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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

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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