernatant was analyzed for monosaccharides (Comino et al., 2013). A sugar recovery standard for monosaccharides was carried through the acid hydrolysis to compensate for degradation of monomers during the hydrolysis step. The acetate content in the supernatant was analyzed at 60 °C using an Aminex HPX-87H column (300 × 7.8 mm) (Bio Rad; California, United States) on an 1100 series liquid chromatography instrument. Elution was performed isocratically with 2.5 mM H_2SO_4 at a rate of 0.5 mL/min. The residual biomass was assayed for acid-insoluble lignin (Sluiter et al., 2008).

Small scale extractions of grape marc were conducted to determine the water soluble carbohydrate content. Briefly, 50 mg of dried, ball milled grape marc was extracted with equal volumes (1.0 mL) of water, 95% v/v ethanol, and 70% v/v ethanol. Extraction for each solvent was conducted at 80 °C for 15 min followed by centrifugation at 10,000g for 10 min and the supernatant collected. Glucose, fructose and sucrose in water extracts were measured by hydrophilic interaction chromatography (HILIC), using a Prevail Carbohydrate ES column (150 × 4.6 mm) (Alltech; Illinois, United States) on an Agilent 1200 series liquid chromatography instrument equipped with an evaporative light scattering detector (Alltech ELSD 800). The mobile phase conditions were modified from those used by Agblevor et al. (2007) and consisted of water (A) and 90% acetonitrile (B) at a flow rate of 1.0 mL/min at 20 °C. Soluble pectins in the water extracts were precipitated and quantified according to Santos et al. (2013). Additional monosaccharides present in the water and ethanol extracts were analyzed using HPLC (Comino et al., 2013), following centrifugal evaporation (Savant SC110 Speed Vac, Thermofisher; Massachusetts, United States) and hydrolysis using 1 M sulfuric acid (H_2SO_4) for 3 h at 100 °C.

Starch was measured in samples using a commercial assay (Total Starch-Megazyme: AOAC Method 996.11; International Ireland Ltd., Wicklow, Ireland), following the method for samples that contain glucose and/or maltodextrins. The elemental content of ball milled grape marc (300 mg) was measured using a closed tube nitric acid/hydrogen peroxide digest and radial view inductively coupled plasma-optical emission spectrometry method (Wheal et al., 2011). The nitrogen value was converted to an estimate of the protein content using the nitrogen factor (NF) 6.25.

2.3. Isolation of polysaccharides

Raw grape marc was extracted overnight at 37 °C with 70% aqueous ethanol. The homogenates were filtered using Miracloth (Calbiochem), and alcohol-insoluble residues (AIRs) were washed with 70% v/v ethanol and 100% v/v acetone. The AIR material was collected after drying at room temperature overnight under constant airflow. To isolate polysaccharides from the marc, the AIR

material was fractionated by sequential extraction (Fig. 1) with 50 mM CDTA (pH 6.5) (cyclohexane-1,2-diamine tetraacetate) and Na_2CO_3 (sodium carbonate) + 25 mM $NaBH_4$ (sodium borohydride) according to Chaplin and Kennedy (1994). Both of these extraction steps were used to extract pectic polysaccharides. Next, 1 M KOH + 25 mM $NaBH_4$ was used to release heteroxylans and other non-cellulosic cell wall polysaccharides. Finally, 4 M KOH + 25 mM $NaBH_4$ resulted in the extraction of a xyloglucan fraction. Following polysaccharide isolation, the fractions were dialyzed and lyophilized (Labconco-Freezone, Kansas City, MO, USA) and hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. The liberated monosaccharides were quantified by HPLC (Fig. 1) (Comino et al., 2013).

2.4. Enzymatic hydrolysis and MS profiling

Three of the whole marc fractions were treated with different enzymes for cell wall analysis (Fig. 1). The CDTA fraction (1 mg) was treated with *endo*-polygalacturonanase M2 (50U) from *Aspergillus aculeatus* (Megazyme, Ireland) with 100 mM NaOAc (pH 4) for 16 h at 37 °C. The 1 M KOH fraction (1 mg) was treated with *endo*-1,4-β-xylanase M6 (45U) from rumen microbacteria (Megazyme) with 100 mM NaOAc (pH 6) for 16 h at 37 °C. The 4 M KOH fraction (1 mg) was treated with recombinant xyloglucan-specific *endo*-β-glucanase (10U) from *Paenibacillus* spp (Megazyme) with 100 mM NaOAc (pH 5.5) for 16 h at 37 °C, or *endo*-1,4-β-xylanase M6 (45U) (as described earlier). The enzymes were inactivated by boiling for 2 min.

The molecular weights of the sodium adducts of oligosaccharides $[M+Na]^+$ or $[M-H]^-$ were determined using a BioTOF UltraFlex II (Bruker Daltonics) mass spectrometer (MS), in positive or negative ion modes. The enzyme hydrolyzates containing oligosaccharides were mixed with 2,5-dihydroxybenzoic acid (10 mg/mL) and 10 mM NaCl in the ratio of 5:5:3 (v/v/v). In MS mode, the spectra were accumulated over an average of 2000 laser shots (Hsieh et al., 2008).

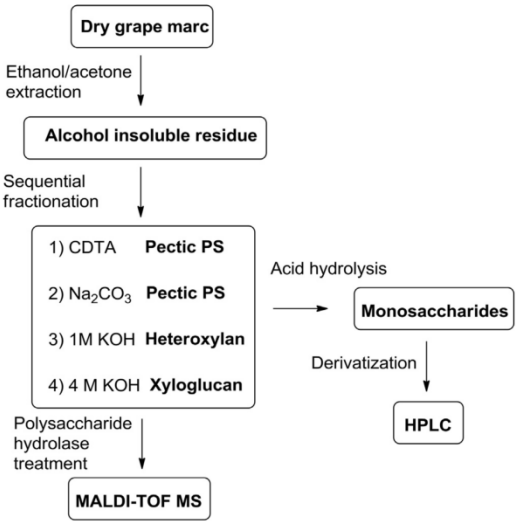

Fig. 1. Flowchart outlining the sequential fractionation of grape marc (AIR) to isolate polysaccharides for characterization.

2.5. Crystalline cellulose

Crystalline cellulose was measured in non-pre-treated and pre-treated grape marc using a modified Updegraff (1969) method as described in Harris et al. (2009). For non-pre-treated samples, the soluble carbohydrates were removed prior to cellulose analysis: dried homogenized grape marc (50 mg) was incubated three times with 70% v/v ethanol at 55 °C for 1 h. The material was washed with 1 mL acetone for 2 min at room temperature and dried overnight under vacuum.

The cellulose content was determined in triplicate using 5 mg of dry non-pre-treated grape marc or pre-treated grape marc boiled in 3 mL acetic-nitric reagent for 30 min. Samples were cooled to room temperature, centrifuged (2500 rpm/10 min) and the supernatant discarded. The residual material was washed twice with 8 mL water, followed by 4 mL acetone, and dried under vacuum. To hydrolyze the cellulose, 1 mL H_2SO_4 (12.5 M) was added, and incubated for 1 h at room temperature. The liberated glucose content was quantified using the anthrone method outlined in Harris et al. (2009) using a spectrophotometer (Thermo Fischer, Waltham, MA, USA) set at a wavelength of 620 nm. Filter paper (20 mg) was carried through the experiment as a control and a standard curve was calculated from known concentrations of glucose (0–50 μg). The cellulose content was calculated by multiplying the glucose value for each sample by the total reaction volume and the hydration factor of 0.9.

2.6. Pre-treatment conditions

Whole marc samples (1 g) were homogenized using an Arthur H Thomas Co. Scientific grinder (Philadelphia, PA, USA) equipped with a 2 mm sieve. Dried, milled grape marc was added to acid or water in a 1:10 (solid:liquid) ratio. Pre-treatments at 100 °C were conducted in an oven and pre-treatments at 121 °C were completed in an autoclave at a maximum pressure of 210 kPa (Tuttnauer 3850 ELC Benchtop Sterilizer). The four different pre-treatment conditions were: (1) 1 h, 121 °C, 0.5 M H_2SO_4; (2) 3 h, 100 °C, 1 M H_2SO_4; (3) 1 h, 121 °C, H_2O; (4) 3 h, 100 °C, H_2O. Following pre-treatment, the slurry was cooled to room temperature and filtered using Whatman GF/C 55 mm glass microfiber filters (Sigma–Aldrich, United States). The undissolved biomass was washed to neutral pH, the cellulose content measured and the material was carried through the saccharification procedure. For comparison, non-extracted (raw, dried and ball milled) grape marc was carried through the NREL compositional analysis method (Sluiter et al., 2008).

2.7. Enzymatic saccharification

The enzyme activity of a Celluclast 1.5 L (cellulase from *Trichoderma reesei*; endoglucanase activity ⩾700 units/g) and Novozyme 188 (cellobiase from *Aspergillus niger*; cellobiase activity ⩾250 unit/g) (Sigma–Aldrich; St. Louis, MO, USA) cocktail mixed 1:1 was measured according to Adney and Baker (1996). Microscale saccharifications were completed using an enzyme concentration of 60 filter paper units and 0.02 g cellulose for non-treated (AIR) and pre-treated samples (Harris et al., 2009). The glucose concentration was measured over 48 h using a Yellow Springs Instrument (YSI) glucose analyzer (Yellow Springs, OH, USA).

2.8. Assumptions for calculating theoretical ethanol yields

Standard constants for ethanol calculations were used: 1.111 kg monomeric C6 sugar per 1 kg polymeric C6 polymer (glucan);

1.1363 kg monomeric C5 sugar per 1 kg polymeric C5 polymer (xylan, arabinan); 0.51 kg of ethanol produced from 1 kg of sugar.

3. Results and discussion

3.1. Characterization of grape marc raw materials and soluble carbohydrates

After the grapes are crushed and processed for winemaking, approximately 20% of the starting material remains as a moisture-dense waste material (Spanghero et al., 2009). The composition of this heterogeneous material is affected by the percentage of seed, skin and stem in the total mass (Fig. 2). For both varieties, the majority of the weight (up to 80% in Sauvignon Blanc grape marc, Fig. 2) is attributable to grape skins, with seeds and stems present to a lesser degree. The moisture content of this waste material was 59–67% w/w (Fig. 2).

The residual carbohydrates that remain in the marc after crushing the grapes are predominantly water soluble monosaccharides, oligosaccharides and polysaccharides and water insoluble structural polysaccharides from the cell wall. The soluble carbohydrates can be extracted with minimal energetic input and may be directly used as a raw substrate for fermentation, whereas cell wall polysaccharides need to be liberated through pre-treatment and saccharification. The water soluble carbohydrates (WSC) in white marc accounts for approximately one-third of the dry weight (37.6% w/w) and 70% of the total carbohydrate content (Table 1). Red grape marc has a much lower percentage of soluble carbohydrates, at 4.6% w/w. The comparatively low amount of WSC detected in the red marc may be due to the processing of the grapes during wine making, as red marc stays in contact with the juice for several days (maceration period) to enhance color and sensory attributes of the wine. During this period, the grapes are subjected to a mild, but prolonged, extraction in which phenolic compounds are released and carbohydrates are partially fermented.

Grape marc is also enriched in soluble pectins. Previous studies have shown that the galacturonan content in the mesocarp of grape berries increases from 26% to 46% of cell wall polysaccharides and becomes more soluble as ripening progresses (Nunan et al., 1998). Soluble pectins were quantified in the water extracts of the grape marc and contributed 4% and 9% w/w of the mass of Sauvignon Blanc and Cabernet Sauvignon, respectively (Table 1).

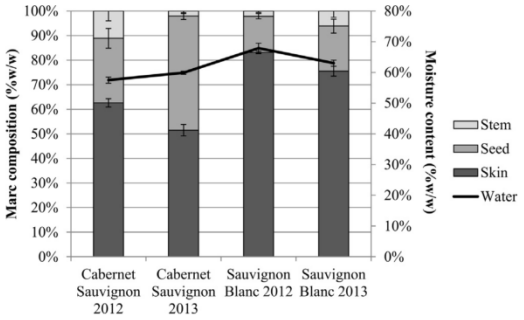

Fig. 2. Grape marc is a heterogeneous material composed predominantly of grape skin. Fresh grape marc from the varieties Cabernet Sauvignon and Sauvignon Blanc (collected in 2012 and 2013) were separated into three fractions. The water content of whole marc was measured for each sample. Data is presented as percent fresh weight (w/w).

Table 1
Mass balance of Cabernet Sauvignon and Sauvignon Blanc grape marc.

	Cabernet Sauvignon (% w/w)	Sauvignon Blanc (% w/w)
Total extractables	20.7 ± 1.0	60.5 ± 4.7
Soluble carbohydrates	**14.8**	**43.0**
*WSC	4.6 ± 1.5	37.6 ± 6.8
Glucose	2.1 ± 0.7	18.6 ± 3.7
Fructose	2.5 ± 0.8	19.0 ± 3.2
*Polysaccharides	8.7 ± 0.2	3.7 ± 1.1
Hydrolyzed monosaccharides	0.2 ± 0.3	0.2 ± 0.2
Ethanol insoluble (pectin-enriched)	8.5 ± 0.5	3.5 ± 0.4
*Ethanol soluble monosaccharides	1.5 ± 1.6	1.7 ± 0.2
Other extractables	**5.9**	**17.5**
Insoluble components (AIR)	**63.9**	**30.6**
*Monosaccharides	16.6 ± 0.3	10.9 ± 0.9
Glucose	6.0 ± 0.4	5.2 ± 0.5
Starch	2.7 ± 0.3	4.0 ± 1.3
Xylose	4.3 ± 0.9	2.4 ± 0.3
Galacturonic acid	1.9 ± 0.2	1.4 ± 0.4
Mannose	1.6 ± 0.3	0.6 ± 0.1
Galactose	1.0 ± 0.1	0.6 ± 0.1
Arabinose	1.8 ± 0.1	0.7 ± 0.1
Acid-insoluble lignin	32.5 ± 2.1	10.5 ± 2.3
Protein	10.8 ± 0.3	7.2 ± 0.8
Acetate groups	1.0 ± 0.1	0.8 ± 0.1
Ash (structural inorganics)	3.0 ± 0.8	1.2 ± 0.2
Total mass balance	**84.6**	**91.1**
Total carbohydrates	**31.4**	**53.9**

The composition of the soluble extracts and alcohol-insoluble residues ($n = 2$). Data are presented as percentage dry weight (% w/w). The soluble carbohydrates include water and ethanol soluble sugars and minerals. Other extractables may include lignin, waxes or soluble proteins. The alcohol-insoluble residue is separated into structural carbohydrates (detected as monosaccharides), total polyphenols, protein, acetate groups and ash. *Indicates values used to calculate carbohydrate sugar content. Bold text indicates values derived from calculation. Unaccounted-for mass is likely to include volatile compounds lost during the drying process, lipids or waxes removed during extraction, and/or unhydrolyzed polysaccharides (pectin or crystalline cellulose).

3.2. Cell wall polysaccharides: characterization, abundance and spatial distribution

Following the extraction (ASE) of soluble components, an insoluble residue (largely cell wall material) remained. This extracted material contains predominantly polysaccharides and the phenolic polymer lignin. The hydrolysis of the polysaccharides was achieved using concentrated sulfuric acid (13.5 M), resulting in liberated monosaccharides, and acetyl groups; the remaining biomass was classified as acid-insoluble lignin (Sluiter et al., 2008).

Monosaccharides liberated from the insoluble material represent a small proportion of the total mass for grape marc at 11–17% w/w (Table 1). Glucose (Glc) is the most predominant monosaccharide (5–6% w/w). Xylose (Xyl), galacturonic acid (GalA), mannose (Man), galactose (Gal) and arabinose (Ara) are detected to lesser extents. Red grape marc has a higher proportion of cell wall polysaccharides (53% of total carbohydrates measured) compared with white marc (20%) (Table 1). The structural complexity of polysaccharides impacts processing methods and costs, but monosaccharide profiling alone does not provide structural information about the polysaccharides present. Intact polysaccharides from the alcohol-insoluble grape marc residue (AIR) were therefore fractionated (Chaplin and Kennedy, 1994) and the relative proportion of monomers quantified (Table 2).

The CDTA and Na_2CO_3 pectic polysaccharide-enriched fractions contained predominantly GalA, Ara and Gal with a minor proportion of Rha. These components could be derived from pectic polysaccharide, such as rhamnogalacturonans. However, the

Table 2
Composition of extracted fractions from grape marc.

	Neutral monosaccharides (mol%)[a]								Uronic acids (mol%)[a]		
	Ara	Fuc	Gal	Glc	Man	Rha	Xyl	Total	GalA	GlcA	Total
Cabernet Sauvignon											
CDTA[b]	21.2	0.0	15.5	10.7	19.9	3.8	7.7	78.8	21.2	0.0	21.2
Na_2CO_3[c]	34.2	0.0	13.8	17.0	8.0	6.4	6.6	86.0	14.0	0.0	14.0
1 M KOH[d]	26.7	0.0	9.1	28.7	13.3	0.0	20.2	98.0	2.0	0.0	2.0
4 M KOH[e]	7.1	2.3	6.8	22.3	7.2	0.0	52.1	97.8	0.0	2.2	2.2
Sauvignon Blanc											
CDTA[b]	30.1	0.0	21.6	2.5	5.6	5.6	7.7	73.1	24.8	2.1	26.9
Na_2CO_3[c]	48.6	0.0	15.8	6.1	0.0	8.6	5.3	84.4	15.6	0.0	15.6
1 M KOH[d]	25.7	0.0	8.2	22.9	1.5	0.0	36.5	94.8	5.2	0.0	5.2
4 M KOH[e]	6.0	1.7	7.5	22.6	6.5	0.0	55.7	100.0	0.0	0.0	0.0

[a] Average of duplicate determinations presented as relative mol%.
[b] Acid hydrolyzed trans-cyclohexane-1,2-diamine-tetraacetate (CDTA) extract.
[c] Acid hydrolyzed Na_2CO_3 + 25 mM $NaBH_4$ extract.
[d] Acid hydrolyzed 1 M KOH + 25 mM $NaBH_4$ extract.
[e] Acid hydrolyzed 4 M KOH + 25 mM $NaBH_4$ extract.

MALDI-TOF-MS analysis of the galacturonanase digest provided no molecular ion detection in the CDTA and Na_2CO_3 fractions (data not shown). The lack of detection for oligomers may be attributed to a highly branched rhamnogalacturonan structure in grape marc causing steric hindrance of the enzyme, or alternatively, the size of digest fragments being above the MALDI-TOF-MS detection threshold. Also, higher levels of glucose and mannose were detected in the Cabernet Sauvignon fractions (Table 2), which may be attributed to contamination of Glc and Man enriched yeast cell wall polysaccharides, as the red marc has gone through a partial fermentation step.

The other two fractions (KOH) were enriched in heteroxylans and xyloglucans. The high abundance of Ara, Glc and Xyl in the 1 M KOH fraction was attributed to heterogenous polymers such as xyloglucan or arabinoxylan (Table 2). The Ara may have originated from arabinoxylan, in which case the arabinose:xylose (A:X) ratio for red marc is 1.3 and 0.7 for white marc. The variation in A:X ratio between varieties has been observed in previous studies, where the A:X ratio was 0.7 for Muscat Gordo Blanco and 1.3 for Ohanez grape mesocarp (Nunan et al., 1997). However, when 1 M KOH fractions were treated with *endo*-1,4-β-xylanase M6, no molecular ions were detected using MALDI-TOF-MS (data not shown). Xylanase M6 enzymatically cleaves between two consecutive unsubstituted xylose residues along the xylan backbone, thus the lack of cleavage indicates that these heteroxylans are highly substituted, consistent with the A:X ratios of 1.3 and 0.7. It is unlikely that (glucurono)arabinoxylan or glucuronoxylan is present in this fraction as no glucuronic acid (GlcA) was detected. Whereas in contrast, the 4 M KOH fractions releases two oligosaccharides with *m*/*z* 759 and *m*/*z* 1023, putatively assigned as $Xyl_4MeGlcA_1$ and $Xyl_6MeGlcA_1$, respectively (data not shown).

The polysaccharides isolated in the 4 M KOH fraction were composed of 52–56% mol xylose and 23% mol glucose, with lesser amounts of galactose, mannose, fucose and arabinose. These monosaccharides are characteristic of xyloglucans with the exception of mannose, which may be an artifact from yeast mannan contamination (Doco et al., 2003). Previous reports indicate the predominant non-cellulosic polysaccharide in grape berries are xyloglucans, accounting for 10% of the polysaccharides in grape berry mesocarp (Nunan et al., 1997).

The xyloglucan extracted in the 4 M KOH fraction of the marc was treated with xyloglucan-specific *endo*-(1,4)-β-D-glucanase and analyzed using MALDI-TOF-MS for structural differences in xyloglucan fine structure (Hsieh et al., 2008). The structure of the hydrolyzate products are similar to those found in xyloglucans from other dicots: XXG (*m*/*z* 791), XXGG (*m*/*z* 953), XXXG (*m*/*z* 1085); XLXG/XXLG (*m*/*z* 1247); XXFG (*m*/*z* 1393); XLLG (*m*/*z* 1409); XLFG (*m*/*z* 1555), where X represents the Xyl(α1,6)Glc unit, L represents Gal(β1,2)Xyl(α1,6)Glc, F represents Fuc(α1,2) Gal(β1,2)Xyl(α1,6)Glc and G represents the backbone Glc in β1,4-linkage (Fry et al., 1993).

The isolated grape marc xyloglucans examined here consisted predominantly of XXXG backbones with a lower proportion of XXGG and XXG oligomers, which may have been generated from XXXG during cell wall maturation and restructuring (Doco et al., 2003). The backbones also contained the fucosylated oligomers XLFG and XXFG, with XLXG/XXLG and XLLG substituents present at a lower intensity.

3.3. Analysis of non-fermentable cell wall components in grape marc

Compared with other dedicated biofuel or waste materials, the amount of lignin present in grape marc is relatively high (Kim and Dale, 2004; Saha, 2003). The acid-insoluble lignin content contributes 11% and 32% w/w of white and red marc mass, respectively (Table 1). However, the high lignin content of grape marc has been attributed to the presence of condensed tannins (22% dry matter in red marc) and resistant proteins present as insoluble protein–tannin complexes or as Maillard products (Llobera and Cañellas, 2007). Thus presence of these compounds could interfere with the quantification of insoluble lignin, resulting in overestimations, but may still be informative when making processing decisions.

In a biofuel context, mineral content is an important consideration, for example, specific elemental levels are essential in the maintenance and progression of fermentation as yeast cannot function effectively outside a narrow range of environmental conditions (Bisson, 1999). The total concentration of elements in red marc is 27% w/w higher than that measured in white marc. Potassium was detected in the highest abundance in both varieties and represented 75% w/w of the total elemental content (Table 3). Phosphorus, calcium, and sulfur are the next most abundant and collectively contribute an additional 20% w/w.

3.4. Comparison of acid and thermal pre-treatments

Pre-treatment is the initial step to convert lignocellulosic biomass from its native recalcitrant state into a form that can be more readily hydrolyzed by enzymes (Mosier et al., 2005). In this study, the effectiveness of four pre-treatments was assessed by comparison with grape marc hydrolyzed following Sluiter et al. (2008).

Table 3
Elemental analysis of grape marc.

	Cabernet Sauvignon (mg/kg)	Sauvignon Blanc (mg/kg)
Aluminum (Al)	31 ± 17	13 ± 14
Calcium (Ca)	3867 ± 611	2170 ± 549
Iron (Fe)	85 ± 21	60 ± 27
Magnesium (Mg)	987 ± 76	710 ± 46
Phosphorus (P)	2733 ± 351	2367 ± 58
Potassium (K)	27,333 ± 2309	20,267 ± 3164
Sodium (Na)	58 ± 15	61 ± 15
Sulfur (S)	1370 ± 44	1027 ± 60
Zinc (Zn)	15 ± 3	9 ± 4
Total	36,479 ± 2267	26,684 ± 2567

Data is presented as mg/kg of dry material, $n = 2$.

Pre-treatment can generate a number of compounds inhibitory to fermenting organisms, such 5-(hydroxymethyl)furfural (HMF; from hexose carbohydrate degradation) and formic or levulinic acids (from the breakdown of HMF) (Palmqvist and Hahn-Hägerdal, 2000). As inhibitory compounds were not quantified in this study, moderate pre-treatment conditions were chosen that would minimize the formation of these secondary byproducts (Panagiotopoulos et al., 2011). The four pre-treatments conditions were: acid/autoclave (1 h, 121 °C, 0.5 M H_2SO_4); acid/oven (3 h, 100 °C, 1 M H_2SO_4); water/autoclave (1 h, 121 °C, H_2O; and water/oven (3 h, 100 °C, H_2O).

Glucose was the most abundant monosaccharide liberated in all pre-treatments (Table 4). Only acid treatments liberated other monomers, predominantly xylose and arabinose, from non-cellulosic polysaccharides (NCPs), consistent with previous studies in which heated water treatment was not severe enough to facilitate NCP solubilization (Sreenath et al., 1999). The acid/autoclave treatment liberated the highest proportion of monosaccharides for both varieties: 58% of the total carbohydrates measured using the NREL method were liberated from the red marc and 84% from the white marc (Table 4). The dilute acid (0.5 M) at higher temperature and pressure was the most effective pre-treatment.

There are advantages to using thermal rather than acid pre-treatments such as lower chemical costs, no need to neutralize the resultant hydrolyzate, and reduced production of inhibitory compounds (Mosier et al., 2005). However, there is a trade off in the increased energy consumption and lower recovery of fermentable sugars. In red marc, only 15–18% of the total glucose was liberated using thermal pre-treatments (2.1% w/w; Table 1) and 85–87% in white marc, indicating that the cellulose in white marc may be less structured and more amenable to hydrolysis. No NCPs were hydrolyzed using thermal pre-treatments, possibly due to the complexity of grape rhamnogalacturonan and xyloglucan. These data suggest that little or no cell wall breakdown occurred with thermal pre-treatments, rendering 35–93% of the carbohydrates in a structural, unfermentable form, thus significantly decreasing ethanol yields.

3.5. Effects of pre-treatment on the saccharification of cellulose

Pre-treatment not only liberates fermentable monomers but also weakens hydrogen bonding within and between glucan chains, which enhances the rate of polysaccharide breakdown into fermentable sugars in the presence of enzymes. Saccharification tests were performed on non-treated AIR, acid/autoclaved and water/autoclaved grape marc based on identical cellulose loadings following pre-treatment. The liberation of glucose from cellulose was monitored over 48 h of enzymatic digestion using a cellobiase preparation (cellobiase activity ⩾250 units/g) from *A. niger* and a commercial cellulase cocktail (endoglucanase activity ⩾700 units/g) isolated from *T. reesei*.

The acid/autoclave-treated grape marc exhibited the highest conversion of cellulose to glucose and for all pre-treatments, the amount of glucose released was higher for white marc than for red marc at all time points (Fig. 3). The greatest amount of glucose liberated was observed for acid/autoclave-treated Sauvignon Blanc samples, with 28% of cellulose hydrolyzed. Thermal pre-treatment did not increase the rate of glucose liberation from white marc compared with non-treated samples, with only 18% glucose present in the hydrolyzate (Fig. 3A). Similar findings have been recorded for sweet sorghum bagasse, in which water did not significantly improve the rate of cellulose hydrolysis yield (11.8%) over 96 h compared with the control (12.6%) (Cao et al., 2012).

However, for red marc, the amount of glucose released was correlated to the severity of the pre-treatment, and both pre-treatments resulted in higher saccharification rates than untreated samples. The maximum glucose liberated from Cabernet Sauvignon was 17% in acid-treated samples, 15% in thermal-treated samples and 13% in non-treated samples over

Table 4
Compositional changes in hydrolyzate after pre-treatment.

Biomass	Method	Glucose	Xylose	Arabinose	Other[a]	Total
Cabernet Sauvignon	NREL	11.0 ± 2.0	4.0 ± 0.9	1.8 ± 0.2	6.4 ± 0.9	23.2
	Acid/autoclave	5.3 ± 1.6	3.1 ± 0.4	1.6 ± 0.1	3.4 ± 0.4	13.4
		48%	78%	89%	53%	58%
	Acid/oven	4.1 ± 1.5	3.1 ± 0.6	1.6 ± 0.1	3.9 ± 0.6	12.7
		37%	78%	89%	61%	55%
	Water/autoclave	2.0 ± 1.2	0 ± 0.0	0 ± 0.0	0 ± 0.0	2.0
		18%	0%	0%	0%	9%
	Water/oven	1.7 ± 1.1	0 ± 0.0	0 ± 0.0	0 ± 0.0	1.7
		15%	0%	0%	0%	7%
Sauvignon Blanc	NREL	24.5 ± 2.6	2.3 ± 0.3	1.1 ± 0.2	3.9 ± 0.4	31.8
	Acid/autoclave	22.1 ± 4.2	1.3 ± 0.4	1.0 ± 0.3	2.2 ± 0.4	26.6
		90%	57%	91%	56%	84%
	Acid/oven	19.0 ± 2.5	1.6 ± 0.2	1.0 ± 0.3	1.9 ± 0.2	23.5
		78%	70%	91%	49%	74%
	Water/autoclave	21.3 ± 4.9	0 ± 0.0	0 ± 0.0	0 ± 0.0	21.3
		87%	0%	0%	0%	67%
	Water/oven	20.8 ± 4.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	20.8
		85%	0%	0%	0%	65%

Four pre-treatments were used and compared to the standard NREL acid hydrolysis method (Sluiter et al., 2008). Data are presented as percent dry weight (% w/w), $n = 2$. The pre-treatment conditions were: acid/autoclave (1 h, 121 °C, 0.5 M H_2SO_4); acid/oven (3 h, 100 °C, 1 M H_2SO_4); water/autoclave (1 h, 121 °C, H_2O); and water/oven (3 h, 100 °C, H_2O).

[a] Other includes additional monosaccharides (galactose, mannose, rhamnose, glucuronic acid and galacturonic acid).

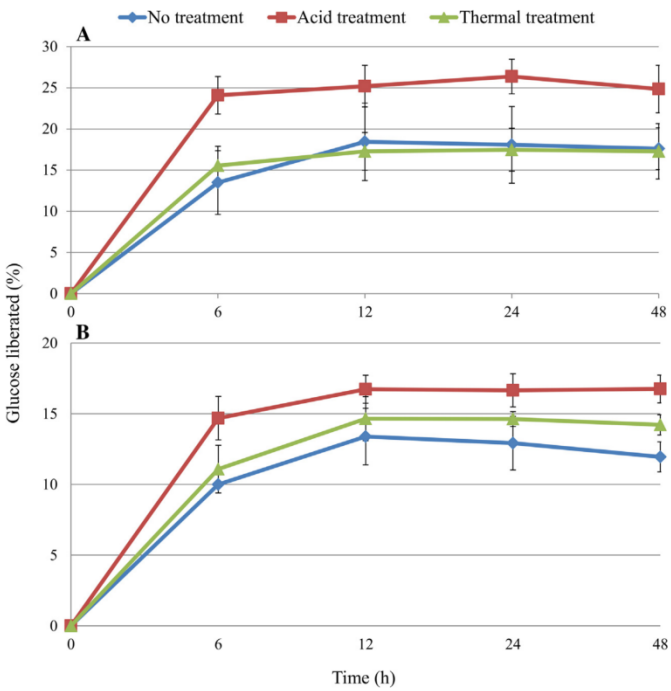

Fig. 3. Pre-treatment of grape marc increases the biochemical conversion of cellulose in the presence of cellulases. Liberation of the monomer glucose in Sauvignon Blanc marc (A) and Cabernet Sauvignon (B) was measured over 48 h. Grape samples were treated with 0.5 M sulfuric acid or water at elevated temperatures (1 h, 121 °C). The rate of saccharification of pre-treated grape marc is compared to non-pre-treated AIR grape marc and expressed as a percentage of cellulose converted into glucose.

Table 5
Theoretical yields of ethanol for agro-industrial waste.

Feedstock	Global production (Mt)[a]	Residue/crop ratio[b]	Waste biomass	Composition (dry % w/w)			Ethanol yield (L/t)
				Cellulose (C6)	NCPs (C5)	Lignin	
Corn (*Zea mays*)	768	1	Stover	40–43[c,d]	21–25[c,d]	17–19[b,c]	441–492
Barley (*Hordeum vulgare*)	140	1.2	Straw	38[e]	37[e]	9–16[b,e]	544
Rice (*Oryza sativa*)	655	1.4	Straw	32–47[d]	19–27[d]	7–12[b,c]	370–536
Wheat (*Triticum aestivum*)	636	1.3	Straw	30–45[c,d]	20–30[d]	16–20[b,c]	362–543
Sorghum (*Sorghum bicolor*)	59	1.3	Straw	32[f]	27[f]	7–15[b,f]	428
Sugarcane (*Saccharum* spp.)	1560	0.6	Bagasse	40[c]	24[c]	15–25[b,c]	463
Cabernet Sauvignon (*Vitis vinifera*)	67*	0.3[g]	Red marc	20.8	8.4	32.5	211
Sauvignon Blanc (*Vitis vinifera*)	67*	0.3[g]	White marc	48.2	7.3	10.5	400
Sauvignon Blanc (*Vitis vinifera*)	67*	–	White marc: WSC	37.6	–	–	270

Average of world production from 2002 to 2012 [a](FAO, 2015) expressed as Mt (megatonne) and the ratio of residue/crop [b](Kim and Dale, 2004) indicates the global availability of raw waste material for bioethanol production. *67 Mt of total grape marc (red and white) is generated annually. The theoretical ethanol yields for agricultural industrial waste materials were calculated based on compositional data from other studies: [c]Saha (2003); [d]Sarkar et al. (2012); [e]Sun and Sun (2002); [f]McIntosh and Vancov (2010); [g]Spanghero et al. (2009).

48 h (Fig. 3B). The slower and lower rate of glucose release observed in Cabernet Sauvignon samples compared to Sauvignon Blanc may be attributed to a higher ratio of fucosylated xyloglucan oligomers (XXFG; 1393 and XLFG; 1555) present in this tissue. The presence of fucosylated xyloglucans has been proposed to facilitate binding to cellulose, thus reinforcing the recalcitrant nature of the cell walls and making the cellulose polymers less accessible to enzymatic hydrolysis (Levy et al., 1997).

Overall, the acid pre-treatments were more efficient at degrading grape marc polysaccharides (Table 4) as well as modifying or weakening the hydrogen bonding of the crystalline cellulose polymers, making them more accessible to enzyme attack. Pre-treatments that increase the number of enzyme binding sites on cellulose microfibrils may increase biofuel yields, although studies have shown that saccharification may be a limiting factor when converting from small to large scale production (Klein-Marcuschamer et al., 2012). As a result, it may be more economically favorable to identify and capitalize on the WSC present in dedicated sources of biomass for biofuel production, such as grape marc.

3.6. Theoretical ethanol yields from grape marc based on compositional data

The agro-industrial waste grape marc has been compared with other lignocellulosic waste materials based on global production, crop/residue ratio, chemical composition, and yield of ethanol per tonne (Table 5). On a dry w/w basis, 31% of the marc from

Cabernet Sauvignon is carbohydrates (15% soluble and 17% insoluble) with more from Sauvignon Blanc at 54% w/w (43% soluble and 11% insoluble) (Table 1).

The global production and subsequently the residue:crop ratio for grapes are lower than feedstocks such as *Zea mays* (corn), *Oryza sativa* (rice) and *Triticum aestivum* (wheat) (Table 5). However, grape marc is a discarded waste residue from an established industry, and would not be grown as a dedicated biofuel feedstock. Thus the utilization of grape marc for bioethanol production is still value-adding, even if lower yields (biomass and ethanol) are achieved. Ethanol yields of 211 L/t can potentially be generated from red marc and 400 L/t from white marc, which is slightly lower than yields predicted for other lignocellulosic biofuel feedstocks such as sugarcane bagasse (*Saccharum* spp.; 463 L/t), rice straw (370–536 L/t) and sorghum straw (*Sorghum bicolor*; 428 L/t) (Table 5). Alternatively, just the WSC in white marc can be extracted and fermented directly, yielding up to 270 L/t. The utilization of only WSC can minimize processing costs associated with liberating fermentable sugars from the cell wall, leaving a polyphenol enriched fraction that may be used in animal feed, applied as a fertilizer or exploited as a source of phenolic compounds. An integrated processing system for grape marc would not only enable multiple products to be refined from the same waste material, increasing its value, and would be environmentally, socially and economically advantageous.

4. Conclusions

Grape marc is a waste material that is rich in carbohydrates. The majority of these carbohydrates are soluble monosaccharides (glucose and fructose) and structurally complex polysaccharides (pectins, heteroxylans, xyloglucan and cellulose). The soluble carbohydrates can be directly converted to ethanol through fermentation, yielding up to 270 L/tonne. Alternatively, ethanol yields may be increased by utilizing the whole material, which benefits from acid pre-treatment followed by enzymatic hydrolysis. Overall, these data suggest that on a weight for weight basis, grape marc has the potential to be a competitive, value-adding waste material for biofuel production, contributing up to 400 L/t ethanol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.06.030.

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