

UNIVERSITY OF ADELAIDE

DOCTORAL THESIS

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

Author:

Kendall R. Corbin

Supervisors:

Dr Natalie S. Betts

Dr Caitlin S. Byrt

Professor Geoffrey B. Fincher

Associate Professor Rachel A. Burton

*A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy*

in the

Australian Research Council Centre of Excellence in Plant Cell Walls

Waite Campus, South Australia, Australia

May 2015

Table of Contents

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

<u>List of Figures</u>	v
<u>List of Tables</u>	vii
<u>Abstract</u>	ix
<u>Project Summary</u>	x
<u>The thesis is based on the following papers</u>	xiii
<u>Candidates' contribution to papers</u>	xiv
<u>Statement of Authorship</u>	xv
<u>Acknowledgements</u>	xvi
<u>Abbreviations</u>	xvii
<u>1.0 Background</u>	1
<u>2.0 Production of bioethanol</u>	3
<u>3.0 Selecting lignocellulosic biomass</u>	6
<u>4.0 Potential feedstocks for bioethanol production in Australia</u>	10
4.1 <i>Agave (A. tequilana and A. americana)</i>	10
4.2 Grape marc derived from <i>Vitis vinifera</i>	12
<u>5.0 Compositional analysis of selected biomass</u>	14
<u>6.0 Pre-treatment solubilizes cell wall polymers</u>	14
<u>7.0 Enzymatic saccharification increases the amount of fermentable monosaccharides</u>	17
<u>8.0 Anaerobic fermentations may be used for bioethanol production</u>	19
<u>9.0 Objectives of the thesis</u>	21
<u>10.0 Materials and Methods</u>	23
10.1 <u>Comparison of water extraction methods</u>	23
10.2 <u>Fluorescent Immuno-microscopy</u>	23
10.3 <u>Monolignol analysis: S/G ratio</u>	24
10.4 <u>Total phenolic and anthocyanin content</u>	24
10.5 <u>Immuno-electron microscopy: grape marc</u>	25
10.6 <u>Screening of yeast: assimilation and biomass accumulation on glucose</u>	25

<u>11.0</u>	<u>Results and Discussion</u>	27
11.1	<u>Paper I: Highlights</u>	27
11.2	<u>Paper II: Highlights</u>	77
11.3	<u>Paper III: Highlights</u>	111
<u>12.0</u>	<u>Thesis Conclusions and Key Findings</u>	149
12.1	<u>Refining the techniques used for biomass processing</u>	150
12.2	<u>Increasing monosaccharide availability and identifying efficient pre-treatments</u>	152
12.3	<u>Ensuring complete utilization of carbohydrates during fermentation</u>	153
<u>13.0</u>	<u>Future Directions</u>	154
13.1	<u>Trialing the scalability of bioethanol production from lignocellulosic residues</u>	154
13.2	<u>Investigating alternative conversion methods</u>	155
13.3	<u>Mining the microbiome of lignocellulosic feedstocks</u>	157
<u>14.0</u>	<u>Appendices</u>	159
14.1	<u>Appendix A: Unpublished data paper I</u>	159
14.2	<u>Appendix B: Compositional analysis of <i>Agave sisalana</i> leaves</u>	167
14.3	<u>Appendix C: Unpublished data paper II</u>	175
14.4	<u>Appendix D: Additional data paper III</u>	180
14.5	<u>Appendix E: Published Paper I</u>	190
14.6	<u>Appendix F: Published Paper II</u>	212
<u>15.0</u>	<u>References</u>	220

List of Figures

Figure 1 Simplified outline of a generalized processing scheme for the conversion of plant biomass to bioethanol	5
Figure 2 Regions of naturalized <i>A. americana</i> and <i>A. sisalana</i> in Australia	11
Figure 3 Grape production in Australia	13
Figure 4 Selected pre-treatment methods differentially affect the breakdown and liberation of polymers from the cell wall	16
Figure P1-1 Flowchart outlining the steps taken to process and analyze <i>Agave</i> leaves	57
Figure P1-2 <i>Agave</i> processing and moisture content	58
Figure P1-3 Different fractions of <i>Agave</i> material	59
Figure P1-4 <i>Agave</i> leaf morphology	60
Figure P1-5 <i>Agave</i> tissue has pectinaceous crystal clusters localized at cell junctions	61
Figure P1-6 Cell wall polysaccharides detected by immunolabelling and transmission electron microscopy	62
Figure P1-7 Cellulose, the most predominant polymer in <i>Agave</i> leaf tissue is degraded by cellulases	64
Figure P1-8 Quantification of juice sugars from <i>A. americana</i> leaves and <i>A. tequilana</i> leaves and stem	65
Figure P2-1 Flowchart outlining the sequential fractionation of grape marc (AIR) to isolate polysaccharides for characterization	100
Figure P2-2 Grape marc is a heterogeneous material composed predominantly of grape skin	101
Figure P2-3 Pre-treatment of grape marc increases the biochemical conversion of cellulose in the presence of cellulases	102
Figure P2-S1 Maldi-TOF-MS spectra of xyloglucan oligomers from grape marc	109
Figure P2-S2 Basic repeating unit of xyloglucans	110
Figure P3-1 Diagram outlining the fractions that can be generated from processing of <i>Agave</i> plants	135
Figure P3-2 Spontaneous fermentation of <i>A. tequilana</i> juice	136
Figure P3-3 Conversion rates of total carbohydrates to ethanol using <i>Agave</i> stem juice	137
Figure P3-S1 <i>Agave</i> juice naturally ferments	147
Figure A-1 Distribution of pectin labelling in an <i>A. tequilana</i> leaf section	162

Figure B-1 Mass balance of <i>A. sisalana</i> leaves: soluble and insoluble components.	169
Figure B-2 <i>A. sisalana</i> leaves are enriched with calcium oxalate crystals	171
Figure B-3 Cell wall polysaccharides in <i>A. sisalana</i> leaves	172
Figure B-4 Quantification of mono- and di-saccharides in <i>A. sisalana</i> leaf juice	173
Figure C-1 Total phenolic and anthocyanin content in grape marc	177
Figure C-2 Distribution of pectin labeling in Sauvignon Blanc grape marc	178
Figure D-1 Variation in the mineral content of extracted <i>Agave</i> juice	182
Figure D-2 Identifying the optimal growth temperature for selected microorganisms	186
Figure D-3 Example of carbon source utilization for selected yeast strains	187
Figure D-4 Dry cell weight of selected yeast strains after 24 h when cultured in glucose	188
Figure D-5 Fermentation profiles of selected yeast in autoclaved <i>A. tequilana</i> leaf juice	189

List of Tables

Table 1 Composition of lignocellulosic biomass (% w/w)	9
Table P1-1 Comparison of potential biofuel feedstocks	67
Table P1-2 Composition of <i>A. americana</i> and <i>A. tequilana</i> leaves	68
Table P1-3 Polysaccharides detected by linkage analysis in <i>Agave</i> leaf	70
Table P1-4 Carbohydrates in fiber-enriched fractions from <i>Agave</i> leaves	71
Table P1-5 Fermentation of <i>Agave tequilana</i> juice using <i>Saccharomyces cerevisiae</i>	72
Table P1-6 Theoretical ethanol yields for lignocellulosic feedstocks	73
Table P1-S1 Monosaccharide linkage analysis data for <i>Agave</i> leaves (mol %)	75
Table P1-S2 Elemental analysis of <i>Agave</i> juice and whole leaf	76
Table P2-1 Mass balance of Cabernet Sauvignon and Sauvignon Blanc grape marc	103
Table P2-2 Composition of extracted fractions from grape marc	105
Table P2-3 Elemental analysis of grape marc	106
Table P2-4 Compositional changes in hydrolyzate after pre-treatment	107
Table P2-5 Theoretical yields of ethanol for agro-industrial waste	108
Table P3-1 Selected fermenting strains and conditions using <i>Agave</i> as reported in literature	138
Table P3-2 Mass distribution and carbohydrate content of <i>Agave tequilana</i> juice	140
Table P3-3 Analysis of <i>Agave</i> juice to quantify total carbohydrate content	141
Table P3-4 Selected microorganisms for fermentation of <i>Agave</i> juice	142
Table P3-5 Comparison of ethanol yields achieved from fermentation of <i>A. tequilana</i> leaf juice	143
Table P3-6 Predicted ethanol yields for <i>Agave tequilana</i>	144
Table P3-S1 pH and minerals in <i>Agave tequilana</i> juice	145
Table P3-S2 Metabolite concentrations in raw, autoclaved and fermented (72 h) <i>A. tequilana</i> leaf juice	146
Table P3-S3 Thermo-tolerance of selected commercial yeast	148
Table A-1 Comparison of four WSC extraction methods using <i>A. tequilana</i> leaves	160
Table A-2 Comparison of monolignol ratios (S/G) in <i>A. tequilana</i> biomass	164
Table A-3 Mass balance of <i>A. tequilana</i> leaf and stem bagasse	166

Table B-1 Linkage analysis of <i>A. sisalana</i> leaves	170
Table B-2 Composition of fibers isolated from <i>A. sisalana</i> leaves	174
Table D-1 Mass distribution is variable between plants of <i>A. tequilana</i> (4.5 y old)	181
Table D-2 Microorganisms sourced from ARS Culture Collection for fermentation of <i>Agave</i> juice	184

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.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Kendall R. Corbin

May 2015

Acknowledgements

I would like to thank any and all individuals who have embarked on this roller coaster ride called a PhD with me. You have supported me in moments of doubt, encouraged me during times of frustration and celebrated with me in moments of triumph. Together we have laughed and we have learned. Although this simple heartfelt acknowledgement does not come close to scratching the surface of the gratitude, respect and honor I have for you all, thank you.

Thanks to all the members of the Plant Cell Walls group for ensuring I didn't go hungry with weekly cakes and keeping the KFC jokes to a minimum. Special thanks to Ashley, Marilyn, Jelle, Quang, Dave, Kuok, Emma, Karen, Yves, Julian, and Wai Li. Thanks to the Wine Microbiology and Microbial Biotechnology Group for adopting me as your own: Vladimir, Paul, Joanna, Nick, Patrick, Simon, Tommaso and Michelle. A special thanks to the AusAgave team for beating the heat and fighting the thorns to provide the plant biomass for this project: Don, Joseph, Markus and Charles. Stefan Bauer and the "Bauer Rangers" thank you for kick starting this project and opening my eyes to the wonderful world of analytical chemistry. Six years later and half a world away, thank you Seth and Jozsef for sharing your love of plant cell walls and go getter attitudes with me. To my biggest supporter, Carlos, thank you for sacrificing your weekends and sanity to help me achieve everything I wanted and more. I am sorry for all the white hairs! To the Corbin clan: thanks for having strong shoulders to lean on, helping me fight procrastination one Skype call at a time, and above all THANK YOU for your unconditional and unwavering love. I am blessed.

Last but certainly not least, a special thanks to my supervisors (Natalie, Caitlin, Rachel and Geoff) for all their guidance, mentorship and lessons in "tightening". You each have taught me valuable lessons in life and research that couldn't have been acquired from any textbook.

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