Bioethanol Production Potential of *Agave tequilana* F.A.C. Weber

By
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A thesis submitted for the degree of Doctor of Philosophy

To
The School of Health, Medical and Applied Sciences
Central Queensland University, Rockhampton, Australia

August 2016

Abstract

The growth potential of ten species of *Agave*, and six cultivars of *Agave tequilana* and *Furcraea foetida*, was evaluated over 5 years at Rockhampton, Queensland, Australia. The purpose of this trial was to assess the use of these genotypes as biofuel feedstock owing to their inherent ability to grow on dry lands. The leaf number and leaf area increment, and the above ground biomass accumulation of the naturalised *Agave* species such as *F. foetida*, *Agave decipiens* and *Agave americana* were either similar or better (13.4, 13 and 11.5 t ha^{-1} yr^{-1} respectively) than those of *A. tequilana* cultivars. Amongst *A. tequilana* cultivars, maximum growth was achieved by the cultivar Tcqu, yielding 12.9 t ha^{-1} yr^{-1} dry above ground biomass (with 7.1 t ha^{-1} yr^{-1} leaf and 5.8 t ha^{-1} yr^{-1} stem).

The two best performing cultivars of *A. tequilana* (Tcqu and L19) were further evaluated for leaf cellulose, hemicellulose and lignin content. These compositions varied with the age and environmental conditions. A hand-held short wave near infrared spectrometer (SWNIR), based on silicon photodiode array (Si-PDA) detector was evaluated and recommended for non-invasive estimation of leaf dry matter to guide harvest timing. A Fourier Transform Near Infrared (FTNIR) spectrometer based on indium gallium arsenic (InGaAs) detector was also evaluated and recommended for non-invasive estimation of cellular composition of dried ground samples of *Agave* leaf, to inform fermentation management. The FTNIR technology was more effective in predicting leaf cellulose (Rp 0.87 and RMSEP 2.9% w/w) than hemicellulose or lignin contents.

The effects of five pretreatment conditions (time, temperature and acid concentrations) were assessed in terms of cellulose digestibility, hemicellulose solubilisation and lignin content of the leaves of 1.5 year-old plants of *A. tequilana* from Rockhampton and 2.5 year-old plants from Kalamia. These leaves were pretreated with 2% H_2SO_4 at 121°C for 60 min, enzyme saccharified with 15% w/w Cellic® CTec2 and fermented using *Saccharomyces cerevisiae* to produce ethanol. Based on this protocol, ethanol yield of 180 L t^{-1} can be obtained from the leaves of *A. tequilana.* For an estimated leaf dry biomass of 7.1 t ha^{-1} yr^{-1} from *A. tequilana*, an ethanol production of 1,278 L ha^{-1} yr^{-1} can be obtained. The stems of *A. tequilana* contain higher sugar content than leaves. Thus, assuming an average ethanol yield of 374 L t^{-1} from the stem, an additional 2,169 L ha^{-1} yr^{-1} of ethanol can be produced. Therefore, from both the leaf and stem biomass, ethanol yield of 3,447 L ha^{-1} yr^{-1} could be produced from *A. tequilana.*

Acknowledgments

I would like to express my sincere gratitude to my principal supervisor Associate Professor Nanjappa Ashwath and all of my co-supervisors: Professor Kerry Walsh, Professor Grant Stanley, Dr Phul Subedi and Dr Tony Vancov for their great support and continuous guidance throughout my PhD program and particularly during thesis writing. I am indebted to A/Prof Nanjappa Ashwath for providing me continuous support throughout the research program and his valuable advice and feedback on all aspects of the research work. My sincere thanks and gratitude goes to Prof Kerry Walsh for his guidance, critical feedback, encouragement, endurance and his great contribution to all aspects of my research. I am very much grateful to Prof Grant Stanley for his inspiration, support, guidance and feedback on my overall research activities. My sincere thanks go to Dr Phul Subedi for his support and guidance on operational and analytical aspects of NIR spectroscopy. My utmost gratitude goes to Dr Tony Vancov and Dr Shane McIntosh for overall guidance in the technical and analytical aspects of processing of agave leaves for bioethanol production and giving me the opportunity to conduct experiments at Department of Primary Industries, Wollongbar, NSW.

I would also like to acknowledge the receipt of the University Post Graduate Research Award (UPRA) from Central Queensland University to conduct this research. I was also supported by a Scholarship from the Australian Government's Research Training Scheme. I gratefully acknowledge the financial support provided by the Australian Government.

This research activity would not have been possible without the support of Mr. Don Chambers of AusAgave, South Australia. I would like to acknowledge his support in supplying the tissue cultured plant materials to establish the trial in Rockhampton and allowing me to collect the samples from Kalamia, Queensland. I would also like to thank Professor Joe Holtum and Don Chambers for granting permission to use photographs and figures from their published papers and supplying dry above ground biomass data from Kalamia site to include in my thesis. I am very much grateful to Ross Bonato for sending agave samples from Kalamia and for his assistance during sample collection.

There are many individuals who have contributed to the research program in many ways and their contributions are very much appreciated. Especially, I would like to thank

Dr Resham Gautam for being a great mentor and Geeta Gautam Kafle for being a great friend and in providing moral support since the beginning of my research. My sincere gratitude goes to Gautam family for hosting me at their home as part of their family during my final stages of thesis writing and in providing me a stress-free environment to focus on writing.

I would like to thank Roshan Subedi for his contribution in data collection in the field and his technical assistance in the laboratory, and to Divya Priya for assisting me in the laboratory for compositional analysis experiment. My sincere thanks go to Vineela Challagulla for being a mentor and moral supporter throughout my study period. I am thankful to Dr Pramod Shrestha for his overall support in collecting data in the field, especially during harvest. I also would like to express my gratitude to Antonia, Dr Dipak Devkota, Lila Devkota, Dr Jay Dhungel and Shanta Dhungel and Dr Bed Khatiwada for giving me their valuable time in data collection in the field. Also, I would like to acknowledge all the friends and staff in former Centre for Plant and Water Science and the school of Medical and Applied Sciences, especially, Graham Fox for assisting me with the experimental set up and maintenance and Linda Ahern for providing great administrative support. This activity would not have been complete without the support of all the technical staffs Giselle Weegenaar, Heather Smyth and Judy Couper for my laboratory work and Judy Couper, Andrew Bryant, Giselle Weegenaar and Ingrid Christiansen during harvesting.

I would not have commenced my PhD without the inspiration, moral support and perseverance of my husband, Amod Rijal. My great gratitude goes to him and my children Adip and Eesha for their ongoing patience and support throughout the study period. Thank you Adip for taking care of Eesha during the difficult times. Thank you all from the bottom of my heart for your belief in me to pursue my dreams. My utmost gratitude goes to my parents: Purna Prasad Dhakal and Late mother Devaki Dhakal, who always gave priority for my studies. My parents have been my councillors and mentors throughout my life and during the study career. My in-laws are my inspiration for my further study. My sincere thanks goes to my father in-law Mr. Badri Prasad Rijal and my mother in-law Devi Rijal, who came all the way from Nepal to look after my family while I was away for a period of two months to undertake research activities in NSW, and in providing me with an opportunity to focus on my research activities. My special thanks

go to Dr Pawan Gupta and Mrs. Punita Gupta for hosting me at their home during my stay in Wollongbar, NSW. I am also very grateful to Sumitra Gautam and Dhana Gautam from Cairns for taking care of my family while I was away in Rockhampton to finalise my thesis. Finally, I would like to thank everyone involved in my journey of research higher degree and their well wishes for the completion of the study.

Statutory Declaration

I hereby certify that “Bioethanol Production Potential of *Agave tequilana* Weber” is a presentation of my original research work and all the sources used were acknowledged by means of complete reference. The material has not been submitted, either in whole or in part, for a degree at this or any other universities.

I acknowledge and certify that I have complied with the rules, requirements, procedures and policy of the Central Queensland University, Australia, relating to research higher degree award. I authorise Central Queensland University to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Name: Deepa Rijal

Signature:

Date: August 2016

Dedication

This work is dedicated to my Late mother Mrs Devaki Dhakal who passed away last year during the course of my study after nine month long battle with lung cancer. My mum was my inspiration for my further study.

Deepa Rijal, 2016

List of Abbreviations and Acronyms

Abs	Absorbance
ABS	Australian Bureau of Statistics
AFEX	Ammonia Fibre Explosion
AGB	Above Ground Biomass
AGDOI	Australian Government Department of Industry
AIR	Acid Insoluble Residue
ANOVA	Analysis of Variance
ARRE	Australian Rural Real Estate
ASD	Analytical Spectral Device
ASL	Acid Soluble Lignin
ASMS	Australian Sugar Milling Council
ASTM	American Society of Testing and Materials
BAA	Biofuel Association of Australia
BOM	Bureau of Meteorology
CAM	Crassulacean Acid Metabolism
CEC	Clean Energy Council
CT	Cellic® CTec2
d^2A	Second Derivative of Absorbance
DAFF	Department of Agriculture, Forestry and Fisheries
DEWS	Department of Energy and Water Supply
DM	Dry Matter
DNS	Dinitrosalicylic
DW	Dry Weight
EMSC	Extended Multiplicative Scatter Correction

EPG	Ethanol Production Grant
FAO	Food and Agriculture Organisation
FTNIR	Fourier Transform Near Infrared
FW	Fresh Weight
GC	Gas Chromatography
HMF	5-hydroxymethyl 2-furaldehyde
HPLC	High Performance Liquid Chromatography
InGaAs	Indium Gallium Arsenide
KAL	Kalamia
LAP	Laboratory Analytical Procedure
LHW	Liquid Hot Water
LOO	Leave One Out
MAP	Months After Planting
MLR	Multiple Linear Regression
MSC	Multiplicative Scatter Correction
MVA	Multivariate Analysis
NIRS	Near Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
NIPALS	Non-linear Iterative Partial Least Squares
NREL	National Renewable Energy Laboratory
PC	Principal Component
PCA	Principal Component Analysis
PDA	Photo Diode Array
PEPC	Phosphophenylpyruvate Carboxylase
PLSR	Partial Least Square Regression

PTFE	Poly- tetrafluorethylene
R/r	Correlation Coefficient
R^2	Coefficient of Determination
RMSEC	Root Mean Square Error of Calibration
RMSECV	Root Mean Square Error of Cross Validation
RMSEP	Root Mean Square Error of Prediction
ROK	Rockhampton
RPD	Ratio of Standard Error of Prediction to Standard Deviation
SD	Standard Deviation
SDR	Ratio of Standard Deviation to Standard Error of Prediction
SEC	Standard Error of Calibration
SEP	Standard Error of Prediction
SG	Savitzky Golay
SNV	Standard Normal Variate
SSC	Soluble Solid Content
SSF	Simultaneous Saccharification and Fermentation
SWNIR	Short Wavelength Near Infrared Region
TAPPI	Technical Association of the Pulp and Paper Industry
TSS	Total Soluble Solids
UV	Ultra Violet

Genotypes of Agave used in the Study

A. ame V	*Agave americana* 'Variegata'
A. ame	*Agave americana*
A. ang M	*Agave angustifolia* 'Marginata'
A. ang	*Agave angustifolia*

A. deci	*Agave decipiens*
A. des V	*Agave desmetiana* ‘Variegata’
A. des	*Agave desmetiana*
A. teq	*Agave tequilana*
ABM	Unknown *Agave* sp. 1 (ABM)
F. foe	*Furcraea foetida*
17	*Agave tequilana* cultivar 17
E9	*Agave tequilana* cultivar E9
L3	*Agave tequilana* cultivar L3
L9	*Agave tequilana* cultivar L9
L19	*Agave tequilana* cultivar L19
Tcqu	*Agave tequilana* cultivar Tcqu

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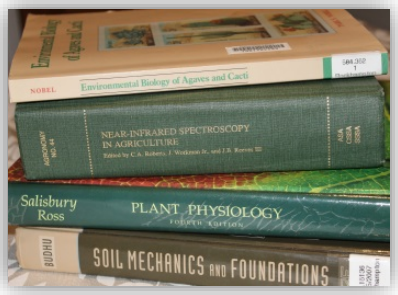
Chapter 1. Introduction and Literature Review



1.1 Introduction

There have been concerns over the use of fossil fuels for energy due to their finite supply, issues surrounding energy security, and their contribution to increased atmospheric CO_2 (Stanley & Dumsday 2010). Reduction of fossil fuel use necessitates the development of alternative sources of energy. While there are a number of alternatives for 'fixed' power production (e.g., electricity production from solar, wind, geothermal), alternatives for transport are more limited. Biofuels offer an alternative to petroleum based fuels (Balat & Balat 2009). The term 'biofuel' refers to any type of liquid, gas or solid fuel that is primarily derived from biomass, e.g., ethanol, methanol, biogas and biodiesel (Demirbas 2008).

First generation biofuels such as biodiesel, biogas, and bioethanol are derived from carbohydrates and lipids of crops such as sugarcane, corn, soybean, sunflower, canola and wheat (Demirbas 2008). The use of these crops for biofuels conflicts with traditional food and feed purposes, and the utilisation of arable land for their cultivation is contentious. This use of edible food crops for biofuels production can cause food shortages and trigger increases in food prices, and is thus controversial on socio-economic grounds (Demirbas 2008).

To overcome direct competition with food uses, second generation biofuels are based on the use of lignocellulosic (non-edible part of plant) biomass residues such as sugarcane bagasse, wheat stalks, corn stover, sorghum residue and wood chips. For example, more than 32 Mt of sugarcane is produced annually in Australia (ABS 2016), generating large quantities of bagasse. These residues can serve as biofuel feedstock although they have existing roles in production systems, including green mulching for erosion and weed control, improvement of soil structure and moisture retention. An alternative is the use of

non-food plants such as *Pongamia*, *Jatropha*, switchgrass and *Eucalyptus* (Limayem & Ricke 2012; Somerville et al. 2010b), and *Calophyllum inophyllum* (Ashwath 2010), preferably on lands not suited to food crop production. For example, approximately 20% of the global land is semi-arid, indicating the potential use of this land for cultivating species that tolerate such conditions and serve as bioenergy feedstock without competing with food crops (Davis, Dohleman & Long 2011).

Third generation biofuels are derived from marine biomass such as macroalgae, or seaweeds, and microalgae (Singh, Nigam & Murphy 2011). Fuels produced from algae include diesel or gasoline from crude oil (lipids), bioethanol from carbohydrates and biogas from anaerobic digestion of algal biomass. However, the production of fuel derived from algae is not yet commercially viable (Chisti 2013).

In Australia, ethanol and biodiesel are blended into regular petroleum products, being marketed as E10 (10% ethanol) and B5 and B20 (5% and 20% biodiesel, respectively). Queensland and New South Wales (NSW) attempted to impose a mandatory requirement of blending a certain percentage of ethanol in regular petrol to achieve the voluntary national biofuels target of 350 ML per annum by 2010, as set by the Government of Australia in 2001(CSIRO, BTRE & ABARE 2003). A requirement for the use of 5% ethanol (on average) in regular petrol was to be implemented from 1 January 2011 in Queensland, but this bill was rejected in October 2014 due to uncertainty in the fuel excise regime. On 1 December 2015, the Liquid Fuel Supply (Ethanol and Other Biofuels Mandate) Amendment Bill 2015 was passed and included a 3% ethanol mandate for petrol in Queensland. Under this mandate, at least one in three litres of non-premium petrol sold must be E10. After 18 months, this mandate will increase to 4% ethanol (DEWS 2015).

In NSW, the Biofuels Act 2007 required legislation of at least 6% of total volume of petrol sold in the state to be ethanol by 2011. However, this Act was removed by the Biofuels Amendment bill in 2012. The requirement was reinstated on 1 July 2015, and NSW is currently the only state to impose mandatory blending of 6% ethanol in all sold petrol (Fair Trading 2015).

In 2008, an Ethanol Production Grant (EPG) program was introduced nationally in Australia. This program provided a full rebate of the 38.143 c/L fuel excise for

domestically produced ethanol for use in the transport sector. Imported ethanol continued to be subjected to a 38.143 c/L custom rate. The main purpose of the EPG was to protect the domestic ethanol industry against imports and to encourage use of ethanol as an alternative transport fuel (AGDOI 2014). Despite the scheme, the production of ethanol declined by 17% from the 2010/11 financial year to 2013/14 (from 319 ML to 265 ML) (BREE 2014). The EPG program was closed in 30 June 2015, but simultaneously, the excise was dropped to a zero rate for one year. From July 1 2016, the fuel excise will increase by 6.554% each year until the final rate reaches 12.5 c/L in July 2020 (BAA 2014). This represents a subsidy of 25.643 c/L compared to imported fuel, which will remain subject to an import duty of 38.143 c/L.

In Australia, total consumption of petrol in 2012–2013 was 18.7 billion litres, and Australian produced ethanol supplied only 265 ML (1.4%) of the total road transport fuel. Ethanol blended petrol; however, accounted for around 13.8% of total petrol sales in NSW and Queensland in 2012–2013, using both locally produced and imported sources (BREE 2014).

The feedstock commonly used for ethanol production in Australia are grain sorghum, molasses (by-product of sugar processing), wheat and waste wheat starch (residue from flour production) (Cuevas-Cubria 2009). There are currently three ethanol-producing plants located in NSW and Queensland, with a total installed production capacity of 440 ML (BREE 2014). The largest producer, the Manildra ethanol plant in Nowra, NSW, uses waste wheat starch to produce ethanol and has a production capacity of 300 ML. The second largest producer of ethanol is the Dalby Bio-refinery in Dalby, Queensland, which uses red sorghum as the feedstock with a production capacity of 80 ML. An ethanol-producing plant located in Sarina, Queensland, is the third largest producer with a capacity of 60 ML (BREE 2014; Farrell 2014). This is the most energy efficient plant in Australia as it uses sugarcane molasses as the feedstock, and sugarcane bagasse as the source of electricity for ethanol production (co-generation). An additional 90 ML of ethanol (based on 2010 figures and a fermentation yield of 88%, distillation efficiency of 99% and ethanol density of 0.789 kg L^{-1}) could be produced if all exported cane molasses (produced in Queensland) were utilised in ethanol production (O'Hara 2010).

Fuel ethanol producers have raised concerns regarding the cost and adequate supply of ethanol-generating feedstock in terms of sustainability of the industry. For example, in

order to double the amount of available feedstock, the crop area to be sown should be more than double, as current production uses productive areas. The amount of available land in areas with adequate rainfall and irrigation may not be sufficient to meet the demand. Therefore, marginal land that requires irrigation and fertilisation would be needed to maintain biomass accumulation at a sustainable rate. Irrigation and fertilisation add to the cost of feedstock and ultimately to bioethanol production cost. According to the Australian Bureau of Statistics (ABS 2009), 14% of all agricultural irrigation in Australia was used in sugarcane farming in the year 2007–2008. The cost of irrigation contributes to approximately one third of the total cost of production for about 60% of sugarcane farmers (Holtum et al. 2010).

Feedstock derived from lignocellulosic biomass is also considered to be a good source for bioenergy production. The most commonly used resources are forest residues from hardwood and softwood species such as eucalypts and pines (McIntosh et al. 2012; Puri, Abraham & Barrow 2012), sorghum straw (McIntosh & Vancov 2010), agricultural residues such as sugarcane bagasse (O'Hara 2010), rice straw (Wi et al. 2013), wheat stubble (McIntosh & Vancov 2011) and corn stover (De Bari et al. 2014). Perennial grasses such as Napier grass (*Pennisetum purpureum*), *Miscanthus* sp. (Somerville et al. 2010b) and giant reed (*Arundo donax*) (Williams 2010) are also considered useful for production of biofuel in Australia. Climatic conditions in Australia are also suitable for producing various woody weeds such as *Camphor laurel*, *Mimosa pigra* and *Acacia nilotica*, which could possibly be used as biofuel feedstock (CEC 2008). Most of the above-mentioned species have low to moderate tolerance to drought and require up to 1200 mm of seasonal water and a nitrogen requirement of 120 kg ha^{-1} yr^{-1} (Table 1.1).

Table 1.1. Estimated productivity, rainfall, and nitrogen requirements of the current or potential bioenergy crops (Somerville et al. 2010b).

Crop	Average productivity Mg $ha^{-1}yr^{-1}$	Ethanol yield L ha^{-1}	Seasonal water requirement mm yr^{-1}	Drought tolerance	Nitrogen requirements kg ha^{-1} yr^{-1}	References
Corn		3800 (total)	500–800	low	90–120	(FAO 2010; Moose 2009; Perrin, Fretes & Sesmero 2009)
Cereal grain	7	2900				
Stover	3–6	900				
Sugarcane	80 (wet)	9900 (total)	1500–2500	moderate	100–160	(Somerville et al. 2010a)
Sugar	11	6900				
Bagasse	10	3000				
***Miscanthus* sp.**	15–40	4600–12400	750–1200	low	0–15	(DEFRA 2007; Jones & Walsh 2001)
Poplar	05–11	1500–3400	700–1050	moderate	0–50	
***Agave* sp. (piña)**	10–34	3000–10500	300–800	high	0–100	(Bandano & Pugnaire 2004; Nobel 2003)

Therefore, to achieve a sustainable bioethanol industry, the use of water-use efficient plants grown on land unfit for current crop production is considered ideal (Holtum et al. 2010). Species belonging to the genus *Agave* could serve as good sources of lignocellulosic biomass, and species of this genus are already cultivated for fibre production (*Agave sisalana*, *Agave fourcroydes*) and alcoholic beverages (*Agave tequilana*, *Agave salmiana*), with established agronomic cultivation practices (Nobel 2003; Somerville et al. 2010b).

Agave spp. are water efficient plants utilising crassulacean acid metabolism (CAM) (see Section 1.4) that grow well in arid and semi-arid environments (Holtum et al. 2010; Nobel 1989; Somerville et al. 2010b). *Agave tequilana* is a drought tolerant species grown commercially in Mexico for production of Tequila, a popular alcoholic beverage. However, Tequila production utilises only soluble sugars that are present in the stem, with harvest destroying the whole plant. Leaves, which account for approximately 38% of the above ground biomass, are left in the field as waste (Iñiguez-Covarrubias et al. 2001). The leaves of *A. tequilana* are high in cellulose and low in lignin content, making them candidate feedstock for fuel ethanol production following cellulose hydrolysis (Li et al. 2012a). The South Australian company AusAgave is a pioneer in introducing *A. tequilana* plants to Australia as a biofuel feedstock (Chambers & Holtum 2010).

The current study will determine the growth pattern and biomass productivity of six cultivars of *A. tequilana*, ten species of *Agave* and *F. foetida* in the Central Queensland region to determine the appropriate time for leaf harvest for maximal fuel ethanol production. The potential for use of near infrared (NIR) spectroscopy for rapid non-destructive assessment of dry matter, total soluble solids, and cellulose, hemicellulose and lignin compositions will also be studied.

1.2 Review of Literature

1.2.1 The *Agave* group

The family Agavaceae consists of 12 genera and 250 species, of which 136 species have succulent leaves (Appendix A). The genus *Agave* is further divided into two sub-genera, Littaea and Agave, according to their respective inflorescence pattern; racemose and umbellate or paniculate (Gentry 1982; Nobel 2003). The natural distribution of the members of the genus *Agave* is the central western highlands of Mexico (Jalisco, Oaxaca

and Puebla), but they are now also naturalised to the dry sub-tropical regions of North America, Mediterranean, Africa, Brazil and Australia (Davis, Dohleman & Long 2011; GRIN 2014).

1.2.1.1 Morphology and Species Description

Classification

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopsida
Subclass:	Lillidae
Order:	Liliales
Family:	Agavaceae
Genus:	*Agave*
Species:	250 species

Agave tequilana Weber var. Azul was classified by German botanist F. Weber in 1905. It belongs to the group Rigidae of the genus *Agave* and is commonly known as blue agave. This plant is used for the production of Tequila, a popular alcoholic beverage from Mexico. *Agave tequilana* (Fig. 1.1) originated in the Jalisco region, Mexico (Bowen & Zapata 2009; Nobel 2003).

Figure 1.1: *Agave tequilana* plants growing in Rockhampton, Queensland.

1.2.1.2 Morphology

Agave tequilana is a surculose (producing suckers) radially spreading plant which can grow up to 1.2 to 1.8 m tall and has short thick stems measuring 30–50 cm tall at maturity (Gentry 1982). The leaves are 90–120 cm long and 8–12 cm wide, lanceolate, acuminate, firm and fibrous. They are mostly rigidly outstretched, concave and widest through the middle, narrowed and thickened toward the base. The colours of the leaves are glaucous green (bluish green or yellowish green) and sometimes cross-zoned. The leaf margins are straight to undulated, teeth generally regular in size and spacing, mostly 3–6 mm long through the mid blade, and the slender cusps are curved or flexed from low pyramidal bases and are light brown to dark brown in colour (Fig. 1.2 A). Spines are generally short; about 1–2 cm long. The leaf bases are broad, dark brown and may or may not be decurrent (Gentry 1982).

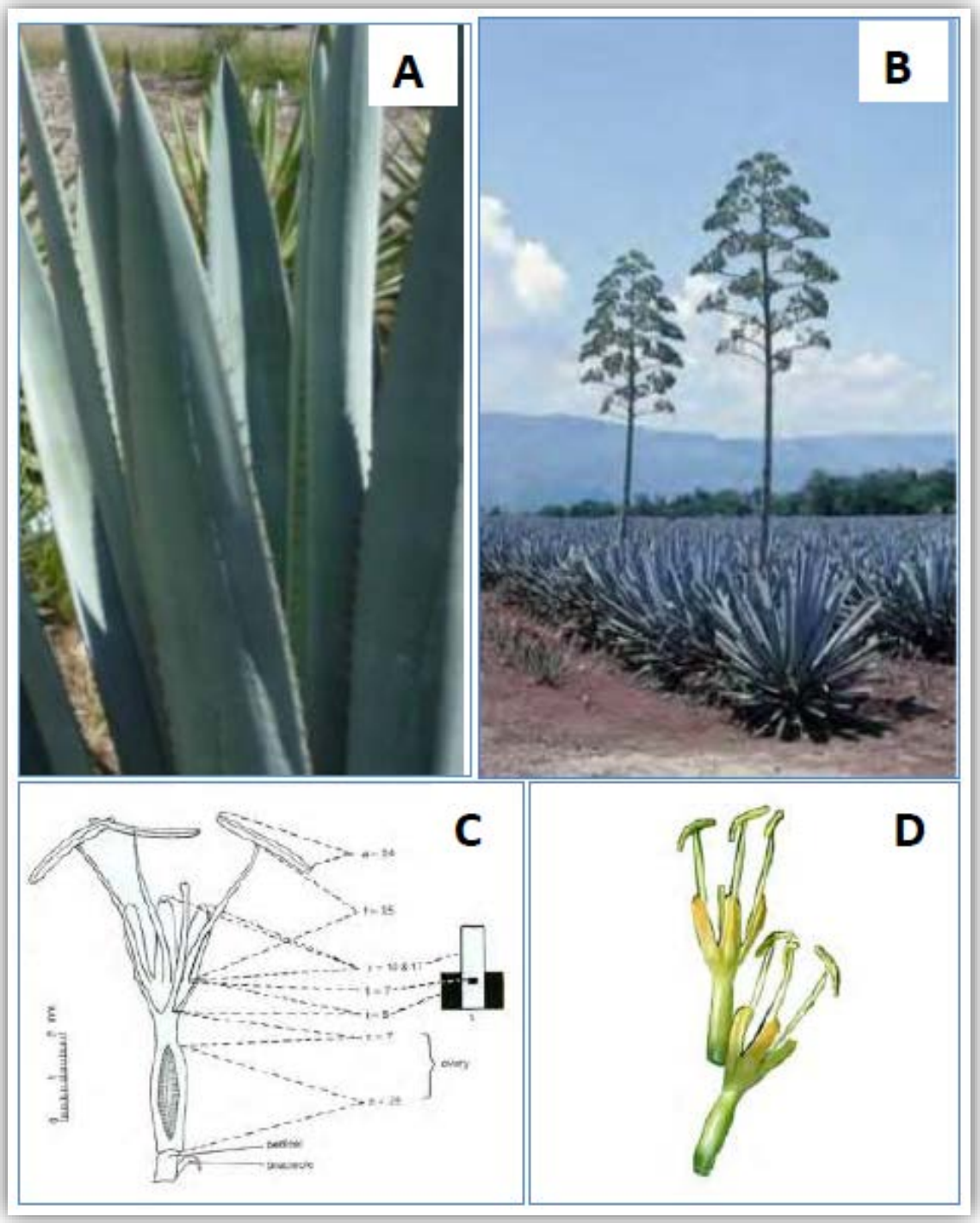


Figure 1.2: (A) Leaves of *Agave tequilana* Weber grown at Rockhampton. (B) *A. tequilana* with flower spike (Valenzuela–Zapata 2008). (C) Cross-section of a stylised *Agave* flower with tube/tepal ideogram denoted by x. Tepal and tube is represented by white column and black columns respectively; a black square represents the insertion of the tube. In the picture, O = body length of ovary; n=neck of ovary; t = length of tube; a = length of anther; fi = filament insertion; s = length of sepal and f = length of filament (Gentry1982). (D) *A. tequilana* flower (Valenzuela–Zapata 2008), Figure modified and sourced with permission from Chambers and Holtum (2010).

According to Gentry (1982), panicles are 5–6 m tall, large and densely branched with 20-25 large diffusive decompound umbels of green flowers with roseate stamens (Fig. 1.2 D); flowers are 68–75 mm long and are borne on small bracteolate pedicels of 3–8 mm long (Fig. 1.2 B).

Ovaries are 25–38 mm long, cylindrical and 6-ridged with an unconstricted short neck and are slightly tapered at the base with linear tubes 10 mm deep and 12 mm wide (Gentry 1982) (Fig. 1.2 C). These are erect and linear but wither quickly during anthesis (the period during which a flower is fully open and functional). The filaments are 45–50 mm long and are bent inward against the pistil and are inserted 7 and 5 mm above the base of the tube with 25 mm long anthers (Fig. 1.2 C).

1.2.1.3 Plant Physiology

The CAM photosynthetic pathway is present in approximately 6% of vascular plants (Holtum, Smith & Neuhaus 2005; Winter & Smith 1996). In CAM photosynthesis, plants assimilate CO_2 during light using C_3 photosynthetic pathways, and assimilate CO_2 in the dark using phosphophenylpyruvate carboxylase (PEPC), with vacuolar storage of C_4 organic acids such as malate (Fig. 1.3). The stomata are thus only open at night. This mechanism results in significant reduction in transpirational water loss, an adaptation which suits the plant for growth in hot dry climates.

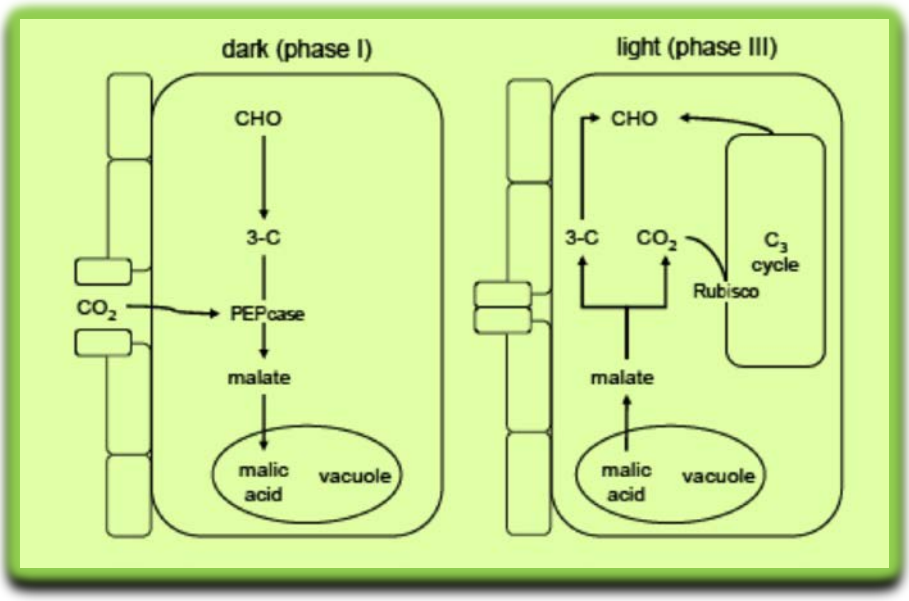


Figure 1.3: The CAM pathway. Dark (phase 1) on the left and Light (phase III) on the right (Chambers & Holtum 2010). Modified and sourced with permission from D Chambers.

In a typical facultative CAM plant (Fig. 1.4), CO_2 is fixed mainly at night (phase I), but CO_2 uptake occurs until early morning (phase II), and in favourable (well-watered) environmental conditions, CO_2 uptake may occur even in the late afternoon (phase IV). During the middle of the day, CO_2 uptake ceases due to closure of stomata (Nobel & Valenzuela 1987b) (Fig. 1.4).

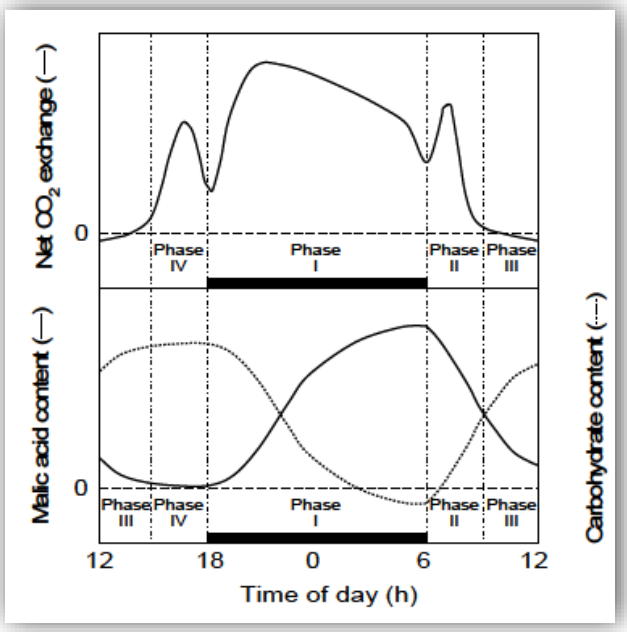


Figure 1.4: The daily cycle of net CO_2 exchange and the reciprocating fluctuations of malic acid and storage carbohydrates for a typical facultative CAM. Solid bar represents darkness (Holtum, Smith & Neuhaus 2005; Osmond, Neales & Stange 2008, as cited in Chambers and Holtum 2010). Sourced with permission from D Chambers.

Agave species utilise the CAM photosynthetic pathway and are thus adapted to arid and semi-arid regions or warm, temperate sub-tropical climates (Iñiguez-Covarrubias et al. 2001). Although most species of *Agave*, such as *A. fourcroydes*, *A. tequilana*, *A. angustifolia*, *A. deserti*, *A. sisalana*, *A. weberi* and *A. americana*, are considered to be constitutive in displaying CAM photosynthetic pathway under natural conditions, *A. deserti* is found to be facultative, switching from CAM to C_3 modes of photosynthesis under well-watered conditions in the laboratory (Hartsock & Nobel 1976). This observation suggests the possibility that a CAM to C_3 transformation may occur in other *Agave* species under favourable conditions. *Agave tequilana* is considered to be a constitutive CAM plant; however, the contribution of daytime (phases II and III) and night-time (phase I) carbon acquisition may alter with environmental conditions

(Pimienta-Barrios, Robles-Murguia & Nobel 2001), suggesting that it can be facultative (Fig. 1.4).

1.2.1.4 Growth and Climatic Conditions

1.2.1.4.1 Soil

In Mexico, *A. tequilana* is cultivated on land unsuited for the production of other crops. Generally, *Agave* grows on well drained, volcanic Cambisols, Luvisols and Lithosols (Krasnozem) with a slightly acidic pH (Holtum et al. 2010). According to Nobel and Valenzuela (1987b), *A. tequilana* grows well in iron-rich, fertile basaltic soils and black soil in the valleys near Tequila, in the state of Jalisco, Mexico (Fig. 1.5).

Figure 1.5: Map of Mexico showing Jalisco, place of origin of *Agave tequilana*.
Source: (GEOATLAS.com 2004)

1.2.1.4.2 Climate

Ruiz-Corral, Pimienta-Barrios and Zanudo- Hernandez (2002) recommend cultivation of *Agave* species in warm tropical and temperate sub-tropical climates with average annual rainfall between 705–1050 mm. *Agave tequilana* production is recommended for areas with a minimum night time temperature of 10–15°C and maximum daytime temperature of 25–35°C (Nobel & Valenzuela 1987a), although it is reported to withstand temperatures up to 50ºC for a limited period, and a day-night temperature difference of

10°C. Planting soon before the onset of wet season is recommended in non-irrigated production systems.

1.2.1.5 History of *Agave*

Agave spp. have been used for food, drink, fibre and shelter for at least 9000 years as evidenced by fossilised human faeces, archaeological specimens and fibre artefacts in North America and Mexico (Gentry 1982). Inflorescence, stems and leaves of agaves were roasted and eaten (Sauer 1965). The roasting converts glucans and other hexose polymers into digestible sugars such as glucose and fructose. According to Crane and Griffin (1958), hard fibre from *Agave lechuguilla* was used 8000 years ago for making sandals. The increased use of agaves in pre-historic times led to their cultivation approximately 6000 years ago. The Hokokam people from south western United States of America began cultivation of agaves 1000 years ago, using rocks as mulch to prevent water loss from soil around plants.

A sugary sap collected after cutting through the central spike of the folded leaf is also consumed directly as aguamiel ('honey water'). Pulque is a fermented beverage produced from aguamiel. Records of its use date back to the late twelfth century when Aztecs (ethnic group of central Mexico) were migrating into Mexico (Gentry 1982). The cultivation of agave for pulque became widespread in the late fifteenth and early sixteenth centuries (Gentry 1982). When the north central part of Mexico was colonised by Spaniards, the Nahuatl Indians were forced to move with them to cultivate *A. salmiana* and other *Agave* species for pulque.

A number of species are used for producing alcoholic beverages: 'Mezcal' from *A. salmiana* and *A. angustifolia* and 'Tequila' from *A. tequilana*. In the sixteenth century, the Europeans introduced distillation techniques to North America, which led to the development of these beverages (Nobel 2003). The production of Tequila, named after the place of its origin (Tequila in Jalisco, Mexico) started in 1621. Tequila is produced in large factories by double distillation, whereas mezcal is produced in cottage industries by crushing the piñas or stems which resemble pineapple when the leaves are cut, using large millstones run by oxen or tractor and then fermenting and distilling the extracts in small stills (Valenzuela 1985).

In the early nineteenth century, fibre-producing agaves, particularly *A. sisalana*, were exported to Indonesia and the Philippines, and in the twentieth century to East Africa. Currently *A. sisalana* is planted in many African countries such as Angola, Mozambique, Kenya and Uganda for fibre production, supplying half of the world's hard natural fibre. Brazil supplies another 20% of the hard fibre from *A. sisalana* (Gentry 1982). Apart from beverages and fibres, agaves are used for a range of other purposes (Table 1.2).

Table 1.2: Various uses of *Agave* spp.

Species	Part of the plant	Uses	References
Agave salmiana	stem leaves	mezcal cattle feed	(Gentry 1982) (Nobel 2003)
Agave tequilana	stem	Tequila	(Gentry 1982; Valenzuela 1985)
	bagasse (waste product of Tequila production)	fuel ethanol	(Chambers & Holtum 2010; Holtum et al. 2010; Somerville et al. 2010a)
	leaves and stems	potential feedstock for fuel ethanol production	
Agave angustifolia	stem	mezcal	(Gentry 1982, cited in Nobel 2003)
Agave sisalana	leaves	fibre	(Gentry 1982, cited in Nobel 2003)
Agave fourcroydes	leaves	fibre	(Sheldon 1980, cited in Nobel 2003)
Agave lechuguilla	leaves	fibre	(Sheldon 1980, cited in Nobel 2003)
Agave atrovirens	flower (waxy layer on epidermis)	food (wrapper for tortilla) sandwiches)	(Felger and Moser 1985, cited in Nobel 2003)
Agave americana	whole plant	ornamental plant	(Gentry 1982, cited in Nobel 2003)
	spines	used to punish juvenile delinquents and runaway slaves	
	stalks of inflorescence	Fencing	
Agave schotti, Agave vilmoriniana	sapogenins from leaves	soaps and shampoos	(Gentry 1982, cited in Nobel 2003)
Agave fourcroydes	pulp of leaves (waste product from fibre industry)	increase protein content of livestock feed	(Blancas et al. 1982, cited in Nobel 2003)
	leaves	fuel ethanol	(Cáceres-Farfán et al. 2008)
Agave sisalana	leaves	biogas	(Mshandete et al. 2005)

1.2.1.5.1 History of Agave *in Australia*

Agaves were commercially grown in Australia in the 1890s, with *A. americana* L., *A. fourcroydes* Lem., *A. rigida* var. *sisalana* (Perrine) Engelm. (*A sisalana* Perrine) grown commercially for fibre production (Anon. 1902, 1904; Gentry 1982; Lock 1969; Turner 1981) as cited in Holtum et al. (2010). Although *A. sisalana* plantings achieved good yields, the industry was not viable due to increasing freight costs imposed due to World War I (Anon. 1913 & 1915; as cited in Holtum et al. (2010)). Figure 1.6 shows an *Agave* field at Gladstone, Queensland, in 1904 with the species described as *A. rigida* var. *sisalana*. The planting density was approximately 1500 plants per hectare and yield was 3.7–4.9 tonne fibre/hectare (Holtum et al. 2010). *Agave tequilana* is a relatively new crop in Australia, introduced by the agronomist Don Chambers of AusAgave P/L, South Australia. This crop is yet to be commercially harvested in Australia, and, has not been reported as a feedstock for biofuel production.

Figure 1.6: *Agave* cultivation field at Gladstone, Queensland in 1904 (Anon. 1904), as cited in Holtum et al. (2010). Figure used with permission from Joseph Holtum and Don Chambers.

Furcraea foetida, a species belonging to family AGAVACEAE and sub-family AGAVOIDEAE has invaded native plant communities around Rockhampton (Fig. 1.7) and hence it has been declared as a local weed.

Figure 1.7: *Furcraea foetida* invasion (Patches of bright green area as shown in the picture) at Mt Archer, Rockhampton (23° 21’ 20.09" S; 150° 34’ 13.21" E (Google earth 2016).

1.2.1.6 Cultivation of *Agave* spp.

The growth rate of *A. tequilana* is relatively slow, taking up to 7 years for a plant to reach maturity (Escamilla-Treviño 2012). However, harvesting of leaves from the plant may be possible at an earlier age, once the plant reaches a certain stage of maturity.

In Mexico, *A. tequilana* is produced traditionally on small farms to large acreages and the farming method is generally passed from one generation to the next. *Agave tequilana* is planted in raised beds, 15 cm deep and 2–4 m apart, intercalated with nitrogen fixing crops such as beans, chickpeas or soybean (Pimienta-Barrios, Robles-Murguia & Nobel 2001), suggesting that these are also grown in good agricultural soil for high productivity. However, these plants can also grow with average productivity in marginal soil in rain-fed conditions (Nobel 2003). Application of fertiliser is based on soil type, age of the plant, and economic ability and resources of the farmer. Urea (46% nitrogen) is a commonly used fertiliser, added at the rate of 250 kg per hectare. In some areas, phosphorus and potassium are also added according to soil type (Holtum et al. 2010). Addition of nitrogen in a field trial of *A. deserti* doubled the number of leaves unfolding (Nobel 2003). However, addition of phosphorus gave only a slight increase in yield, and that of potassium and calcium did not have any effect on growth (Nobel 2003).

1.2.1.6.1 Propagation

In Mexico, *A. tequilana* is traditionally planted using suckers (ramets derived from root buds). Propagation by suckers is cheap in comparison with planting using tissue cultured material or seed. Use of tissue cultured plantlets (Figs 1.8A and 1.8B), however, provides uniformity of seedlings at planting.

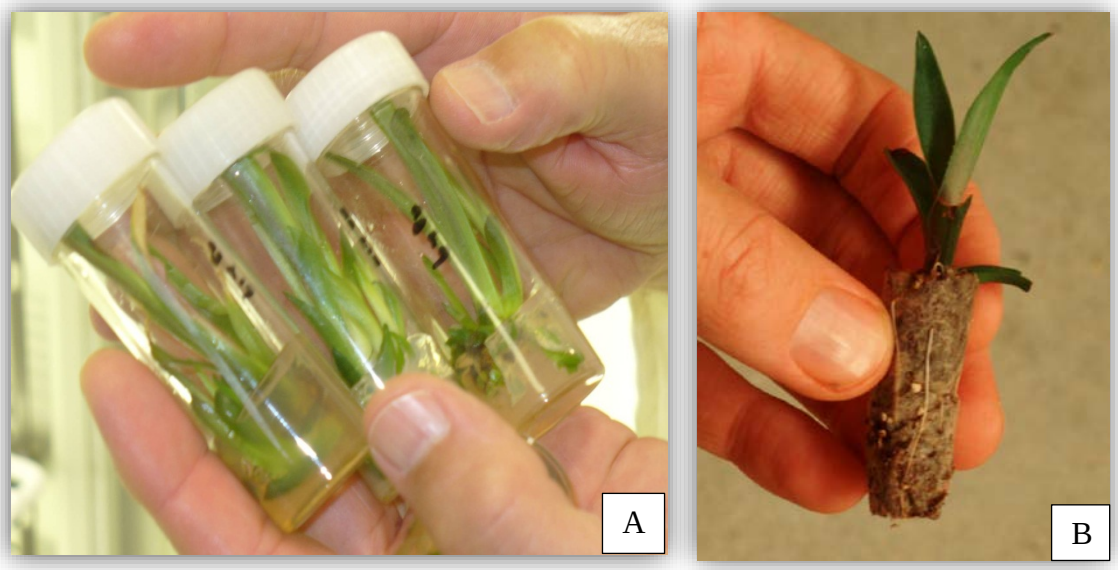


Figure 1.8: (A) Tissue cultured plants of *Agave tequilana* before deflasking and (B) after deflasking. Source: (Holtum et al. 2010). Figure used with permission from Joe Holtum and Don Chambers.

1.2.1.6.2 Planting density

In Mexico, *A. tequilana* is typically grown in raised beds at a density of 2000–4000 plants per hectare, with a row width suitable for hand labour and harvest. The raised beds help with drainage during wet seasons (Holtum et al. 2010). The first trial in Australia was established in July 2009 at Kalamia, North Queensland. *Agave tequilana* was planted at Kalamia at an initial planting density of 4000 plants per hectare in 15 cm wide beds, with a row spacing of 1.8 m x 1.6 m and using plastic mulching for weed control (Fig. 1.9).

Figure 1.9: First trial of *Agave tequilana* Weber var. Azul in Australia at Kalamia estate, Queensland. (a) First day of planting July 2009 (b) March 2010 during crops first wet season. Photographs (a) and (b) Source: Holtum et al. (2010); (c) September 2011 and (d) May 2014. Source: Figure supplied by and used with permission from Joe Holtum and Don Chambers.

However, it was later determined that the planting density impeded access to plants. Therefore, the plants in alternate rows were removed using an excavator (Chambers 2013), thus achieving a final planting density of 2000 plants per hectare (3.6 m x 1.6 m spacing).

1.2.2 Growth Evaluation

Agave productivity varies with the species and growth conditions. Dry biomass yields of up to 34 Mg ha^{-1} $year^{-1}$ under rain-fed conditions with an annual rainfall between 427-981 mm have been reported (Nobel, García-Moya & Quero 1992) (Table 1.3).

Yields of agave can be improved by appropriate agricultural management such as weeding, pruning, irrigation, fertiliser application and pest control, as was evident in a trial conducted with species such as *Agave mapisaga* and *A. salmiana* in which yields of 38 and 42 Mg ha^{-1} yr^{-1} were achieved for total above ground biomass of leaf and stem

(Nobel, García-Moya & Quero 1992). Without irrigation and other agricultural inputs, *A. mapisaga*, *A. salmiana* and *A. tequilana* had annual above ground biomass productivity of 25–26 Mg ha^{-1} in the semi-arid conditions of Mexico (Nobel 1991) (Table 1.4). With appropriate irrigation and fertigation, biomass yield of *Agave* exceeds the yield of some other bioenergy crops such as *Panicum virgatum* (switchgrass) and *Zea mays* (corn) (Table 1.3).

Table 1.3: Above ground biomass of various *Agave* species and bioenergy crops at different locations with varying rainfall conditions.

Location	Species	Annual rainfall (mm)	Annual productivity (Mg ha^{-1})	Reference
Southwestern USA	*Agave vilmoriniana*	<200	<1	(Idso & Kimball 1995)
San Louis Potosi, Mexico	*Agave salmiana*	320	10	(Nobel & Meyer 1985)
Mazatlan, Mexico	*Agave salmiana* *Agave lechuguilla*	427	10 3.8	(Nobel 1990; Nobel 1985; Nobel 1991)
Sonora desert, California, USA	*Agave deserti*	430	7	(Nobel 1990; Nobel 1985; Nobel 1991)
Morelia, Mexico	*Agave tequilana*	764	24	(Nobel 1990; Nobel 1985; Nobel 1991)
Tacubaya, Mexico	*Agave mapisaga* *Agave salmiana*	848	32 34	(Davis, Dohleman & Long 2011; Nobel, García-Moya & Quero 1992)
Yucatan, Mexico	*Agave fourcroydes*	981	16	(Nobel 1985)
Jalisco, Mexico	*Agave tequilana*	1080	25	(Nobel & Valenzuela 1987a)
Minnesota, USA	*Zea mays*	500–800	3–10 (grain) 3–8 (stover)	(Johnson et al. 2013; Somerville et al. 2010b)
Texas, Arkansas and Louisiana, USA	*Panicum virgatum* var. Almao	600–1300	11–20	(McLaughlin & Adams Kszos 2005)
Guyana, Hawaii, USA; Queensland, Australia	*Saccharum officinarum*	ns*	50–85	(ASMC 2016b; Nobel 1991)
California, USA	*Sorghum bicolor*	ns*	47	(Somerville et al. 2010b)

Note: *ns = Not specified.

Agave mapisaga is reported to yield 32 Mg ha^{-1} (stem and leaf) in regions with annual rainfall of 848 mm, whereas *A. lechuguilla* was reported to yield only 3.8 Mg ha^{-1} yr^{-1} at a site with an annual rainfall of 427 mm (Table 1.3). An annual yield of 10 Mg ha^{-1} was recorded for *A. salmiana* in an arid cold climate, and 34 Mg ha^{-1} in a semiarid region with moderately warm daytime temperatures (Table 1.3) (Davis, Dohleman & Long 2011).

The growth of agave can be measured by counting the number of unfolded leaves (Nobel 2003). This non-destructive approach is determined by identifying the number of leaves unfolding from the central spike of folded leaves over a particular period of time.
A practical method for monitoring new leaves involves clipping the dead tip of the unfolded leaves (Garcia-Moya, Romero-Manzanares & Nobel 2011; Nobel 1989; Quero & Nobel 1987). In a comparison planting of *A. angustifolia*, *A. fourcroydes*, *A. mapisaga*, *A. deserti*, *A. lechuguilla*, *A. tequilana* and *A. salmiana*, *A. tequilana* had the best growth rate (46 leaves over a period of one year). *Agave deserti* and *A. lechuguilla* had the lowest growth rate (five and seven leaves, respectively) (Table 1.4).

Table 1.4: Number of leaf unfolding of native, naturalised and introduced species of *Agave* at various geographical locations.

Species	Location	Age (years)	No. of plants studied	Annual leaf unfolding per plant	Reference
Agave angustifolia	Oxaca, Mexico (plantation)	3 6	20 20	19.6 24.9	(Garcia-Moya, Romero-Manzanares & Nobel 2011)
Agave deserti	Southern California, USA (native)	-	50	5.2	(Nobel 1984)
Agave fourcroydes	Yucatan, Mexico (plantation)	4 6	20 20	22.5 26.8	(Nobel 1985)
Agave lechuguilla	Coahuila, Mexico	-	52	6.8	(Nobel & Quero 1986)
Agave mapisaga	Tlaxcala and Mexico Valley, Mexico (plantation)	10	120	6.9–9.9	(Nobel, Garcia-Moya & Quero 1992)
	Tequesquinahuac, Mexico (plantation)	5	40	5.2	(Nobel, Garcia-Moya & Quero 1992)
Agave salmiana	San Luis Potosi, Mexico		73	4.4	(Nobel & Meyer 1985)
	Hidalgo Mexico (plantation)	10	120	7.2–10.7	(García-Moya & Nobel 1990)
		5	40	4.9	(Nobel, García-Moya & Quero 1992)
Agave tequilana	Jalisco, Mexico (plantation)	3 6	20 20	34.7 45.6	(Nobel & Valenzuela 1987b)

The age of the plant, season and the environmental conditions (e.g., shading) affected plant growth. For example, more leaves were produced during the wet summer season than during the dry winter season, and shading of the plants was associated with a 30% reduction in the number of leaves (Garcia-Moya, Romero-Manzanares & Nobel 2011). Six-year-old plants of *A. angustifolia*, *A. fourcroydes* and *A. tequilana*, respectively, had 27%, 19% and 32% more leaves unfolded compared to the 3-year-old plants (Table 1.4) (Garcia-Moya, Romero-Manzanares & Nobel 2011).

Plant CO_2 uptake is impacted by light index (PAR), water and temperature. The environmental productivity index (EPI) is represented as the product of PAR, temperature index, water index, nutrient index and CO_2 index (Nobel 2003), with each index usually ranging from 0.0 to 1.0. An index of 0.0 indicates complete inhibition of CO_2 uptake, while an index of 1.0 indicates non-limiting, optimal behaviour (Garcia-Moya, Romero-Manzanares & Nobel 2011). The CO_2 index can be more than 1.00 if the atmospheric CO_2 is increased. EPI has been used effectively to predict the number of new leaves of *A. fourcroydes* ($r^2 = 0.85$) (Nobel 1985), *A. salmiana* ($r^2 = 0.95$) (Nobel & Meyer 1985), *A. lechuguilla* ($r^2 = 0.83$) (Nobel & Quero 1986) and *A. deserti* (Nobel 1984; Nobel & Hartsock 1986).

For *A. tequilana*, leaf dry weight increased approximately with the cube of leaf length until the leaf length increased to 70 cm ($r^2 = 0.95$), which suggests that leaf length, width and thickness all increased proportionately up to 70 cm. For leaves longer than 90 cm, dry weight increased linearly with leaf length ($r^2 = 0.95$). Thus, leaf length, area and number are appropriate parameters to measure the productivity of agave (Nobel & Valenzuela 1987a).

1.2.3 Chemical Composition

1.2.3.1 Compositional analysis

The composition of lignocellulosic plant biomass is complex, but consists of three major fractions - cellulose, hemicellulose and lignin. The relative content of these fractions vary with species and environmental conditions (Table 1.5). Cellulose, the main structural constituent in the plant cell wall, consists of long chains of cellobiose units that are linked to D-glucose sub-units through β-(1, 4)-glycosidic bonds. Hemicellulose is composed of branches of short lateral pentose (xylose, rhamnose and arabinose) and hexose (glucose,

mannose and galactose) monosaccharides (Jung, Kim & Chung 2015). Lignin is a complex structure that consists of cross-linked polymers of phenolic monomers that provide structural support to the plant but are impermeable and recalcitrant in nature (Jung, Kim & Chung 2015). Biomass with high cellulose and low lignin content is considered ideal for bioenergy crops for fuel ethanol production (Li et al. 2014), as cellulose microfibrils surrounded by lignin and non-cellulosic cell wall structural polysaccharides (xylose, arabinose, rhamnose, galactose, collectively known as hemicellulose) interfere with enzymatic (cellulose) hydrolysis.

The major insoluble components in the leaves of *Agave* species are cellulose, hemicellulose and lignin, which vary with the species and growing conditions (Li et al. 2012a; Yan et al. 2011), similar to other commonly used biofuel feedstock (Table 1.5) (Hernández-Salas et al. 2009; McIntosh et al. 2012; Vancov & McIntosh 2011). Lignin composition in *Agave* is 17% as compared to 28–30%, 26% and 28% in eucalypts, poplars and pines, respectively (Table 1.5). Thus, agaves have potential to liberate high amounts of sugar following biochemical processing (discussed in section 1.10 and Chapter 5).

Table 1.5: Composition of various lignocellulosic biomass feedstock and agave. Extractives were obtained after water and ethanol extraction.

Plant species	Plant part	Cellulose	Hemicellulose	Lignin	Extractives	References
***Miscanthus giganteus* (*Miscanthus* sp.)**	leaves	31	25	19	26	(Jung, Kim & Chung 2015)
***Panicum virgatum* (Switchgrass)**	leaves	32	25	18	23	(Jung, Kim & Chung 2015)
***Sorghum bicolor* (Sweet sorghum)**	leaves	30	23	19	25	(Jung, Kim & Chung 2015)
	whole	28	16	13	27	(Wolfrum et al. 2013)
	straw	32	27	10	30	(Vancov & McIntosh 2012)
***Phragmites australis* (Reed grass)**	leaves	26	22	25	27	(Jung, Kim & Chung 2015)
***Acacia* sp. (Black locust)**	-	42	18	27	7	(Hamelinck, Hooijdonk & Faaij 2005)
***Eucalyptus* sp.**	-	50	13	28	4	(Hamelinck, Hooijdonk & Faaij 2005)
Eucalyptus dunnii	residue (plantation thinnings)	42–44	24–25	30	7–8	(McIntosh et al. 2012)
***Populus* spp. (Hybrid Poplar)**	stem	45	19	26	7	(Hamelinck, Hooijdonk & Faaij 2005)
Pine	stem	45	22	28	3	(Hamelinck, Hooijdonk & Faaij 2005)
***Saccharum officinarum* (Sugarcane)**	bagasse	42	25	20	-	(Kim & Day 2011)
***Oryza* sp. (Rice)**	straw	40	18	6	-	(Prasad, Singh & Joshi 2007)
***Triticum aestivum* (Wheat)**	stubble	36	26	7	19	(Vancov & McIntosh 2011)
***Zea mays* (Corn)**	whole plant leaf	33 31	25 23	22 24	-	(Liu et al. 2010)
***Agave* spp.**	stem residue from Tequila brewing	31	17	17	7	(DOE 2004)

The leaves of mature *A. tequilana* plants may be suitable for bioethanol production because they contain up to 42% w/w structural carbohydrates (cellulose and hemicellulose) and only 12% w/w lignin (Li et al. 2012b). Corbin et al. (2015) have also reported that dry leaf fibre of *A. americana* and *A. tequilana* 2–3 year-old plants consisted of crystalline cellulose (47% and 50%, w/w), non-cellulosic polysaccharides or hemicellulose (22% and 16%, w/w) and total lignin (9.3% and 12.7%, w/w), respectively (Table 1.6).

Table 1.6: Chemical composition of *Agave* spp. and other biomass feedstocks (g/100 g biomass or % w/w).

Plant species	Plant part	WSC	Cellulose	Non-cellulosic cell-wall polysaccharide (hemicellulose)			K-lignin	
			Glu	*Xyl*	*Galact*	*Arab*		*Reference*
Agave americana	leaves	6.5	33.8	8.2	5.2	3.2	8.2	(Li et al. 2014)
Agave sisalana	leaves	7.9	32.1	8.5	4.0	3.1	9.8	(Li et al. 2014)
Agave tequilana	leaves	4.4	33.7	8.8	2.0	2.0	11.9	(Li et al. 2014)
Agave tequilana	leaves	15.3	50	16*			9.1	Corbin et al. (2015)
Agave americana	leaves	9.1	47	22*			5.3	Corbin et al. (2015)
Agave americana	heart (stem)	17.0	22.8	7.7	9.9	3.9	7.3	(Li et al. 2014)
Populus trichocarpa	-	-	51.4	22.9	-	-	23.4	(Li et al. 2014)
Panicum virgatum	-	-	36	24	-	-	18.8	(Li et al. 2014)
Eucalyptus dunnii	forest thinning residues	-	47.5	17.3	1.2	0.5	27	(McIntosh et al. 2012)

Note: WSC = water soluble carbohydrates, Glu = Glucose, Xyl = Xylose, Galact = Galactose, Arab = Arabinose and K-lignin = Klaosin lignin; * Total non-cellulosic cell wall polysaccharide consisting of (xylose, mannose, rhamnose, galactose, glucuronic acid and galacturonic acid).

Thus, *A. tequilana* biomass is high in cellulose and low in lignin, and meets the criteria required for an ideal feedstock for bioethanol production (Table 1.6). In addition, up to 4.4% w/w water soluble carbohydrates are present in the leaf juice (Li et al. 2014).
In another study, Corbin et al. (2015) found that the water soluble carbohydrates of *Agave*

leaf consisted mainly of glucose, fructose and sucrose (3% w/w in raw untreated juice and increased to 4.1% by treating with fructanase enzyme).

The determination of non-structural carbohydrates can be accomplished using (i) high performance liquid chromatography (HPLC), (ii) gas chromatography (GC), (iii) anthrone colorimetry, and (iv) dinitrosalicylic (DNS) methods (Mc Clements 2003). The four commonly used methods for compositional analysis of the lignocelluloses are: (i) Van Soest method of forage fibre analysis (Van Soest, Robertson & Lewis 1991), (ii) Technical Association of the Pulp and Paper Industry method (TAPPI), (iii) American Society for Testing and Materials method (ASTM 2012), and (iv) National Energy Renewable Laboratory (NREL) method (Sluiter et al. 2008b). Of these methods, the most commonly used method for determination of structural carbohydrates and lignin and non-structural carbohydrates in the context of bioenergy work is the method developed by NREL. This method can be applied to woody materials, agricultural residues, grasses and herbaceous feedstock (Sluiter et al. 2010).

In the NREL method, samples are first subjected to a laboratory analytical procedure (LAP) for determination of extractives in biomass using a two-step Soxhlet extraction using water and ethanol to recover water and ethanol soluble material. The water and ethanol soluble extractives are removed prior to compositional analysis because they interfere with the characterisation of carbohydrates and lignin in the biomass (Sluiter et al. 2008a; Sluiter et al. 2008b). The extractive free biomass obtained after removing non-structural materials is used for subsequent compositional analysis.

The NREL method described in LAP for determination of structural carbohydrates and lignin in biomass uses a two-step acid hydrolysis (Sluiter et al. 2008b). In this procedure, samples are hydrolysed with 72% w/v H_2SO_4, followed by autoclaving at a dilution of 4% w/v H_2SO_4 in a sealed vessel. This step degrades the biomass into the more easily quantifiable forms of hexose and pentose sugars. This is a micro method using only 300 mg of sample, where the sugars are separated and quantified using simple isocratic HPLC separation (soluble sugars separated with water as the mobile phase), and identified with a refractive index detector (Sluiter et al. 2010). With acid hydrolysis, the lignin separates into acid soluble and acid insoluble material. The acid soluble lignin is measured using an UV-vis spectrophotometer and acid insoluble lignin is measured gravimetrically (Sluiter et al. 2008b).

1.2.3.2 Use of NIR spectroscopy in compositional analysis

When light is irradiated upon a sample, it may be either absorbed, reflected or transmitted. Visible light (400–750 nm) absorption is due to electronic transitions which we perceive as colour (Owen-Reece 1999). Near infrared (NIR) (780–2500 nm) absorption is generally due to bond movements (Agelet & Hurburgh 2010; Workman & Shenk 2004). Near infrared radiation is strongly absorbed by water between 1200-2500 nm, and therefore has an effective pathlength around 1 mm within biological tissue. There is less absorption by water in the short wavelength NIR region (SWNIR) (700–1100 nm), which therefore allows this region to be used for chemical composition to a greater effective depth in fresh biological material (Workman & Shenk 2004).

However, it is not only water that absorbs radiation in the NIR spectrum but also various other compounds in biological materials. When material containing molecular bonds such as C-H, O-H, C-O, S-H, N-H is irradiated by NIR frequencies, there will be vibrational energy change of these molecular bonds in characteristic wavelengths (Cen & He 2007; Workman & Shenk 2004). The information provided by NIR spectroscopy is complex due to the repetition of information on one particular molecular bond over a narrow region of electromagnetic spectrum as overtones and combination bands. For example, the information on the C-H bond is repeated eight times; i.e, from first through fourth overtones and in combination band regions between each overtone (Cen & He 2007; Workman & Shenk 2004) (Fig. 1.10).

Therefore, although NIR gives unique information on chemical and physical properties quickly and non-invasively, the information cannot be used directly without the use of chemometric analysis (Workman & Shenk 2004; Xu et al. 2013). Calibration is achieved using spectra collected from numerous samples (representing the range of sample matrices to be analysed in future) and actual compositional attributes estimated using a reference method. Thus the NIR technology is a secondary method of analysis, requiring a primary reference method for calibration and validation (Workman & Shenk 2004).

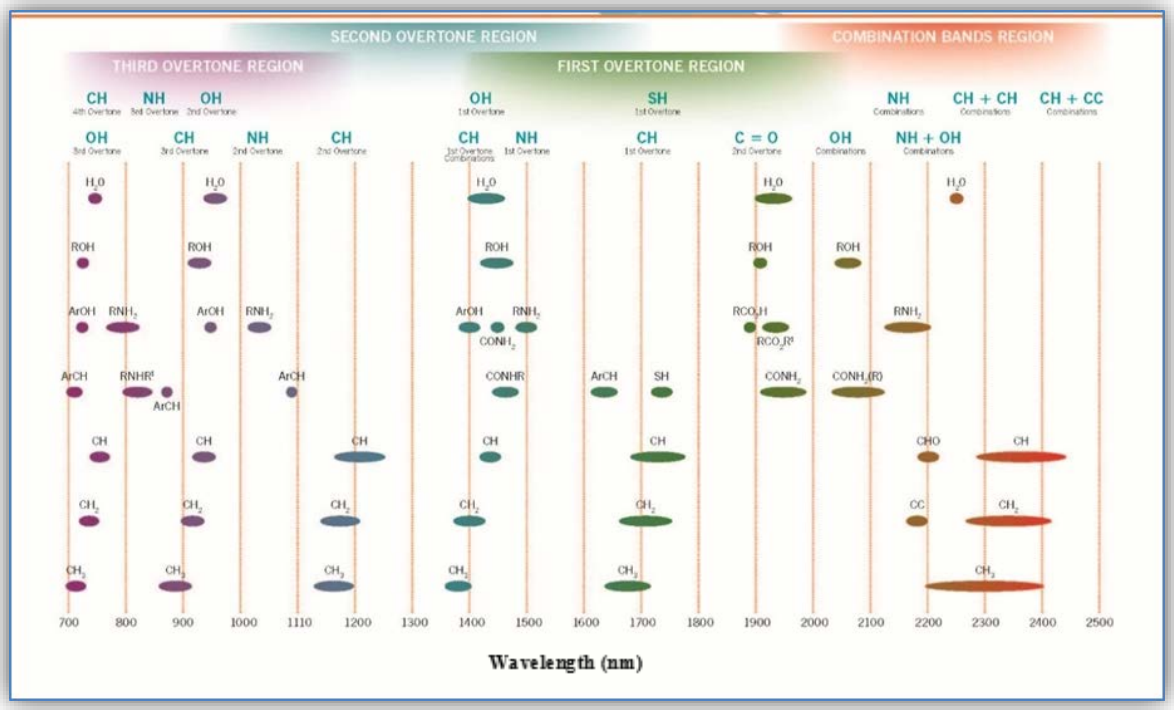


Figure 1.10: Band assignment in near infrared spectra (Ellis 1928; Goddu & Delkar 1960; Goddu 1960; Weyer & Lo 2002 and Workman 2000 as cited in NDC (2013)). Modified and sourced with permission from NDC (2013).

The Fourier Transform NIR (FTNIR) Spectrometer and scanning grating instruments (e.g., Thermo Antaris and Foss NIRSystems 6500) record the full NIR spectrum (up to 2500 nm) with good wavelength precision and signal to noise ratio, but are expensive ($50,000–$100,000) and are not field ready. In contrast, diode array spectrometers are cheaper and allow for in-field measurement. However, instruments based on silicon photodiode array detectors are limited to the visible-SWNIR range (400–1100 nm) while the technology based on indium-gallium arsenide (InGaAs) photodiode arrays operate to 2500 nm but are more expensive. The Integrated Spectronics (Nirvana)-Felix Instruments (F750) portable instrumentation based on silicon photodiode array detectors have been used in determination of the dry matter, total soluble solids (TSS) of various fruits such as mango, apple and mandarin (Subedi 2007; Subedi 2011; Subedi, Walsh & Owens 2007). Rapid and non-invasive assessment of dry matter and leaf chemical constituents is also of interest for selection of material in bioethanol production.

The NIR region was discovered by Herschel in 1800 (Davies 2000). The application of NIR spectroscopy to food and agriculture became familiar through the work of Ben-Gera and Norris (1968) and Norris and Hart (1996). Near infrared spectroscopic technology

has been used widely in the food and agriculture industry to predict protein, fat, fibre and moisture content of plant material (Davies & Grant 1987; Workman & Shenk 2004), and subsequently in the fields of medicine, pharmaceuticals (Roggo et al. 2007), forensic sciences (Pereira 2015), medical imaging and diagnostics (Afara, Singh & Oloyede 2013), petrochemical industries (Reboucas et al. 2010) and forestry (Downes et al. 2012; Downes et al. 2011).

Near infrared spectroscopy was first used commercially to assess the quality of food in the agricultural and food industries (Cen & He 2007). Recently, there has been wide use of NIR application for various wood and forest industry products (Castillo et al. 2012; He & Hu 2012, 2013; Kelley et al. 2004b; Sanderson et al. 1996), and herbaceous lignocellulosic feedstock such as corn stover, switchgrass, sorghum and rice straw (Jin & Chen 2007; Liu et al. 2010; Wolfrum et al. 2013; Ye et al. 2008) (Table 1.7). Near infrared spectroscopy has been used to measure the nitrogen, cellulose and lignin content of fresh and dried leaves (Downes et al. 2011; Kelley et al. 2004a; Martin & Aber 1994; Newman et al. 1994), and the content of glucan, xylan, arabinan, galactan, mannan, lignin and extractives of various lignocellulosic feedstock (Table 1.7).

In summary, scanning grating and FTNIR technologies have been used with various dried ground herbaceous and woody biomass feedstock. These technologies operate over a spectral range of 400–2500 nm and have been used in the estimation of glucan (cellulose) (RMSEP = 0.44–2.7%, R^2p = 0.89–0.96); xylan (hemicellulose) (RMSEP = 0.57–1.03% w/w, R^2p = 0.89–0.94) and lignin (RMSEP = 0.48–1.4% w/w, R^2p = 0.86–0.96) contents of various species (Table 1.7).

Table 1.7: An overview of NIR applications to estimate chemical compositions of various wood and biomass feedstock in recent years.

Source	Sample/ type	Parameter	Instrument	Geometry/ Wavelength Resolution	Spectral range (nm)	Spectral processing	Regres -sion	[a] RMSEP/ [b] RMSECV (%)	[a] R^2/[b] R	Reference method
Sanderson et al. (1996)	Woody and herbaceous feedstock, Dry powder	Glucose Xylose Arabinose Lignin Extractives	Pacific Scientific Model 6250 scanning Monochromator	Diffuse reflectance/ 2 nm	1100–1400	SNV-D	PLS	-	0.92–0.98 [a]	NREL (Sluiter et al. 2008b; Sluiter et al. 2008a)
He and Hu (2013)	Various wood species, Dry powder	Hot water soluble extractives Pentosan Cellulose	Bruker FTNIR	Diffuse reflectance/ 8 cm^{-1}	800–2778	1 Der MSC 1 Der Norm 1 Der MSC	PLS	0.32 [a]/0.37 [b] 0.49 [a]/0.49 [b] 0.46 [a]/0.44 [b]	0.96 [a] 0.99 [a] 0.96 [a]	TAPPI standard test method
Ye et al. (2008)	Corn stover, Dry powder	Glucan Xylan Galactan Arabinan Mannan Lignin Ash	Excalibur 3100, Varian Inc.	Diffuse reflectance/ 8 cm^{-1}	1000–2500	EMSC	PLS	0.92 [a] 1.03 [a] 0.17 [a] 0.27 [a] 0.21 [a] 1.12 [a] 0.57 [a]	0.97 [b] 0.93 [b] 0.92 [b] 0.96 [b] 0.91 [b] 0.94 [b] 0.90 [b]	NREL (Sluiter et al. 2008b)
Liu et al. (2010)	Switch grass, Dry powder	Glucan Xylan Galactan Arabinan Mannan Lignin Ash	Excalibur 3100, Varian Inc.	Diffuse reflectance/ 8 cm^{-1}	1000–2500	EMSC	PLS	0.65 [a] 0.57 [a] 0.12 [a] 0.19 [a] 0.11 [a] 0.48 [a] 0.26 [a]	0.97 [b] 0.96 [b] 0.97 [b] 0.95 [b] 0.87 [b] 0.93 [b] 0.96 [b]	NREL (Sluiter et al. 2008b)
Liu et al. (2010)	Switch grass+corn stover,	Glucan Xylan Galactan	Excalibur 3100, Varian Inc.	Diffuse reflectance/ 8 cm^{-1}	1000–2500	EMSC	PLS	0.84 [a] 0.87 [a] 0.26 [a]	0.97 [b] 0.94 [b] 0.88 [b]	NREL (Sluiter et al. 2008b)

Source	Sample/ type	Parameter	Instrument	Geometry/ Wavelength Resolution	Spectral range (nm)	Spectral processing	Regres -sion	[a] RMSEP/ [b] RMSECV (%)	[a] R^2/[b] R	Reference method
	Dry powder	Arabinan Mannan Lignin Ash						0.33 [a] 0.18 [a] 1.31 [a] 0.61 [a]	0.91 [b] 0.89 [b] 0.90 [b] 0.88 [b]	
Wolfrum et al. (2013)	Sorghum, Dry, knife milled, <2 mm.	Glucan Xylan Lignin Starch	FTNIR (Antaris, Thermofisher	Diffuse reflectance	1111–2500	2nd Der S. Golay	PLS	1.24 [b] 0.85 [b] 0.86 [b] 2.33 [b]	0.95 [a] 0.89 [a] 0.91 [a] 0.91 [a]	NREL (Sluiter et al. 2008b; Sluiter & Sluiter 2008)
Castillo et al. (2012)	*Eucalyptus globulus*, Dry powder	Glucans Hemicellulose Lignin		Diffuse Reflectance/ 2 nm	1000–2500	Mean centering, MSC	PLS	3.4 [a] 2.1 [a] 2.7 [a]	0.95 [a] 0.94 [a] 0.96 [a]	NREL (Sluiter et al. 2008b)
Jin and Chen (2007)	Rice straw, Dry powder	Moisture Hemicellulose Cellulose Klaoson lignin	Nicolet Nexus-FTNIR, Thermo	Diffuse reflectance/ 8cm^{-1}	1000–2500	Karl Norris Derivative filter	PLS	-	0.89 [a] 0.90 [a] 0.93 [a] 0.86 [a]	Van Sorest method
Hou and Li (2011)	Poplar and Eucalyptus, Dry powder	Lignin Hollocellulose α- cellulose		Diffuse reflectance	400–2500		PLS	0.21 [b] 0.34 [b] 0.53 [b]	0.98 [a] 0.98 [a] 0.98 [a]	NREL Modified sodium chlorite procedure (Yokoyama, Kadla & Chang 2002)
Kelley et al. (2004b)	Loblolly pine wood, Dry powder	Lignin Glucose Xylose Mannose Galactose Extractives	Analytical Spectral Device (ASD)	Diffuse reflectance	500–2400	Nil	PLS	1 [a]/1.1 [b] 2.7 [a]/2.4 [b] 0.6 [a]/0.6 [b] 1.3 [a]/0.8 [b] 1.0 [a]/1.0 [b] 2.3 [a]/2.3 [b]	0.76 [b] 0.78 [b] 0.56 [b] 0.58 [b] 0.80 [b] 0.85 [b]	ASTM standard method for whole biomass analysis

Source	Sample/ type	Parameter	Instrument	Geometry/ Wavelength Resolution	Spectral range (nm)	Spectral processing	Regres -sion	[a] RMSEP/ [b] RMSECV (%)	[a] R^2/[b] R	Reference method
Kelley et al. (2004b)	Loblolly pine wood Dry powder	Lignin Glucose Xylose Mannose Galactose Extractives	Analytical Spectral Device (ASD)	Diffuse reflectance	650–1150	Nil	PLS	1.4 [a]/1.5 [b] 2.3 [a]/2.6 [b] 0.6 [a]/0.54 [b] 1.0 [a]/0.69 [b] 0.8 [a]/0.83 [b] 2.2 [a]/2.4 [b]	0.67 [b] 0.84 [b] 0.54 [b] 0.69 [b] 0.83 [b] 0.88 [b]	ASTM standard method for whole biomass analysis

The components of *A. tequilana* leaf and stem bagasse of interest in bioethanol production are cellulose (glucan), hemicellulose (xylan, arabinan) and lignin, similar to other lignocellulosic feedstock. There is potential use of NIR spectroscopy for prediction of these attributes in the chemical composition of *A. tequilana* leaves in a rapid and non-invasive manner, although there has been no such application to date.

1.2.4 Use of *A. tequilana* as a Feedstock for Fuel Ethanol Production

The conversion of lignocellulosic biomass to ethanol (fermentable sugars) involves a common methodology with a series of different steps (Fig. 1.11). The lignocellulosic materials are pretreated at high temperature using acids or alkalis to facilitate the accessibility of cell wall polysaccharides to hydrolysing enzymes. The next step after pretreatment is enzymatic hydrolysis. Through enzymatic hydrolysis, polysaccharides are converted into monomeric sugars, which are finally converted into ethanol through the process of fermentation (Stanley & Hahn-Hagerdal 2010).

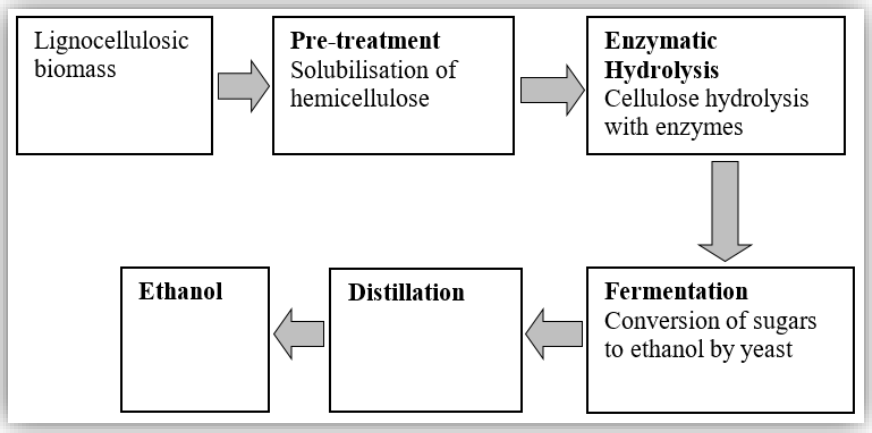


Figure 1.11: Flow chart of conversion of lignocellulosic biomass to ethanol. Source: Modified from Stanley and Hahn-Hagerdal (2010).

1.2.4.1 Pretreatment and enzyme saccharification

Efficient utilisation of lignocellulosic biomass requires pretreatment to liberate cellulose from its lignin seal and disrupt its crystalline structure (Fig. 1.12), before effective enzymatic hydrolysis can take place. This is generally achieved through either chemical or physical methods or a combination of both. Commonly used pretreatment methods for various biomass feedstock and their characteristic features are summarised in Table 1.8.

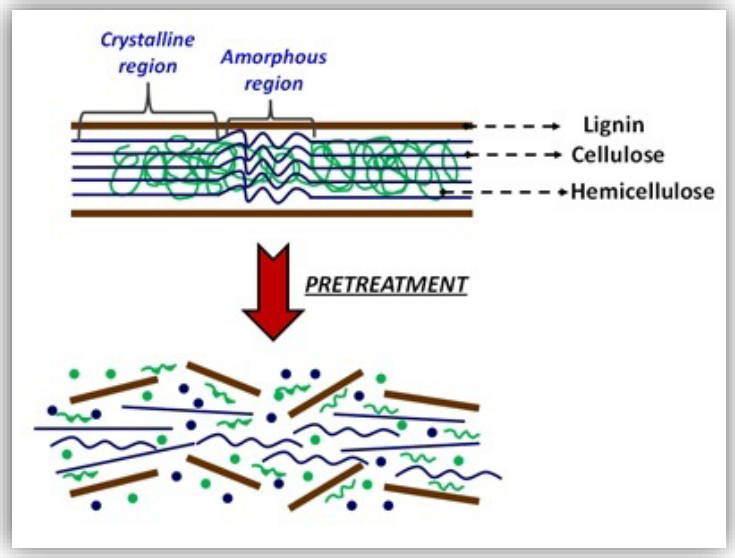


Figure 1.12: Schematic representation of the effect of pretreatment on lignocellulosic biomass Source: Modified from (Bhatia, Johri & Ahmad 2012; Mosier et al. 2005).

The various pretreatment methods (Table 1.8) have merits and demerits in terms of glucose and xylose recovery and operational costs. The outcome of pretreatment is also biomass feedstock dependant (Karimi & Taherzadeh 2016). Of all these methods, dilute acid pretreatment (H_2SO_4) is one of the most commonly used low-cost methods for various feedstocks (Chaturvedi & Verma 2013; Mosier et al. 2005; Sluiter et al. 2008b).

Several pretreatment methods have been reported for hydrolysis of agave bagasse, such as dilute acid (HCl 1.2–2% v/v) (Hernández-Salas et al. 2009; Saucedo-Luna et al. 2011), dilute alkali (NaOH 2% w/v) (Hernández-Salas et al. 2009) and ionic liquid (1-ethyl-3- methylimidazolium acetate [C2mim][OAc]) (Perez-Pimienta et al. 2013), followed by enzymatic saccharification. A comparative acid-alkali pretreatment of *Agave atrovirens* bagasse showed that dilute acid pretreatment (1.2% HCl) was far less effective than its alkali counterpart (2% NaOH) in producing sugars after enzyme saccharification (Hernández-Salas et al. 2009). The dilute acid treatment yielded between 5–9.9% w/w reducing sugars from bagasse of agave piña and whole biomass (piña + leaves) while the alkaline treatment yielded 12–58% reducing sugars (Hernández-Salas et al. 2009). In contrast, Saucedo-Luna et al. (2011) demonstrated greater yields with the piña bagasse from *A. tequilana* using a dilute acid approach.

Table 1.8: Effect of pretreatment methods on different feedstock and key characteristics.

Pretreatment	Feedstock	Pretreatment conditions	Yield	Advantages	Drawbacks and disadvantages	References
Dilute acid	Sugarcane bagasse	2–6% H_2SO_4; 100–128°C, 0–300 min	Maximum xylose recovery in 24 min at 122°C and 2% H_2SO_4 (~ 90% of hemicellulose hydrolysis)	Practical and simple technique Effectively removes and recovers hemicellulose as dissolved sugars, only a small portion of soluble lignin is present after dilute acid pretreatment	Generates toxic inhibitors such as hydroxymethyl furfural, furfural and acetic acid. These compounds, if present in high concentration, cause adverse effects on fermentation Requires recovery steps to neutralise pretreated slurry	(Aguilar et al. 2002; Haghighi et al. 2013; McIntosh et al. 2012; Tao et al. 2011; Vancov & McIntosh 2012)
	Eucalyptus residue	0–0.5% v/v H_2SO_4; 175–195°C and 2–7 min	93% (theoretical) recovery of xylose at 0.25 % v/v H_2SO_4 at 185°C, 5 min)			
	Sorghum bicolor straw	0–2% v/v H_2SO_4, 60 and 121°C and 30–90 min	Maximum recovery of xylose (hemicellulose solubilisation) at 2% H_2SO_4, 60 min at 121°C			
	Switch grass	0.5–2% H_2SO_4, 120-200°C, <60 min	76% monosaccharides yield at 140°C in 40 mins			
Alkaline pretreatment	*Sorghum bicolor* straw	2% NaOH, 60°C for 60-90 min	Increment (4-fold) in total sugar at 2% NaOH, 60°C in 90 min	Delignifies biomass by disrupting the ester bonds cross-linking lignin and xylan, fractionating cellulose and hemicellulose fibrils	Long pretreatment resident time Neutralisation of pretreated slurry	(Haghighi et al. 2013; Kim et al. 2003; McIntosh & Vancov 2010)
	Corn stover	Aqueous ammonia, (ammonia recycled percolation method)	Reduced lignin content by 70–80%			
Steam explosion with/without catalyst	Wheat straw	0.2% H_2SO_4 at 190-210°C, 2–10 min. Dilute H_2SO_4 as a catalyst	Recovery of glucose 102% and xylose 96% after pretreatment at 190°C for 10 min	Limited use of chemicals, low energy consumption and short reaction time Effective for agricultural residues and hardwood Solubilisation and removal of high hemicellulose fraction with a catalyst (dilute H_2SO_4 or SO_2)	Incomplete disruption of lignin-carbohydrate matrix Production of degradation products with high temperature	(Chandra et al. 2007; Haghighi et al. 2013; Linde et al. 2008; Lloyd & Wyman 2005; Pengilly et al. 2015)
	Sweet sorghum	200°C for 5 mins	Solubilisation of xylose resulting in water insoluble fraction cellulose and lignin			
	Corn stover and hybrid poplar	170–215°C, 5–9 min, with or without 3% SO_2 as catalyst (pretreatment conditions with low, medium and high severities)	Recovery of 86% glucan and 78% xylan content using steam pretreatment at medium severity (190°C, 5 min and 0% SO_2)			

Pretreatment	Feedstock	Pretreatment conditions	Yield	Advantages	Drawbacks and disadvantages	References
Liquid hot water (LHW)	Switch grass	200°C, 10 min, Water: DM ratio 5:1	61% recovery of monosaccharides	Makes cellulose accessible to enzymes Minimises degradation products	High water consumption and energy input, residual soluble lignin is distributed homogeneously in the cell wall which has negative impact on enzymatic hydrolysis	(Mosier et al. 2005; Tao et al. 2011; Zhuang et al. 2016)
	Corn fibre	120 °C for 20 min	Recovery of 54% xylose and 74% arabinose			
Ammonia fibre explosion (AFEX)	Switch grass	NH_3:water:DM = 1.52:0.81:1; 150°C, 30 min	76% monosaccharides yield	Does not form any toxic compounds, recovers 99% sugars Ammonia can be recycled and reused	Not very effective for biomass with high lignin content High cost of ammonia High pressure requirement	(Haghighi et al. 2013; Tao et al. 2011; Teymouri et al. 2005)
	Corn stover	NH_3:DM = 1:1 moisture content 60% (DW basis), 90°C for 5 mins	Yield of 98% glucan and 80% xylan vs 29 and 16% for AFEX untreated biomass of corn stover during enzymatic hydrolysis (60 FPU/g enzyme loading).			
Organosolv	Hybrid poplar chips	180 °C, 60 min., 1.25% H_2SO_4, and 60% ethanol, (20 FPU/g cellulose)	Recovery of 82% cellulose as monomeric glucose in 24 h 72% of total xylose and 74% lignin as ethanol organosolv lignin fraction (EOL) present in untreated wood were recovered	Organic solvents are recovered easily by distillation and can be recycled More viable for bio-refineries of lignocellulosic biomass as all the components of biomass can be utilised	Expensive for biomass pretreatment Needs washing with water after pretreatment to remove organic solvents to avoid precipitation of dissolved lignin	(Pan et al. 2006; Zhao, Cheng & Liu 2009)
Ionic liquid	Wheat straw and steam exploded wheat straw (SEWS)	Ionic liquid 1-ethyl-3-methylimidazolium chloride, water as control	Hydrolysis rate of wheat straw and SEWS reached 70% and 100% with ionic liquid while only 43% and 67% were achieved with water	Enhances digestibility of lignocellulosic biomass, good dissolution of cellulose in the presence of ionic liquid	High cost of ionic liquid	Liu and Chen (2006)

They pretreated the bagasse with 1–3% H_2SO_4 (w/w) and, following enzymatic saccharisation, were able to recover 41 g L^{-1} fermentable sugar (73.6% theoretical). Similarly, *A. tequilana* stalk bagasse pretreated with a modified organosolv method (combination of water, ethanol and H_2SO_4 at 10% w/v) led to a theoretical recovery of 91% of total fermentable sugars (0.51 g g^{-1}) following saccharification with cellulase and beta-glucosidase (Caspeta et al. 2014). Also, *Agave* bagasse pretreated with ionic liquid ([C2mim][OAc]) at 160°C with solid loading of 15% w/w resulted in 45.5% delignification and significant improvement in sugar recovery, releasing 14 mg mL^{-1} compared to 7 mg mL^{-1} from untreated bagasse (Perez-Pimienta et al. 2013).

1.2.4.2 Fermentation

Fermentation of lignocellulosic biomass after pretreatment, followed by enzymatic hydrolysis and/or simultaneous saccharification and fermentation (SSF) involves the use of different organisms for different lignocellulosic feedstock (Olofsson, Bertilsson & Lidén 2008). The theoretical ethanol yield (Y_E) from glucose is calculated according to the following equation:

$$\% \text{ Theoretical ethanol yield } Y_E = [E]/ (0.51 \times [G]) \times 100 \quad (1)$$

Where [E] is the final ethanol concentration and [G] is the initial glucose concentration (g L^{-1}).

Organisms used in ethanol production are selected based on their ability to give high ethanol yield (Von Sivers & Zacchi 1996). Ethanol-fermenting organisms should also have inhibitor tolerance, temperature tolerance, ability to utilise multiple sugars and tolerance to pH (Olofsson, Bertilsson & Lidén 2008).

The commonly used organisms are *Saccharomyces cerevisiae* (Baker's yeast), mostly used for fermenting hexose sugars (glucose, fructose) and recombinant *S. cerevisiae* to ferment pentose (xylose, arabinose) and hexose sugars (glucose and fructose) (Bettiga et al. 2009; Hahn-Hägerdal, Tjerneld & Zacchi 1988), recombinant *Zymomonas mobilis* to ferment hexose and pentose sugars (Rogers et al. 2007), and *Pichia stipitis* to ferment pentose sugar (material with high xylan content) (Grootjen, van der Lans & Luyben 1990). The wild type of *Z. mobilis* cannot ferment pentose sugar and the organism is not as robust as *S. cerevisiae* (Rogers, Lee & Tribe 1979).

For starch or sugar based ethanol production, *S. cerevisiae* is commonly used, producing ethanol of >0.45 g g^{-1} substrate at optimal conditions. In addition, these organisms are very robust against inhibitory compounds and tolerant to a high concentration of ethanol (>100 g L^{-1}), low pH and low to high sugars (Verduyn et al. 1990). Therefore, *S. cerevisiae* is considered ideal for fermentation of lignocellulosic biomass (Olsson & Hahn-Hägerdal 1993).

For conversion of agave bagasse to ethanol, Saucedo-Luna et al. (2011) reported the use of dilute acid pretreatment accompanied by enzyme saccharification at 10% w/w solid loading to produce 18.3 g L^{-1} ethanol. In this study, the fermentation was based on the use of native yeast, *Pichia carribica* (UM-5 strain), which could ferment both hexose and pentose sugars, with an overall theoretical ethanol yield of 56.8% w/v.

In a study reported by Hernández-Salas et al. (2009), alkaline pretreated/enzymatic saccharified agave bagasse yielded only 6.6 g L^{-1} ethanol from 56.4 g L^{-1} glucose (23% w/v theoretical yield) following fermentation with a non-recombinant strain of *S. cerevisiae* (Hernández-Salas et al. 2009). Higher ethanol yields were reported by Caspeta et al. (2014) through enzymatic hydrolysis of the bagasse of *A. tequilana* stalk at high-solids loading following dilute acid pretreatment, and use of an industrial strain of *S. cerevisiae* demonstrated a maximum ethanol yield of 0.25 g g^{-1} of dry agave bagasse, corresponding to 86% of maximum theoretical yield (0.29 g g^{-1}).

Villegas-Silva et al. (2014) reported that *Kluveromyces marxianus* produced up to 13.7 g L^{-1} ethanol from treated *A. fourcroydes* juice (50% theoretical) initially, and then showed that the yield could be substantially increased (80%) by liberating fructose from inulin via enzyme (inulinase) saccharification. Therefore, organisms with the ability to ferment both C5 and C6 sugars, such as genetically engineered *S. cerevisiae*, could produce greater ethanol yields (Bettiga et al. 2009). In studies focusing on agave juice fermentation, the use of inulinase enzyme prior to fermentation with a mixture of two yeasts, *K. marxianus* and *S. cerevisiae*, increased ethanol yield by 30% (Villegas-Silva et al. 2014). Therefore, *S. cerevisiae* is used at an industrial scale for ethanol production due to its favourable characteristics for ethanol production, such as quick and efficient conversion of carbohydrates into alcohol (high speed fermentation), tolerance to acidic media, high cell viability and ability to produce a high concentration of ethanol, tolerance to low oxygen concentration and ability to withstand high temperatures

(Andrietta et al. 2007). Of the many established technologies for conversion of lignocellulosic biomass to ethanol, the current study will explore the most suitable and commonly used process technologies for conversion of leaves of *A. tequilana* grown at different environmental conditions in Queensland through pretreatment, enzymatic hydrolysis and fermentation.

1.3 Proposed Work

There are many gaps in available information relevant to the establishment of an agave-bioethanol production system in central Queensland. In this thesis, studies were undertaken to address some of these gaps, with the view that the information would assist future investment decisions, either in further research and development and/or in the pursuit of commercial production.

A primary need is species-specific information on yield and recommendations for management. These issues are addressed in Chapter 2.

A second need is for information on changes in dry matter, TSS and major chemical composition of *A. tequilana* leaves with respect to plant growth and maturity, in order to optimise harvest timing. In support of this goal, the use of NIR spectroscopy as a rapid and potentially non-invasive measurement technology was considered. Three technologies were examined, viz., FTNIR (high wavelength resolution), scanning grating (high signal to noise) and silicon diode array short wave NIR (portable) (Chapters 3 and 4).

Further, a review of the literature reveals only a few studies on the use of *A. tequilana* leaves for biofuel production, focussing on chemical composition, cellulose characterisation, pretreatment and enzymatic hydrolysis (Corbin et al. 2015; Li et al. 2012b; Li et al. 2014). Work on the suitability of bagasse derived from *A. tequilana* leaves at different stages of maturity and from different localities as feedstock for bioethanol production was therefore undertaken (Chapter 5). Specifically, Chapter 5 examines and reports on the major chemical constituents of *A. tequilana* leaf biomass from two locations in Queensland and details the use of dilute acid pretreatment and enzyme saccharification options for *A. tequilana* leaf bagasse. The fermentation potential of recovered sugars was also examined using an ethanol fermenting *S. cerevisiae* strain.

Overall, this study aims to ascertain whether production of ethanol from agave leaf is technically feasible with consideration given to plant production rates, management systems and use as a feedstock in bioethanol production.

Chapter 2. Genotypic variation of different species of *Agave* and selected cultivars of *A. tequilana*

Abstract

Agave is a potential crop for central Queensland (CQ), as the climatic conditions of several parts of Queensland are similar to those of Mexico, the place of origin for *Agave* species. The growth and ethanol production potential of *Agave* species in CQ was studied over 5 years. The rate of leaf number and leaf area production and above ground biomass of *F. foetida*, *A. decipiens* and *A. americana* were either similar or better than those of *A. tequilana* cultivars. For example, the above ground biomass of the best performing cultivar of *A. tequilana* was 12.9 t ha^{-1} yr^{-1}, whereas the above ground biomass (excluding inflorescence) of *A. decipiens and F. foetida* were 13 and 13.4 t ha^{-1} yr^{-1} respectively. The full maturity (producing stalks or inflorescence) for both *A. decipiens* and *F. foetida* were earlier than those of *A. tequilana*. Thus, *F. foetida*, *A. decipiens* and the best performing cultivars of *A. tequilana* are recommended for leaf compositional analysis to assess their potential for bioethanol production.

2.1 Introduction

The current bioethanol industry uses sugar and starch for bioethanol production and relies on crops that are used for food production. Therefore, the biofuel industry is exploring alternative sources of feedstock such as lignocellulosic biomass for biofuel production (Stanley & Dumsday 2010; Villegas-Silva et al. 2014). Agaves are potential biofuel crops for semi-arid conditions. Well-developed cultivation practices in Mexico have been established for *Agave* spp. (Somerville et al. 2010b) for production of alcoholic beverages (for example,

A. tequilana for Tequila and *A. salmiana* for Mezcal) and fibre (*A. sisalana* and *A. fourcroydes*) (Gentry 1982; Nobel 2003). The genus *Agave* exhibits enormous diversity among its species regarding growth and biomass production, and a few species are reported to be better than the currently used bioenergy crops such as poplar, switchgrass and *miscanthus* (Corbin et al. 2015; Somerville et al. 2010b). Several studies have been undertaken to determine the growth potential of *Agave* species in Mexico and North America (Nobel & Valenzuela 1987a; Nobel 1984, 2003; Nobel, García-Moya & Quero 1992); however, no published research can be found regarding growth assessment of various *Agave* species for biofuel production in Australia, and particularly in the central Queensland (CQ) region.

Agaves take up to 5–8 years to mature. The crop is generally harvested just before flowering for Tequila production (Valenzuela 2010). The above ground biomass consists of succulent leaves and stem (piña). The soluble component of piña of agaves contains non-structural carbohydrates (oligofructans) such as inulin, neoinulin, levan and highly branched fructans (Cedeño Cruz & Alvarez-Jacobs 1999; López-Alvarez et al. 2012; Mancilla-Margalli & Lopez 2006; Wang & Nobel 1998) that are hydrolysed to simple sugars in the production of Tequila.

Agave leaves account for approximately 38% w/w of the above ground biomass. This material is generally left in the field as a waste in traditional agave growing areas of Mexico, with only the stem utilised for production of alcoholic beverages (Cedeño Cruz M 2003; Iñiguez-Covarrubias et al. 2001). The leaf material has high cellulose (30–49.5% w/w) and low lignin content (11.9–14.8% w/w) (Corbin et al. 2015; Li et al. 2012a; Rijal et al. 2016). In addition, the mature leaves contain 15–29% dry weight (DW) water soluble carbohydrates comprising of glucose, fructose, fructans and sucrose (Corbin et al. 2015; Nobel 1990). Leaves can be harvested at regular intervals after 2–3 years, with the plant being allowed to continually produce new leaves (Nobel 2003). Therefore, determination of leaf growth is necessary in assessment of the suitability of different agave species and genotypes for biofuel production.

Agave productivity varies with species and growth conditions. Dry above ground biomass yields range from 3.8 to 34 t ha^{-1} yr^{-1} at sites with an annual rainfall of 427 and 848 mm for *A. lechuguiilla* and *A. salmiana*, respectively (Nobel, García-Moya & Quero 1992). The above ground biomass productivity of *A. tequilana* was 25 t ha^{-1} yr^{-1} at a site that received an annual rainfall of 1080 mm (Nobel & Valenzuela 1987a). Application of nitrogenous fertilisers has been reported to be beneficial for enhancing plant growth and in particular to increase leaf number (Nobel 2003). Agronomic practices such as weeding, leaf pruning, irrigation, fertiliser application and pest control can also enhance agave growth (Nobel et al. 1992). Semi-arid regions with moderately warm daytime temperatures are considered most suitable for agave, whereas a climate with relatively cooler day temperatures substantially hamper growth, thus leading to low biomass production (Davis et al. 2011).

One method for measuring agave growth involves counting the number of unfolded leaves over a particular period of time (García-Moya & Nobel 1990; Garcia-Moya, Romero-Manzanares & Nobel 2011; Nobel 2003). A practical method for monitoring new leaves involves clipping of the dead tips of the unfolded leaves (Garcia-Moya, Romero-Manzanares & Nobel 2011; Nobel 1989; Quero & Nobel 1987). The rate of leaves unfolding is affected by the age of the plant, and seasonal and environmental conditions. For example, more leaves are produced during wet summer than in dry winter. A reduction in unfolding of leaves of up to 30% was observed due to shading (Garcia-Moya, Romero-Manzanares & Nobel 2011). The age of the plants also influences the number of leaves unfolding as found in six-year-old plants of *A. angustifolia*, *A. fourcroydes* and *A. tequilana* which showed 27, 19 and 32% more leaves unfolding, respectively, compared to 3 year-old plants (Garcia-Moya, Romero-Manzanares & Nobel 2011). The productivity is also determined by measuring the leaf length, width and thickness as evident in the study conducted by Nobel & Valenzuela (1987a) in *A. tequilana*, where leaf dry weight increased proportionately with the cube of leaf length up to 70 cm ($r^2 = 0.95$). Therefore, leaf length, leaf area and leaf numbers are appropriate parameters to measure the growth of *Agave* spp. in the field (Nobel & Valenzuela 1987a).

2.2 Materials and Methods

2.2.1 *Agave* field trial at Rockhampton

Six cultivars of *A. tequilana*, nine other *Agave* spp. and a species of another genus (*Furcraea*) from the sub-family Agavoideae were established at Central Queensland University, Rockhampton (23.32°S,150.52°E) on a black cracking clay soil that had a pH of 6.7–7.3, electrical conductivity (1:5 H_2O) of 210–330 dS/m (Fig. 2.1). The experimental plot was established on a slope (10%) and the plants were grown on raised beds (Appendix B).

The tested genotypes included *A. tequilana*, *Agave americana*, *A. americana* 'Variegata', *Agave decipiens*, *A. angustifolia*, *A. angustifolia* 'Marginata', *A. sisalana*, *Agave attenuata*, *Agave desmetiana*, *Agave desmetiana* 'Variegata', an unidentified genotype *Agave* sp. 'ABM' and *Furcraea foetida*, a species closely related to *Agave*. This last species was chosen as it was observed growing well in the Rockhampton area. The saplings of *A. tequilana* cultivars were grown from tissue cultured material and were sourced from AusAgave, South Australia. The other genotypes were procured from local nurseries or from woodlands around Rockhampton as suckers or offshoots from mature plants. All plants were grown at a site in Rockhampton which had average annual rainfall of 1000 mm (2010–2015) and mean minimum and maximum temperature of 17°C and 29°C over 5 years (Fig. 2.2). These plants survived heavy rainfall during December 2010 (524 mm), January 2013 (555 mm) (Fig. 2.2) and during cyclone Marcia in February 2015 (282 mm).

The saplings or offshoots were planted during September–October 2010. The cultivars of *A. tequilana* were approximately 1 year-old at the time of planting (Fig. 2.1 A). The saplings were planted in raised beds that were spaced at 1.5 m, with a plant-to-plant spacing of 1 m. The *A. tequilana* cultivars were planted in blocks of five plants in replicate plots. The other genotypes had one to two replications (due to limited availability of saplings) with five plants in each replication. The raised beds were covered with plastic sheets (strawberry mulch) to minimise weed competition (Fig. 2.1 A).

Figure 2.1: Progressive growth of various *Agave* genotypes at Rockhampton, Queensland A = September 2010 (on the day of planting), B = February 2011 (5 months after planting (MAP)), C = June 2012 (20 MAP), D = September 2012 (24 MAP), E = June 2013 (33MAP), F = February 2014 (41 MAP), G = May 2015 (56 MAP), and H = May 2015 (56 MAP).

The bed was drip-irrigated once to three times a month. The quantities of water added and the rainfall received were monitored (Fig. 2.2). The plants were provided with all-purpose controlled release Osmocote (w/w; N = 15%, P = 9%, K = 12%, Mg = 1%, S = 2.3% + Trace

elements) released over 5–6 months (30 g/plant) at planting. This was equivalent to NPK of 30, 18 and 24 kg ha^{-1}. From March 2012 (18 MAP), the irrigation water was supplemented with liquid controlled release fertiliser NPKomplete (SJB Ag-Nutri Pty Ltd, Australia) (w/w; N = 10%, P = 4%, K = 8%, Mg = 1%, S = 3% + trace elements) 10 kg ha^{-1} via drip irrigation at an interval of every 6 months until 33 MAP. The experimental site was sloping (10%) with the rows running across the slope. Thus, the first replication was located at the top of the plot and the fifth replication at the bottom of the experimental site (Appendix B).

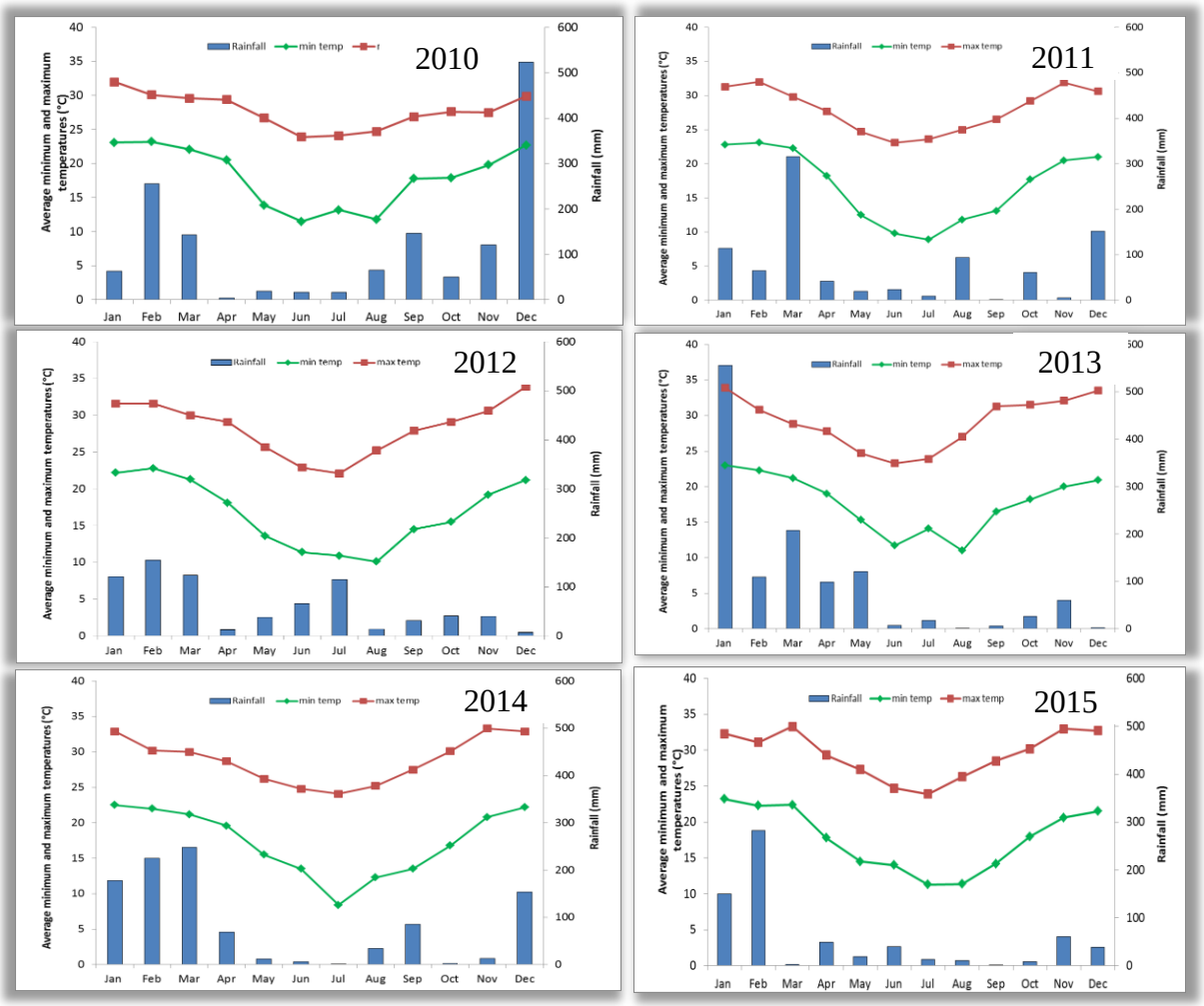


Figure 2.2: Average monthly rainfall and minimum and maximum temperature of Rockhampton from 2010–2015.

2.2.2 Soil analysis

Soil samples were collected from the agave field in June 2011 (9 MAP) from top (see Appendix B; rows 1–5), middle (rows 6–10), and bottom (rows 11–15) positions of the experimental plot. The samples were collected to a depth of 0–10 cm using a 10 cm diameter soil auger. The soil core samples (4–6) were taken separately from ridges and furrows. The cores were mixed to produce two samples (a ridge and a furrow sample) per position. The resulting samples (2 locations x 3 positions) were air-dried and ground to pass through a 2 mm sieve and were sent to CSBP Soil and Plant Analysis Laboratory, Perth, Australia.

2.2.3 Plant growth measurement

Plant growth was measured by counting the number of leaves per plant, and estimating the area of tagged leaves at 4–6 month intervals, starting from one MAP until 33 MAP. The youngest leaf that had separated from the central cylinder was tagged at the beginning and the same leaf was measured at every sampling, until it stopped expanding. At each measurement, the length and width of the tagged leaves were measured to calculate individual leaf area. Total leaf area was calculated using the formula:

Total leaf area at a given time (cm^2) = total number of leaves x leaf width x leaf length

The correction factor was determined by measuring 10 leaves and comparing the area of those leaves with the actual area of the leaves (traced on paper). The correction factor was close to 1 (0.9), and therefore was not used in calculating the total leaf area.

2.2.4 Plant harvest and estimation of above ground biomass

The leaves and stems were harvested at 58 MAP. The above ground biomass was determined as follows:

Plant biomass (kg ha^{-1}) = Plant density (6700 plants ha^{-1}) x (leaf dry weight+stem dry weight)

Agave attenuata was excluded from biomass measurement as this species barely survived in the experimental trial, although it is growing well as an ornamental plant in the Rockhampton region.

2.2.4.1 Leaf harvest

Leaves were harvested using a modified shovel (Fig. 2.3), cut to the same shape as the 'Coa' that is commonly used for harvesting leaves in Mexico. A long handled branch cutter was also used to cut the dead and dried leaves. At harvest, the leaves were counted and the total fresh weights were recorded immediately after harvest. A representative sub-sample of 10–25 leaves were taken, the fresh weight determined, and the sub-sample transferred to an oven. The samples were dried in a fan-forced oven at 70°C until no change in weight occurred. The whole plant leaf weight was estimated using the dry weight of the sub-samples.

Figure 2.3: A tool similar to the 'Coa' was used for harvesting the leaves of agave genotypes.

2.2.4.2 Stem harvest

The stems were harvested using a chainsaw and the fresh weight was determined soon after harvest. A sub-sample was taken and the fresh weight determined. The sub-sample was dried in a fan forced oven at 70°C to constant weight. The whole plant stem weight was calculated based on the sub-sample weight.

2.2.5 Statistical analysis

Data were analysed using Analysis of variance (Genstat statistical package Version 16.1, VSNI Ltd, UK) after verifying the data for normality, outliers and homogeneity of error variances. A value of $P \leq 0.05$ was considered as significant and all interpretations were made based on this criterion. Where the F-test was not significant, standard errors are provided, with an indication of the number of observations.

2.3 Results and Discussion

The initial size of the saplings of different *Agave* genotypes varied, as they were sourced from field, glasshouse or different nurseries. Considering the length of time these plants were grown (~5 years), and the agronomic practices used, these initial differences are deemed negligible. For example, the saplings of *F. foetida* were very small at planting (<10 cm), yet they exceeded the other species within one year (Figs 2.1D and 2.4). The saplings of *A. desmetiana* were large (~25) cm tall at the time of planting, but their growth rate was much slower than many other genotypes (Fig. 2.4).

2.3.1 Performance of *Agave* genotypes

Four of nine species produced flowering stalks (reached full maturity) within 5 years (*A. decipiens*, *A. desmetiana*, *A. desmetiana* 'Variegata' and *F. foetida*). *Agave desmetiana* was the first species to reach fully maturity at 32 MAP (Fig. 2.1E), followed by *A. decipiens* which produced stalk at 41 MAP. The rest of the genotypes had reached maturity at around 50 MAP (Fig. 2.1 G and H). Based on the approximate age of the plant at initial planting, *A. desmetiana* reached maturity in 4.5–5 years. Therefore, it can be stated that *A. decipiens* was the first species to reach maturity within 3.5 years. Full maturity is reached usually between 5 and 8 years in *A. tequilana* and the average maturity time for most *Agave* species is between 8–12 years (Cedeño Cruz & Alvarez-Jacobs 1999).

Amongst all the genotypes tested, *A. angustifolia* 'Marginata' and *A. angustifolia* produced very high numbers of leaves at 33 MAP (233 and 192, respectively). Most other species produced around 100 leaves per plant, with *Agave* sp. 1 (ABM) having the lowest number of leaves (22 per plant) at 33 MAP (Fig. 2.4). For leaf area, *F. foetida* ranked the highest as it produced the maximum leaf area (2275 cm^2 per leaf) at 33 MAP (Fig. 2.4). Coincidentally, this species also had the longest leaves among all genotypes (data not shown). The species *A. americana*, *A. sisalana* and *A. americana* 'Variegata' also had larger leaves, but their size was nearly half that of *F. foetida*. This was because the leaves of *F. foetida* were longer as well as broader than the other species. The leaf size of *A. tequilana* (535 cm^2) was of medium size compared to that of *A. attenuata* which had the smallest leaves (340 cm^2).

The rate of increase in leaf size varied markedly between the species. *Furcraea foetida*, *A. americana*, *A. sisalana* and *A. decipiens* showed faster increases in leaf size with their maturity, whereas the other species such as *A. angustifolia* and *A. attenuata* had slower leaf expansion rates (Fig. 2.4). Again, *F. foetida*, *A. sisalana* and *A. americana* had higher growth rates and *A. tequilana* remained the moderately growing species.

Comparable published data is lacking; however, past research shows that the rate of leaf unfolding varies with species (Nobel 1984). Annual leaf unfolding can be as high as 45.7 for *A. tequilana* (Nobel & Valenzuela 1987a) and as low as 5.2 for *Agave deserti* (Nobel 1984). *Agave tequilana* produced approximately 32 leaves annually (Fig. 2.4), indicating that its growth rate at Rockhampton was approximately 30% less than reported for it in Jalisco, Mexico by Nobel and Valenzuela (1987a).

Furcraea foetida was the fastest growing species at Rockhampton. The current study has shown that it can grow faster than all other *Agave* species at Rockhampton, suggesting that this species may be used in biofuel production as it is resilient to infertile soil conditions and grows fast in these soils.

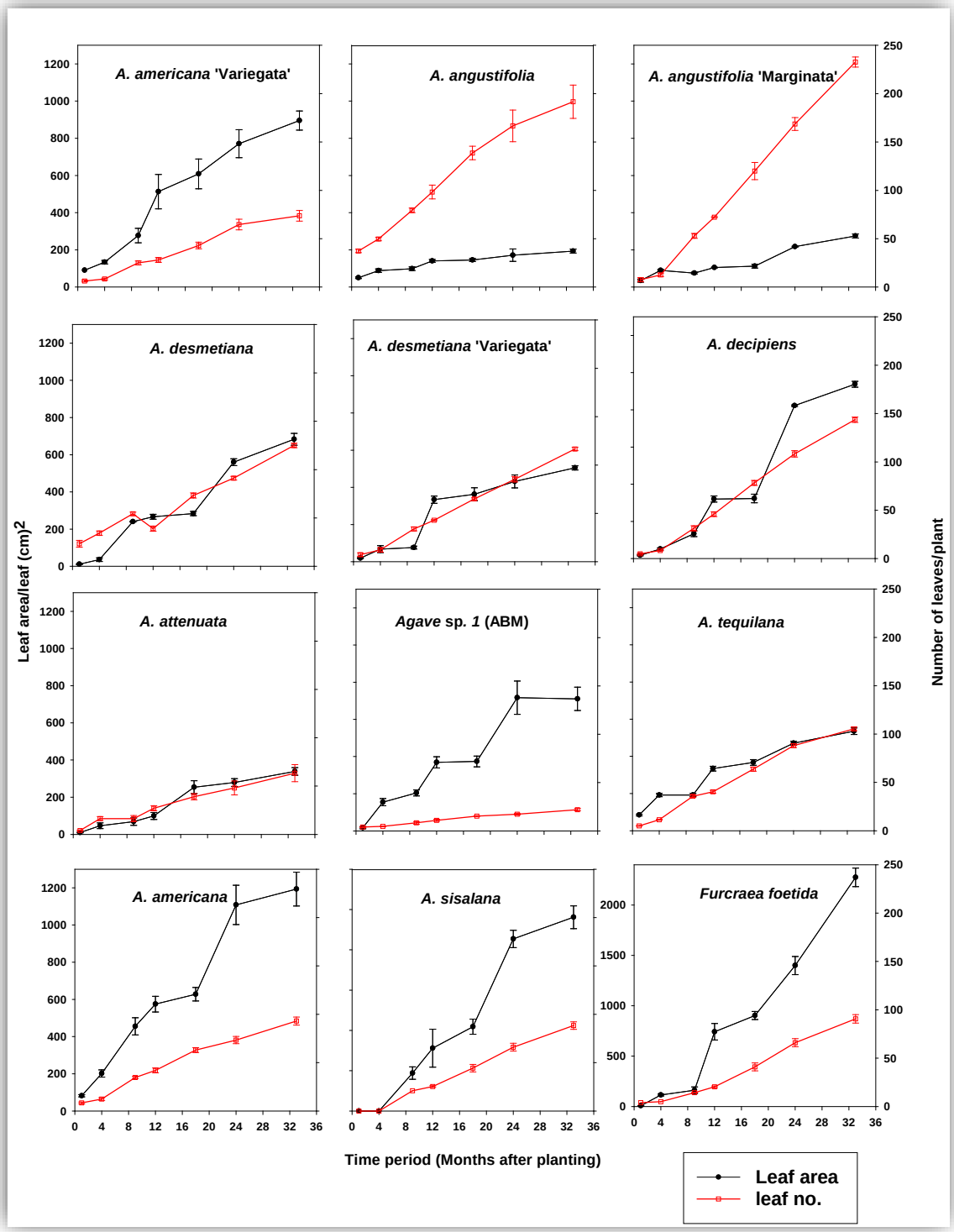


Figure 2.4: Number of leaves and leaf area/leaf of different genotypes of *Agave* grown at Rockhampton from 1 to 33 MAP. Data represents mean values of 3 plants (n=3) except *A. tequilana* (Tcqu), where values are the means of 12 plants. Bars represent ± SE.

At 33 MAP, *Agave* species produced up to 20 m^2 of leaves per plant. Again, there were large differences between the cultivars, with *F. foetida* producing the highest leaf area (Fig. 2.5). *Furcraea foetida* had ceased growth about 6 months prior to harvesting as shown by the production of flowering stocks (Fig. 2.1 G). *Agave decipiens* and *A. americana* were the two other species that showed higher leaf area and their growth was much superior to *A. tequilana*, which produced nearly half the leaf area of the above species. The total leaf area of *F. foetida* was nearly twice those of *A. decipiens* and *A. americana* and 4 times more than *A. tequilana*, indicating that this species could serve as a highly useful species for biomass production in Rockhampton (Fig. 2.5).

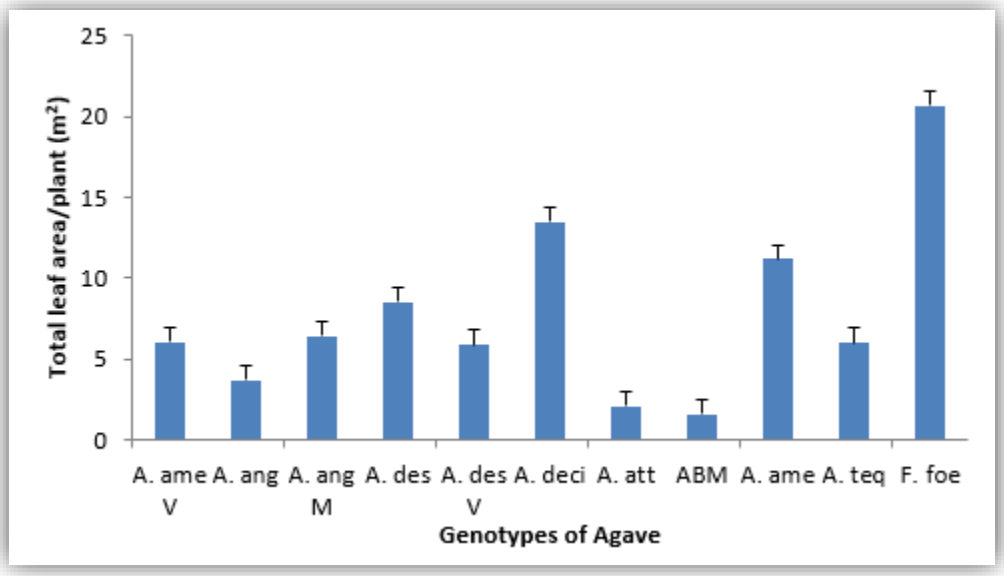


Figure 2.5: Total leaf area among different genotypes of *Agave* at 33 months after planting (Bars represent l.s.d. (P <0.05). A. ang M = *A. angustifolia* 'Marginata', A. ang = *A. angustifolia*, A. deci = *A. decipiens*, A. ame = *A. americana*, A. ame V = *A. americana* 'Variegata', ABM = unknown *Agave* sp. 1, A. des = *A. desmetiana*), A. des V = *A. desmetiana* 'Variegata', F. foe = *F. foetida*, and A. teq = *A. tequilana* (cultivar Tcqu).

2.3.1.1 Leaf senescence amongst Agave genotypes

There was wide variation between agave genotypes in the leaf number and distribution of dead and live leaves (leaf senescence) at harvest (58 MAP). The number of leaves ranged from as low as 30 to a maximum of 304. This corresponded to 6 and 63 leaves unfolding every year on average in *Agave* sp. 1 (ABM) and *A. angustifolia* 'Marginata', respectively (Table 2.1, Fig. 2.6). *Agave tequilana* had an annual leaf unfolding of 32 (Fig. 2.6).

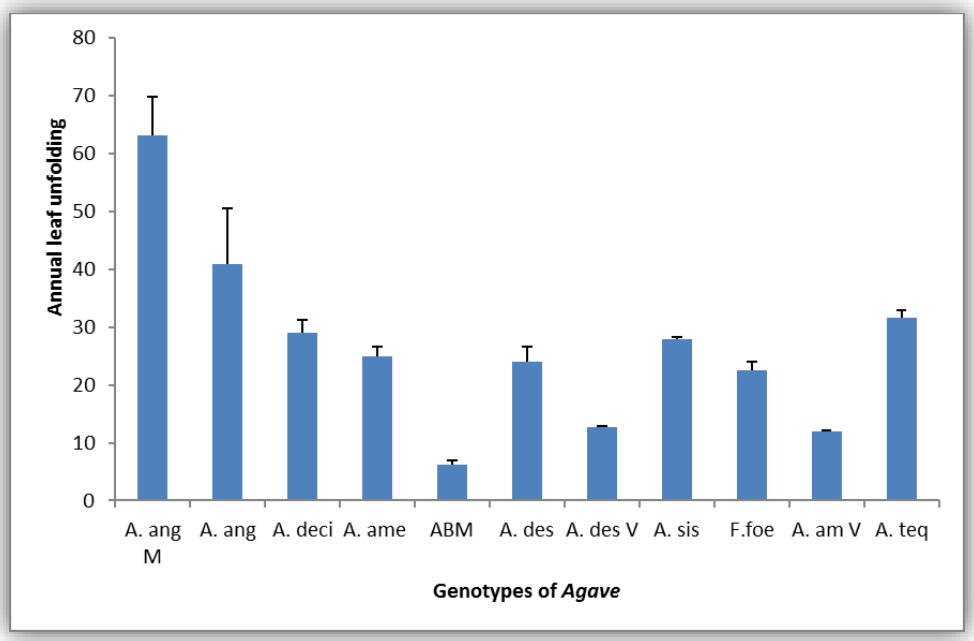


Figure 2.6: Annual leaf unfolding of different genotypes of *Agave* at harvest (58 MAP). Genotypes compared are: A. ang M = *A. angustifolia* 'Marginata', A. ang = *A. angustifolia*, A. deci = *A. decipiens*, A. ame = *A. americana*, A. ame V = *A. americana* 'Variegata', ABM = unknown *Agave* sp. 1, A. des = *A. desmetiana*, A. des V = *A. desmetiana* 'Variegata', F. foe = *F. foetida*, and A. teq = *A. tequilana* (cultivar Tcqu). Bars represent ± SE (n = 2); except for *A. angustifolia* 'Marginata', *A. decipiens*, *F. foetida* and *A. tequilana* (cultivar Tcqu), where n=4.

At harvest, the proportion of live and dried/dead leaves varied between the genotypes, with *A. decipiens*, *A. sisalana*, *A. americana* and *A. tequilana* maintaining larger proportions of live leaves compared to the other genotypes. The percentages of dried/dead leaves were 40-69% for the different species. *Agave angustifolia* and *F. foetida* had the high proportions of dead/dry leaves of 69% and 59%, respectively (Table 2.1). The faster growth of *F. foetida*, associated with its larger proportions of dried/dead leaves, indicate that this species is metabolically very active as compared to the other genotypes. This suggests that there is potential for this genotype to be exploited for biomass energy production.

Table 2.1: Number of leaves at harvest (58 MAP). Data represents average values of (n=2 ± SE) for all except *A. angustifolia* 'Marginata', *A. decipiens*, *F. foetida* (n=4), and *A. tequilana* (n=6) at 58 MAP.

Genotypes	living leaves	dead/dried leaves	Total leaves	% living leaves
***A. angustifolia* 'Marginata'**	155±42	148±3	304±46	51
A. angustifolia	62±37	135±10	197±47	31
A. decipiens	84±8	56±20	139±13	60
A. americana	69±2	51±7	120±9	58
***Agave* sp. 1**	15	15±3	30±3	50
A. desmetiana	57±7	59±6	116±13	49
***A. desmetiana* 'Variegata'**	34±3	27±1	61±2	56
A. sisalana	81±6	54±8	134±2	60
F. foetida	44±3	64±7	108±10	41
***A. americana* 'Variegata'**	29±3	28±2	57±1	51
***A. tequilana* (Tcqu)**	91±5	61±2	152±6	60

2.3.1.2 Above ground biomass per plant of different genotypes

The above ground biomass includes weight of old dead leaves, recently dried leaves, green leaves at harvest and stem. The dry biomass of the inflorescence was measured for two species, viz., *F. foetida* and *A. decipiens*, as they were the only species that produced the inflorescence at harvest. The dry weight of the inflorescence accounted for an average of 26% and 28% of the total above ground biomass for *F. foetida* and *A. decipiens*, respectively. However, the dry biomass of flowering stalk was not included in Table 3 to allow equal comparison with other species that had not produced flowering stalks. The *Agave* genotypes had very high moisture content at harvest as shown by the fresh weight to dry weight ratio which varied from 7:1 to 4:1 (or 85% to 75% moisture), respectively, in *A. sisalana* and *A. angustifolia*. Since it is proposed that the fresh leaves and fresh stems are used in ethanol production and the moisture content of the leaves make a significant contribution to transportation costs, it is important to consider this aspect in *Agave* cultivation. Compared to most high performing genotypes such as *A. sisalana* and *A. decipiens*, *A. tequilana* had nearly 40–50% of the maximum obtainable fresh biomass, but had similar dry biomass.

The highest above ground fresh biomass was produced by *A. sisalana* with 71.5 kg/plant at 58 MAP (Fig. 2.7) followed by *A. decipiens* and *A. americana*. Although *F. foetida* was the fourth ranked species for fresh biomass, it produced the highest dry biomass with 9.6 kg/plant (Fig. 2.7).

The genotypes can be ranked based on the dry above ground biomass: (*F. foetida* = *A. decipiens* = *A. tequilana* = *A. sisalana*) >*A. americana* >*A. angustifolia* 'Marginata' >*A. americana* 'Variegata' > (*A. angustifolia* = *A. desmetiana* = *Agave* sp. 1 (ABM)) >*A. desmetiana* 'Variegata (Fig. 2.8).

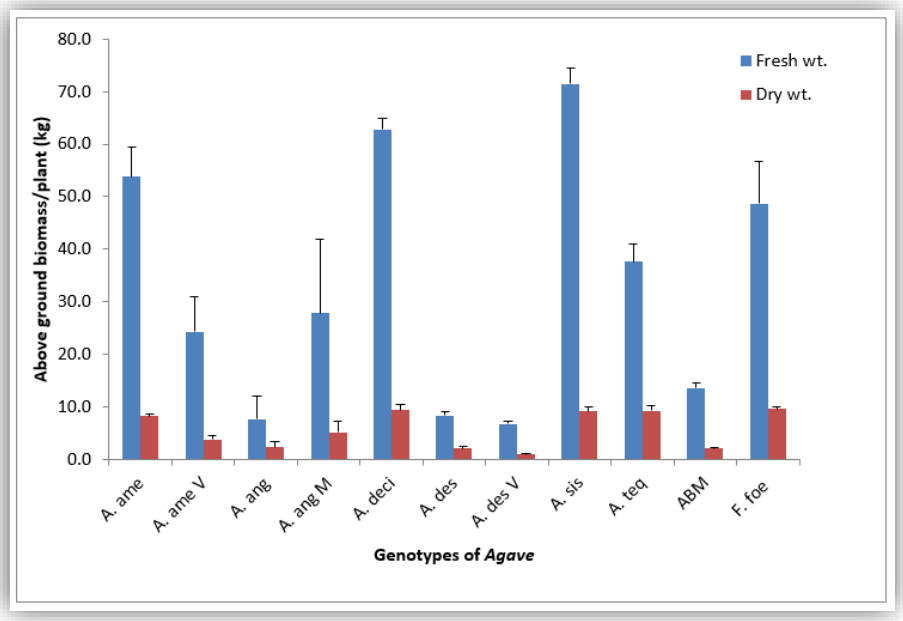


Figure 2.7: Total above ground biomass (leaves+stem)/plant produced by different species of *Agave* at harvest (58 MAP); A. ang M = *A. angustifolia* 'Marginata', A. ang = *A. angustifolia*, A. deci = *A decipiens*, A. ame = *A. americana*, A. ame V = *A. americana* 'Variegata', ABM = unknown *Agave* sp. 1, A. des = *A. desmetiana*, A. des V = *A. desmetiana* 'Variegata', *A. teq* = *A. tequilana* and F. foe = *F. foetida*. Bars represent ±SE n=2 except *A. decipiens*, *F. foetida*, *A. angustifolia* 'Marginata' and *A. tequilana* (n=4).

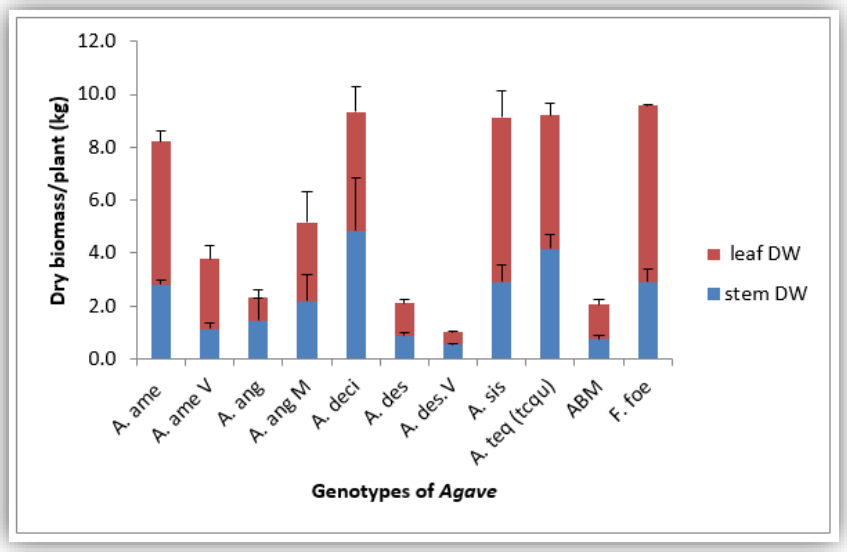


Figure 2.8: Total dry above ground biomass (leaves+stem)/plant produced by different species of *Agave* at harvest (58 MAP); A. ang M = *A. angustifolia* 'Marginata', A. ang = *A. angustifolia*, A. deci = *A. decipiens*, A. ame = *A. americana*, A. ame V = *A. americana* 'Variegata', ABM = unknown *Agave* sp. 1, A. des = *A. desmetiana*), A. des V = *A. desmetiana* 'Variegata' and F. foe = *F. foetida*. Bars represent ±SE, n=2 except *A. decipiens*, *F. foetida*, *A. angustifolia* 'Marginata' and *A. tequilana* (n=4).

The species with similar dry biomass yield/plant were *F. foetida*, *A. decipiens*, *A. tequilana* and *A. sisalana* (ranged from 9.1 to 9.6 kg/plant). *Agave decipiens* and *A. tequilana* had high stem biomass proportions (4.8 and 4.2 kg/plant, respectively), whereas *F. foetida* and *A. sisalana* had high proportions of leaf biomass (6.7 and 6.2 kg/plant, respectively) per plant (Fig. 2.8).

The leaf biomass ranged from 41–70% and stem biomass from 30–59% of the total above ground biomass. *Agave angustifolia* had the lowest leaf biomass fraction of only 41% whereas *A. americana* 'Variegata' and *F. foetida* had the maximum leaf biomass fraction of 70% (Table 2.2). Leaf to stem ratio was greatest for *F. foetida* (2.4), whereas *A. angustifolia* had the least leaf to stem ratio (0.79; Table 2.2).

Table 2.2: Leaf and stem dry biomass of different genotypes of *Agave*. (n=2 ± SE) for all except *A. angustifolia* 'Marginata', *A. decipiens, F. foetida* and *A. tequilana* (n=4) at 58 MAP.

Species	Leaf dry wt	Stem dry wt	Leaf: stem ratio
***A. angustifolia* 'Marginata'**	0.58 ± 0.02	0.42 ± 0.02	1.38 ± 0.08
F. foetida	0.70 ± 0.04	0.30 ± 0.04	2.40 ± 0.31
A. decipiens	0.49 ± 0.15	0.51 ± 0.15	1.23 ± 0.46
A. sisalana	0.67 ± 0.10	0.33 ± 0.10	2.33 ± 0.96
***Agave* sp. 1 (ABM)**	0.63 ± 0.09	0.37 ± 0.09	1.84 ± 0.69
***A. americana* 'Variegata'**	0.70 ± 0.00	0.30 ± 0.00	2.33 ± 0.0
A. angustifolia	0.41 ± 0.07	0.59 ± 0.07	0.71 ± 0.21
A. americana	0.66 ± 0.04	0.34 ± 0.04	1.95 ± 0.32
A. desmetiana	0.58 ± 0.00	0.42 ± 0.00	1.37 ± 0.01
***A. desmetiana* 'Variegata'**	0.46 ± 0.01	0.54 ± 0.01	0.84 ± 0.04
A. tequilana	0.55 ± 0.02	0.45 ± 0.02	1.22 ± 0.10

2.3.2 Above ground biomass of different genotypes

The above ground biomass (AGB) production of various species of *Agave* differed in fresh and dry biomass of both leaves and stems. The fresh biomass of leaves was highest for *A. sisalana* with 67.2 t ha^{-1} yr^{-1}. The dry biomass of the leaves was highest for *F. foetida* at 9.3 t ha^{-1} yr^{-1} (Table 2.3). The results for fresh leaf biomass of *F. foetida* are similar to those reported for *Furcraea gigantea* by the Food and Agriculture Organisation (FAO) (2007). *Furcraea gigantea* was reported to produce dry fibre of 1–1.5 t ha^{-1} yr^{-1} (FAO 2007). The current results for the dry leaf biomass of *A. sisalana* of 8.7 t ha^{-1} yr^{-1} were high compared to 2.5 t ha^{-1} yr^{-1} that was reported by Smith (1928; cited in Nobel (2003)) and 5.1 t ha^{-1} yr^{-1} reported by Lock (1969). The fresh leaf biomass was lowest in *A. angustifolia*, which produced only 4.2 t ha^{-1} yr^{-1} (Table 2.3).

In terms of fresh AGB, *A. sisalana* produced a maximum of 99.7 t ha^{-1} yr^{-1}. However, in terms of dry biomass, *F. foetida* and *A. decipiens* were the species with the maximum AGB of ~13.3 and 13 t ha^{-1} yr^{-1}, respectively (Table 4). Other species such as *A. sisalana* and *A. americana* had total AGB of ~12.7 and 11.5 t ha^{-1} yr^{-1}, respectively. The current findings for *A. americana* are higher than those reported by Davis et al. (2015) who tested growth potential in Arizona, south western USA. Davis et al. (2015) showed that biomass produced was variable with respect to total irrigation supplied, ranging from 2–4 t ha^{-1} yr^{-1} (300 mm

rainfall) to a maximum of 9.3 t ha^{-1} yr^{-1} (530 mm). The dry biomass of *A. americana* (11.45 ha^{-1} yr^{-1}), as determined in the current study, is 23% more than the maximum biomass identified by Davis et al. (2015). The other genotypes produced dry biomass between 1.4 and 7.2 t ha^{-1} yr^{-1}, with the lowest production shown by *A. desmetiana* 'Variegata'. With regard to stem biomass, the maximum fresh and dry biomass was produced by *A. decipiens* with 41.1 and 6.8 t ha^{-1} yr^{-1}, respectively (Table 2.3).

Table 2.3: Above ground biomass production of different genotypes of *Agave* grown in black cracking clayey soil at Rockhampton. The values are means of n=2, except A deci, F. foe and A. ang M with n=4 ± SE.

	A. ang M	F. foe	A. deci	A. sis	ABM	A. am V	A. ang	A. ame	A. des	A des V
FW leaves (t ha^{-1} yr^{-1})	25.37	46.87	46.48	67.24	11.03	22.33	4.19	50.49	7.64	5.83
SE	13.30	10.02	14.94	11.2	0.00	6.28	2.51	6.32	0.73	1.29
DW leaves (t ha^{-1} yr^{-1})	4.12	9.29	6.28	8.65	1.81	3.66	1.19	7.54	1.69	0.64
SE	1.60	0.04	1.31	1.97	0.38	0.73	0.43	0.77	0.27	0.03
FW piña (t ha^{-1} yr^{-1})	13.50	21.02	41.11	32.49	7.96	11.62	6.53	24.67	3.98	3.56
SE	6.28	1.20	11.86	6.87	1.40	2.83	3.73	1.43	0.28	0.56
DW piña (t ha^{-1} yr^{-1})	3.06	4.06	6.75	4.07	1.05	1.57	2.02	3.92	1.23	0.77
SE	1.38	0.69	2.81	0.86	0.18	0.31	1.18	0.25	0.19	0.07
FW AGB (t ha^{-1} yr^{-1})	38.87	67.89	87.59	99.73	18.98	33.95	10.71	75.17	11.62	9.39
SE	19.58	11.22	3.07	4.33	1.40	9.11	6.25	7.75	1.01	0.73
DW AGB (t ha^{-1} yr^{-1})	7.18	13.35	13.04	12.73	2.86	5.23	3.21	11.45	2.93	1.41
SE	2.98	0.73	1.50	1.11	0.20	1.05	1.61	0.51	0.46	0.10

2.3.3 Performance of *A. tequilana* cultivars

The cultivars of *A. tequilana* studied were: 17, E9, L3, L9, L19 and Tcqu. Closer examinations of the *A. tequilana* cultivars show large variations between cultivars for leaf area expansion and the number of leaves per plant. The cultivars 17, L19 and Tcqu had higher leaf area than other cultivars. They also produced an average of 150 leaves after 5 years of growth.

All six cultivars of *A. tequilana* had similar number of leaves (3–6) at the start, but differed in leaf size as they grew (Fig. 2.9). The cultivars showed significant differences with time in both leaf number and leaf area increment (Fig. 2.9). For example, the cultivar Tcqu had the highest number of leaves, and the leaves were larger than those of L9. The average leaf unfolding per year for three of the better performing cultivars, Tcqu, L19 and 17, were 39, 36 and 33 calculated at 33 MAP. However, at harvest (58 MAP; 4.8 years), the annual leaf unfolding rate was reduced to an average of 31 for these cultivars (Fig. 2.10). This result is close to those reported by Garcia-Moya, Romero-Manzanares and Nobel (2011) for a 3-year-old *A. tequilana* plant which produced 34.7 leaves per year. Leaf area differed significantly ($P < 0.05$) among the cultivars (Fig. 2.9). The cultivars Tcqu, L19 and 17 showed better performance compared to E9 and L9 (Fig. 2.9). The cultivars L9 and E9 were found to be the slowest growing cultivars.

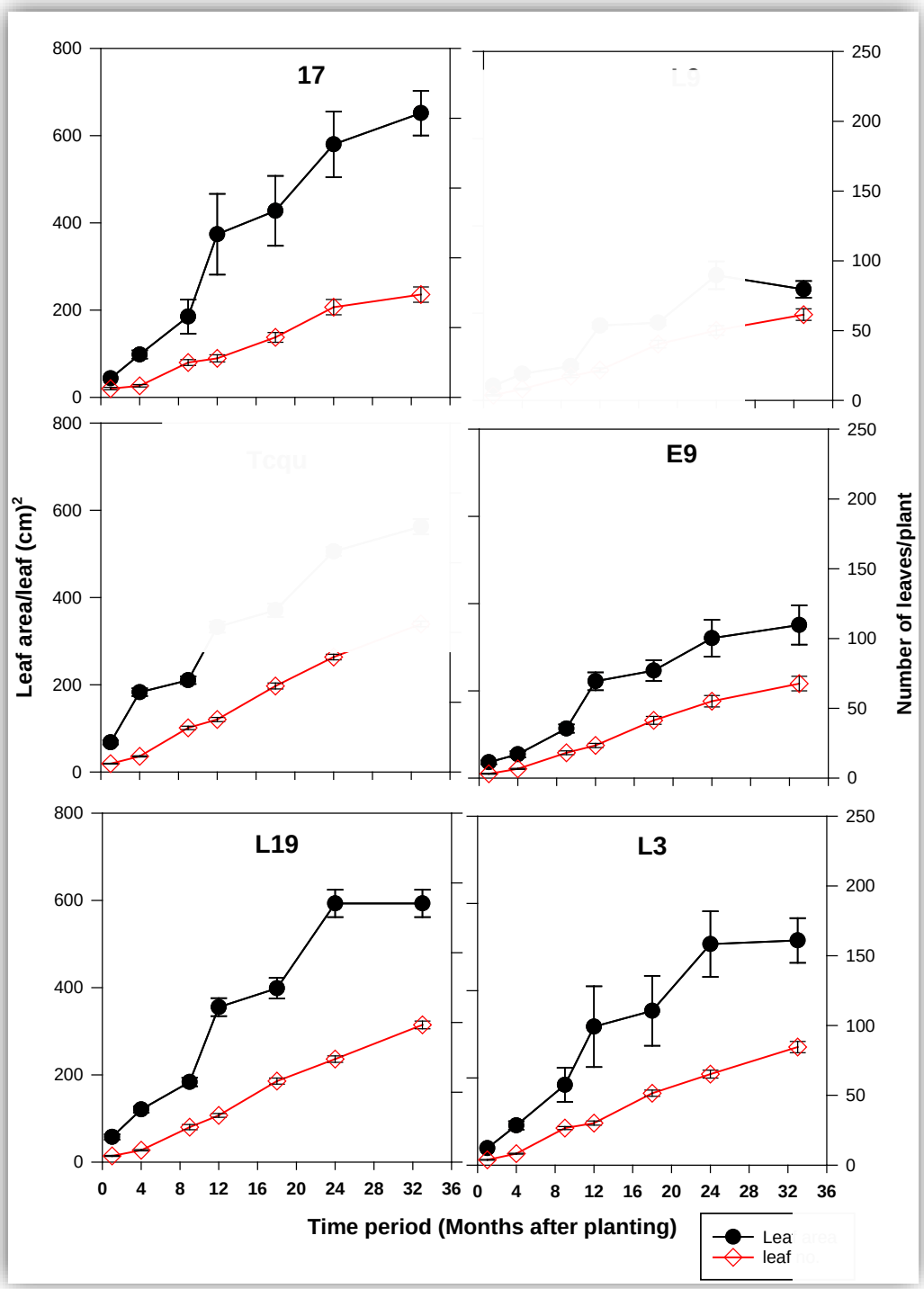


Figure 2.9: Number of leaves per plant and leaf area/leaf of six cultivars of *A. tequilana* grown at Rockhampton up to 33 MAP. Bars represent SE (n=12) for both leaf number and leaf area.

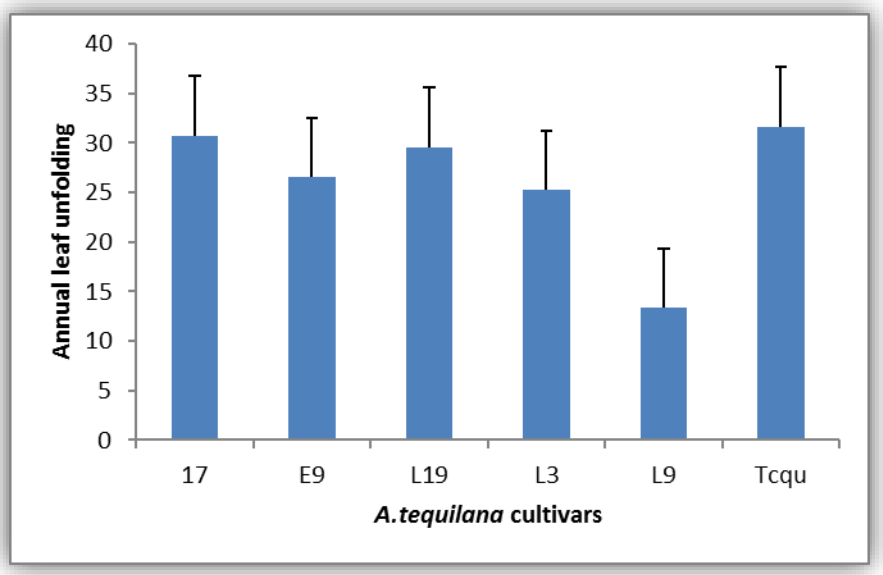


Figure 2.10: Number of annual leaves unfolding per plant calculated over a period of 4.8 years (58 MAP) of six cultivars of *A. tequilana* grown at Rockhampton. Bars represent l.s.d (n=6).

The total leaf area per plant revealed similar performance of the cultivars 17, L19 and Tcqu, and the lowest performance in L9, which produced only a quarter of the leaf area as compared to Tcqu. The cultivars L3 and E9 had moderate growth and they also differed significantly in their total leaf area production (Fig. 2.11). The overall performance of the cultivars can be ranked based on total leaf area as (Tcqu = 17 = L19) >L3 >E9 >L9.

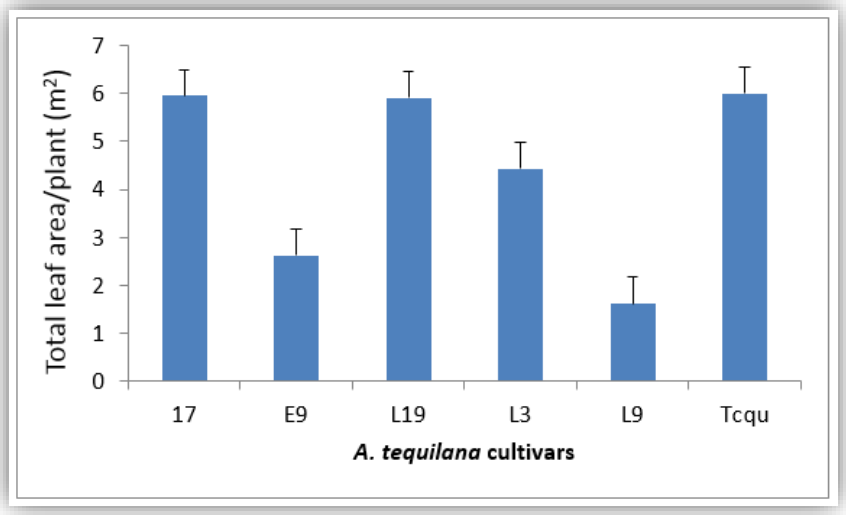


Figure 2.11: Total leaf area among different cultivars of *A. tequilana* at 33 MAP. Bars represent l.s.d. (P <0.05).

The total fresh AGB varied significantly (P<0.001) among different cultivars of *A. tequilana*. Cultivars 17 and Tcqu had the highest fresh AGB of 41 and 37.6 kg/plant, respectively (Fig. 2.12). The average fresh weight of the mature *A. tequilana* plant (~7 years) from Mexico, reported by Iñiguez-Covarrubias et al. (2001), was 53.2 kg/plant. Therefore, the current results are approximately 25% less than that reported by Iñiguez-Covarrubias et al. (2001) for the fresh weight of plants. The average fresh weight of *Agave* piña (stem) for *A. tequilana* (Tcqu) of the current study was only 13.3 kg/plant (56% less) as compared to 30.7 kg/plant reported by Iñiguez-Covarrubias et al. (2001). The variation in plant fresh weights could be due to different factors such as soil characteristics, temperature, hours of sunlight and plant health at different locations (Iñiguez-Covarrubias et al. 2001). The weight of the stem also varies with plant age, as stems of 2, 4 and 6.5-year-old *A. tequilana* weighed 4.5, 32.6 and 90.9 kg, respectively (Arrizon et al. 2010).

The dry AGB was similar between the above cultivars (9.2 and 8.4 kg/plant) and were 5.7, 6.2 and 7.4 kg/plant in E9, L3 and L19, respectively. The lowest dry AGB (3.6 kg/plant) was obtained in L9. The cultivar Tcqu produced dry AGB of 9.2 kg/plant, which was the highest amongst all cultivars. Even the high biomass producing cultivar of in current study had produced 65% less dry biomass per plant than those reported by Nobel (1990) as cited in Yan

et al. (2011) for 7-year-old plants from Mexico which could be due to early harvest i.e., ~5 years in the present study.

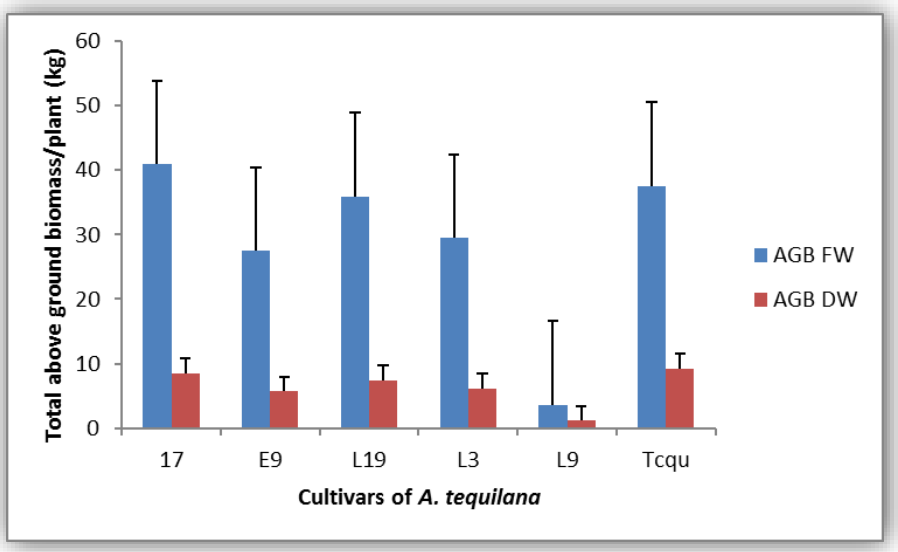


Figure 2.12: Total above ground biomass/plant of all cultivars of *A. tequilana.* Bars represent l.s.d.

2.3.3.1 Leaf and stem ratio

There was a significant difference (P <0.05) in the stem and leaf biomass fractions between L9 and 17, E9, L19 and L3. The leaf and stem biomass fractions ranged from 39–41% and 59–61% in cultivars 17, E9, L19 and L3. The cultivar Tcqu had 55% and 45% of leaf and stem biomass fractions (Table 2.4). On average, the leaf and stem biomass fractions were 58% and 42% in all the cultivars. The ratio of leaf to stem varied significantly (P <0.05), ranging from 1.11 (L9) to 1.62 (17 and L19) (Table 2.4). Nobel (1990) also determined a leaf to stem ratio of 1.13 in a study conducted with 7-year-old *A. tequilana* plant in Jalisco, Mexico.

Table 2.4: Leaf and stem dry biomass fractions of *A. tequilana* cultivars. Along the row the means sharing the same superscript do not differ significantly (P <0.05).

Cultivar	17	E9	L19	L3	L9	Tcqu	l.s.d
Leaf weight fraction (LMF)	0.61^{b}	0.59^{b}	0.61^{b}	0.6^{b}	0.52^{a}	0.55^{ab}	0.063
Stem weight fraction (SMF)	0.39^{a}	0.41^{a}	0.39^{a}	0.4^{a}	0.48^{b}	0.45^{ab}	0.064
Leaf wt to stem wt ratio	1.62^{b}	1.45^{ab}	1.62^{b}	1.57^{b}	1.11^{a}	1.23^{ab}	0.378

The highest fresh and dry biomass of leaves, respectively, was produced by cultivar 17, which had 39.2 and 7.2 t ha^{-1} yr^{-1}, followed by Tcqu with 34.2 and 7.5 t ha^{-1} yr^{-1}, respectively. Regarding stem, the maximum fresh and dry biomass of 18.3 and 5.81 t ha^{-1} $yr^{-1}$was produced by Tcqu (Table 2.5). The wet biomass is close to those reported by Nunez, Rodriguez and Khanna (2010) for *A. tequilana* (12–28 t ha^{-1} yr^{-1}) grown in Jalisco, Mexico, in soils with high oxides and potassium, good drainage and exposure to sunlight. Similar to leaves, the cultivar with the least fresh and dry biomass of stem was L9, which produced only 2.36 and 0.73 t ha^{-1} yr^{-1} respectively. The total fresh AGB, including both leaves and stem, was highest in cultivar 17 with 57.04 t ha^{-1} yr^{-1}; however, the highest dry AGB was produced by the cultivar Tcqu with 12.85 t ha^{-1} yr^{-1}. The least fresh and dry biomass was obtained from L9 with approximately 5 and 1.6 t ha^{-1} yr^{-1} (Table 2.5).

The cultivars L9 and E9 (marked by the red circle in Fig. 2.13) were infected with *Fusarium oxysporium* (leaf curl disease) during the early phase of growth. The infected plants showed leaf spots, and leaf drying and curling symptoms. It is possible that this infection may have contributed to their poor growth in addition to their low genetic potential.

Amongst the six cultivars, the three cultivars that produced high dry AGB were Tcqu, 17 and L19, which yielded 12.9, 11.7 and 10.3 t ha^{-1} yr^{-1}, respectively. These results are comparable to another trial established by AusAgave at Kalamia in northern Queensland on a very fertile and high rainfall area. The AGB of plants at Kalamia was 14 t ha^{-1} yr^{-1} when planted at a planting density of 1900 plants ha^{-1} (pers comm. Don Chambers, 2016).

Comparison of the dry biomass of different cultivars of *A. tequilana* with those of other *Agave* species, reveal that the maximum fresh biomass of *A. tequilana* was low (about 25%), but the dry AGB was similar. In the current study, the species with maximum fresh AGB did not necessarily produced the maximum dry AGB. This presumably represents variation in structural and non-structural carbohydrates and lignin content (Arrizon et al. 2010; Corbin et al. 2015; Li et al. 2012b).

Figure 2.13: The agave plot at 41 MAP (February 2014) showing cultivars L9 and E9 which were affected by the fungus *Fusarium oxysporium*.

The dry AGB for *A. tequilana* obtained in the current study (12.9 t ha^{-1} yr^{-1}) (Table 2.5), is markedly lower than that reported in literature (25 t ha^{-1} yr^{-1}) (Nobel 2003). The AGB at 58 MAP (~5 yr) of the best performing cultivar Tcqu was only 50% of the value reported by Nobel (2003). Although the climatic conditions are similar between Rockhampton and Mexico (Chambers & Holtum 2010), the productivity was low in Rockhampton, even with higher planting density (~6700 plants ha^{-1}), as compared to 3000–3300 plants ha^{-1} in most *A. tequilana* plantations in Mexico (Nobel & Valenzuela 1987a; Nunez, Rodriguez & Khanna 2010).

Table 2.5: Above ground biomass (AGB) production of various cultivars of *Agave tequilana.* Along the row, means sharing the same superscript do not differ significantly (P <0.05).

	17	E9	L19	L3	L9	Tcqu	l.s.d	P value
Leaf FW* (t ha^{-1} yr^{-1})	39.24^{b}	26.54^{b}	34.2^{b}	27.67^{b}	2.62^{a}	34.2^{b}	13.56	<0.001
Leaf DW (t ha^{-1} yr^{-1})	7.64^{c}	4.86^{b}	6.61^{bc}	5.18^{bc}	0.85^{a}	7.05^{bc}	2.51	<0.001
Stem FW (t ha^{-1} yr^{-1})	17.8^{b}	11.88^{b}	15.95^{b}	13.5^{b}	2.36^{a}	18.32^{b}	6.09	<0.001
Stem DW (t ha^{-1} yr^{-1})	4.1b^{c}	3.07^{b}	3.69^{b}	3.41^{b}	0.73^{a}	5.81^{c}	1.82	<0.001
AGB FW (t ha^{-1} yr^{-1})	57.04^{b}	38.42^{b}	50.15^{b}	41.18^{b}	4.97^{a}	52.52^{b}	18.61	<0.001
AGB DW (t ha^{-1} yr^{-1})	11.74^{bc}	7.93^{b}	10.3^{bc}	8.59^{bc}	1.58^{a}	12.85^{c}	1.94	<0.001
FW:DW ratio	4.85	4.84	4.86	4.79	3.14	4.08		

***FW; fresh weight, DW; dry weight**

The current agave trial was established on a black cracking clayey soil which may not have been optimal for *Agave* species as it is widely grown in well-drained volcanic and red Cambisols, Luvisols and Lithosols in Mexico (Holtum et al. 2010). Agaves are reported to prefer a slight acidic pH (Holtum et al. 2010). The soil pH (1:5 H_2O) in the current study was neutral to slightly alkaline (6.7–7.3) (Table 2.6). Based on the recommendations of Horneck et al. (2011) and Hughes et al. (2016), the soil used in this study had the following features; soil nitrate nitrogen ranged from low to normal (3–11 mg kg^{-1}), Colwell phosphorus was low (14–19 mg kg^{-1}) and Colwell potassium ranged from low to marginal (109–155 mg kg^{-1}). The sulphur content (21.8–35.8 mg kg^{-1}), Pentetic or diethylenetriaminepentaacetic acid (DTPA) extractable manganese and chloride, and exchangeable magnesium and calcium were high while the exchangeable potassium was low compared to the normal range (Horneck et al. 2011; Hughes et al. 2016). The chloride levels were medium to excessive (17.7–130.1) (Table 2.6). Comparing the nutrient levels of the present soil to that of the optimal level required for agaves for nitrogen, phosphorus, potassium and boron, the levels of phosphorus and potassium were lower than the required range, and those of nitrogen and boron were in the recommended range (Nobel 1989).

Table 2.6: Physico-chemical properties of the soil collected from *Agave* field site at Rockhampton 21 MAP (June 2011). Bold numbers indicate either high or low levels than the normal range.

Site	R1F	R1R	R2F	R2R	R3F	R3R	Soil interpretation guideline (Horneck et al. 2011; Hughes et al. 2016; Reid & Dirou 2004)	Nutrient index for agaves (Nobel 1989)
Ammonium nitrogen (mg kg^{-1})	21	18	17	20	17	26	2–10 (preferred level)	3
Nitrate nitrogen (mg kg^{1})	**3**	9	11	**3**	**3**	**3**	<4 low, 4–8 marginal, 9–12 adequate, 13–20 high, >20 very high	
Phosphorus: Colwell (mg kg^{-1})	14	17	19	18	15	17	20–100	60
Potassium: Colwell (mg kg^{-1})	**115**	131	155	126	159	**109**	<120 low, 120–180 marginal, 181–300 adequate	250
Sulphur (mg kg^{-1})	30.5	34.3	34.3	29.1	21.8	35.8	<2 Low, 2–10 medium, >10 Sufficient	
Copper; DTPA (mg kg^{-1})	**4**	**4.08**	**3.99**	**4.02**	**4.31**	**4.61**	0.1–0.3 low, 0.6–1 sufficient, >1 high	
Iron; DTPA (mg kg^{-1})	53.9	48.7	61.8	59.5	**129.7**	70.6	5–10 low, 11–69 medium, >70 high	
Manganese; DTPA (mg kg^{-1})	**51.2**	**51.8**	**53.7**	**44.5**	**73.9**	**62.9**	<1 low, 1–9 medium, >10 high	
DTPA Zinc (mg kg^{-1})	3.23	6.1	5.13	1.95	5.07	5.79	>1.5 sufficient	
Boron; hot $CaCl_2$ (mg kg^{1})	0.53	**0.44**	0.61	0.6	0.95	0.88	0.2– 0.5 low, 0.5–1 medium, 1–2 high	1
Chloride (mg kg^{-1})	**27.4**	**17.7**	**46**	**59.2**	**110.6**	**130.1**	0–5 very low, 5–10 low, 10–20 medium, 20-50 high, >50 excessive	
Exc. Aluminium ($meq100g^{-1}$)	<.001	<.001	<.001	<.001	<.001	<.001		
Exc. Calcium (meq $100g^{1}$)	**17.17**	**20.11**	**17.26**	**22.1**	**14.75**	**17.65**	<5 low, 5–10 medium, >10 high	
Exc. Magnesium ($meq100g^{-1}$)	**8.07**	**6.22**	**7.21**	**7.34**	**9.11**	**8.84**	<0.5 low, 0.5–2.5 medium, >2.5 high	
Exc. Potassium ($meq100g^{-1}$)	0.26	0.33	0.28	0.39	0.28	0.39	<0.4 low, 0.4–0.6 medium, 0.6–2 high	

Site	R1F	R1R	R2F	R2R	R3F	R3R	Soil interpretation guideline (Horneck et al. 2011; Hughes et al. 2016; Reid & Dirou 2004)	Nutrient index for agaves (Nobel 1989)
Exc. Sodium (meq100g^{-1})	**1.26**	0.55	**1.81**	0.93	**3.2**	**2.27**	<1.0 (preferred level)	
Exc. sodium (mg100g^{-1})	28.98	12.65	41.63	21.39	73.6	52.21		150
Organic Carbon (%)	**1.96**	**1.8**	**1.82**	2.18	**1.82**	2.51	>2% (preferred level)	
Conductivity (dS m^{-1}; 1:5 H_2O)	0.217	0.211	0.258	0.21	0.332	0.278	<0.15 dS m^{-1}	
pH (1:5 $CaCl_2$)	6.1	6.3	6.3	6.5	6.3	6.5	5.0–5.5 (preferred level)	
pH (l:5 H_2O)	6.7	6.8	7	7.1	7	7.3	<5.4 acidic, 5.5–6.4 moderately acidic, 6.5–6.9 slightly acidic; 7 neutral, 7.1–7.5 slightly alkaline, 7.6–8.3 moderately alkaline, >8.4 strongly alkaline	

Note: R1, R2 and R3 represents three sample locations (top, middle and bottom) of the experimental sites, where R1 = rows 1–5, R2 = rows 6–10 and R3 = Rows 11–15 in the field (See Appendix 2); F = furrow, R = ridge.

2.4 Species Selection

The growth and productivity of *F. foetida* and ten *Agave* spp., including six cultivars of *A. tequilana,* were assessed at Rockhampton. *Furcraea foetida* produced the highest dry AGB (leaves+stem) (13.4 t ha^{-1} yr^{-1}). This species also produced flowering stocks, and if these were included, its performance will be much more superior to other species such as *A. sisalana, A. decipiens* and *A. tequilana.* The next highest dry AGB were produced by *A. decipiens* followed by *A. tequilana* and *A. sisalana.*

Of all 11 genotypes tested, the following were found highly suitable for establishment as biofuel feedstocks, as they were ranked as follows for their above ground biomass production. The species ranking was (*A. foetida ~ A. decipiens ~ A. sisalana*) >(*A. americana ~ A. tequilana*).

Of the six cultivars of *A. tequilana* trialled, Tcqu, 17 and L19 produced the highest numbers of unfolding leaves (>30) and >10.3 tons of dry biomass per year. These cultivars should be further evaluated as potential feedstocks, not only for bioethanol but also for other forms of biofuel (pyrolysis or gasification).

Of all the genotypes evaluated, *A. tequilana* was selected for further investigation, as this species is reported to accumulate higher concentrations of soluble sugars in its stems (Cedeño Cruz & Alvarez-Jacobs 1999), and the technology for converting this sugar into bioethanol is very well understood (Hernández-Salas et al. 2009; Mielenz et al. 2015).

Agave tequilana had an annual leaf unfolding of 30–32 leaves. These leaves can be harvested after two years of establishment at regular intervals, possibly once every 6 months. The final harvest of both leaf and stem can be done in five years. This way approximately 40% of the leaves that would have died during plant maturity can be utilised as a feedstock. In addition, the regular harvesting of leaves would allow more sugars to be accumulated in the stem, leading to an increase in bioethanol production (Nobel 2003). Until now, only regular pruning of the leaves is being practiced in Agave production and this is done to enhance the growth of stem or piña for Tequila production with manual labour. It is not yet known if periodic harvesting of the leaves using mechanical harvesters is feasible and this would be a new area of research for Agave.

The two best performing cultivars of *A. tequilana* (Tcqu and L19) that were identified in this study were chosen for further assessment of their chemical compositions in context of bioethanol production (Chapters 3 and 4), and in terms of leaf processing options for fuel ethanol production (Chapter 5).

Chapter 3. Quality estimation of *Agave tequilana* leaf for bioethanol production[1]

Abstract

Agave tequilana is a potential biofuel crop, for which the characters of juice total soluble sugar contents (TSS), dry matter content (DM), cellulose, hemicellulose and lignin content are quality criteria. Spectra of leaves were obtained using a hand-held portable near infrared spectrometer with a wavelength range 300–1100 nm and a FTNIR spectrometer with a wavelength range 1100–2500 nm. Fresh leaves were harvested at different maturity stages, in different seasons and from two locations in Queensland during 2012-2014. Partial Least Square Regression models were developed for DM and TSS of fresh leaf, and cellulose, hemicellulose and lignin of dried material, with models tested on a population of independent samples collected in different years, seasons and locations. Prediction statistics for DM using SWNIR were r^2 = 0.49–0.87, RMSEP = 2.36–1.44 %, and for FTNIR were r^2 = 0.53–0.66, RMSEP = 2.63–2.18% (across different years, seasons and locations).

Prediction statistics for TSS using SWNIR were r^2 = 0.53–0.69, RMSEP = 1.70–1.91. With increased sample diversity in the calibration set, NIR technology could be used in estimation of DM and TSS in fresh *Agave* leaves.

Key words: *Agave tequilana*, Total soluble solids, Dry matter, FTNIR, SWNIR, cellulose, hemicellulose and lignin.

[1] This chapter is an unabridged version of a revised manuscript submitted to JNIRS for publication. To strengthen this publication some results on leaf compositional analysis (Tables 3.10 and 3.11) were included, giving a small amount of crossover to thesis chapter 4.

3.1 Introduction

The stem of *Agave tequilana* is used in production of alcoholic beverage (Tequila^{TM}), with harvest destroying the whole plant. Periodic leaf harvests after 2-3 years of planting could support bio-ethanol production (Chambers & Holtum 2010). An above ground dry biomass yield of 13 t ha^{-1} year^{-1} was achieved with an annual rainfall of 811 mm (Rockhampton, Queensland), and leaf material accounted for 55% of total biomass (Chapter 2, Table 2.4 and 2.5). Therefore, there is potential to use 7 t ha^{-1} year^{-1} leaf as feedstock for biofuel production.

The value of this leaf material for ethanol production is dependent on leaf attributes such as total soluble solids (TSS), dry matter (DM), and the sugar, cellulose, hemicellulose and lignin contents of the dry matter. For example, we have observed *Agave* leaf TSS to vary from 3.8 to 17.3% and DM from 9.3 to 21.9% in 1.5 to 4-year-old plants. DM is an index of structural and non-structural carbohydrates and thus an approximation of fermentable material, with composition varying with age and environmental conditions. The timing of harvest of agave leaves for bioethanol production should be guided by field measurement of leaf DM and/or TSS, and/or laboratory measurement of dry matter cellulose, hemicellulose and lignin. To influence the agronomic decision on when to harvest, a rapid assessment of these attributes is required.

There have been numerous reports on the use of NIR spectroscopy to predict DM of agricultural products (Ben-Gera & Norris 1968; Cen & He 2007; Kelley et al. 2004b; Norris & Hart 1996) and TSS and DM of fruit (Subedi, Walsh & Owens 2007; Walsh, Golic & Greensill 2004). Indeed, a Scopus search on the key words NIR and dry matter for the period 2000 to 2015 returned 140 results. Similarly, NIR spectroscopy has been used in estimation of glucans (cellulose), xylan (hemicellulose) and lignin (e.g., rice straw, agricultural residues, wood) (Jin & Chen 2007; Downes et al. 2012; Jones et al. 2006; Kelley et al. 2004b). These applications are based on the O-H absorption features found at 1940, 1440 and 960 nm, and C-H features between 1690–1755, 1127–1170 and 910 nm. (Workman 2014). However, the development of a given application requires consideration of appropriate instrumentation. For example, scanning grating technology coupled to silicon and lead sulphide detector technology (e.g., Foss NIRSystems 6500) has been a workhorse ‘standard’ of the NIR agricultural community for assessment of dried and ground samples, offering a wide wavelength range (400–2500 nm) and high

signal to noise. FTNIR offers the advantage of higher wavelength resolution than can be achieved with dispersive technologies, a feature that is suited to some applications (e.g., the presence of sugars on cellulose cotton fibres) (Barton et al. 2005). However, due to the strong absorption of longer wavelengths by water, assessment of intact biological products is generally undertaken using the wavelength range below 1100 nm (short wave NIR or SWNIR). Instruments operating in this wavelength range are generally smaller and less expensive, features suited to an application that involves field assessments.

In the current study, NIR spectroscopy is applied to fresh intact leaves for the estimation of DM and TSS and dried, ground leaf material for the estimation of cellulose, hemi-cellulose and lignin contents. A comparison is made of the suitability of FTNIR (1000–2500 nm) and portable silicon photodiode array (300–1100 nm) instrumentation for the measurement of DM and TSS in intact leaf, and FTNIR and scanning grating (400–2500 nm) NIR technologies for assessment of cellulose, hemicellulose and lignin in ground *Agave* leaf samples.

3.2 Materials and Methods

3.2.1 Plant material

Plants of two cultivars (Tcqu and L19) of *A. tequilana* were established in Rockhampton, Queensland (23.32°S, 150.52°E) in September 2010 and at Kalamia, Queensland (19.58°S, 147.41°E) in June 2009. Four leaves at the same whorl (rosette) of approximately same size and age (3 months) were tagged on each of 20 plants (10 from each cultivar Tcqu and L19) at the Rockhampton site, with sampling of one leaf per plant on four occasions from May 2012 through to August 2013. A total of 42 leaf samples were also collected at Kalamia (Ayr, Queensland) in February 2013 and May 2014.

3.2.2 Spectral acquisition of intact leaf

In summary, spectra were acquired of intact leaves at three locations on each leaf (base, middle and tip), following which the leaves were dried and ground and re-scanned. The ground material was solvent extracted, re-dried and rescanned. Two spectrophotometers were compared for both intact leaf and ground material, as described below.

Spectra (absorbance and interpolated second derivative) were obtained of intact leaves using a portable silicon photodiode instrument (“Nirvana” spectrophotometer, Integrated

Spectronics, Sydney Australia) and a Fourier Transform Near Infrared (FTNIR) instrument (Nicolet Antaris Near IR Analyzer, Thermo, USA). The silicon photodiode unit employed a Zeiss MMS1 spectrometer and a tungsten halogen lamp in an interactance geometry (the 'shadow probe' geometry described by Greensill and Walsh (2000)), collecting spectra between 300 nm to 1100 nm at approximately 3.3 nm steps. In this instrument, references (dark and white) are taken associated with every sample measurement. FTNIR spectra were acquired using the fibre optic interactance probe accessory and the unit was operated over the range 1000–2500 nm at a spectral resolution of 8.0 cm^{-1} with 64 scans per sample, with a background spectrum (averaged from 64 scans) taken using the in-built polytetrafluoroethylene (PTFE) tile every 4 hours.

3.2.3 Determination of DM and TSS

A 3 cm diameter core of leaf was taken using a cylindrical corer at the point of spectra acquisition and dried to constant weight at 65^{o}C for DM estimation. For cultivar L19 at Rockhampton, a second (adjacent) scanned area was also cored; with juice extracted using a garlic press and TSS assessed using a refractometer (DFM 320, Bellingham and Stanley).

3.2.4 Spectral acquisition of dried, ground leaf

The remaining portions of the leaves were cut into approximately 2 cm pieces and dried in an oven at 65°C until constant weight was obtained. Dried material was ground to pass a 530 µm sieve using a laboratory grinder (Mikro Feinmuhle Culatti, Janke and Kunkel GmbH and Co, Staufen, Germany). Powdered samples (approx. 5 g) were loaded into quartz spinning cups and reflectance spectra recorded using a scanning grating NIR spectrometer (NIRSystems model 6500, FOSS, VIC, Australia) using the spinning cup accessory, with averaging of 32 scans per spectra. The sample was then transferred to an Antares spinning cup and spectra acquired with the Antaris FTNIR unit (described above).

Sample material was then subjected to Soxhlet extraction using boiling water (Milli Q, http://www.emdmillipore.com/Milli_Q) for 12 hours (Sluiter et al. 2008a). Once dried (to constant weight at 65°C), Soxhlet extracted samples were scanned again using the FTNIR (Antaris) and scanning grating (NIRS 6500) units.

3.2.5 Determination of cellulose, hemicellulose and lignin

Soxhlet extracted samples (10 g DW, n=104) were analysed for cellulose, hemicellulose and lignin following the National Renewable Laboratory Protocol (NREL) (Sluiter et al. 2008b). The procedure entailed mixing 300 mg of sample (in triplicate) with 3 mL 72% v/v H_2SO_4 and incubation in a water bath at 30°C for 60 min, stirring every 15 min to avoid sample clumping. The samples were then diluted to 4% v/v H_2SO_4 with 84 mL pure water (Milli-Q, www.emdmillipore.com/Milli_Q) and then autoclaved at 121°C for 60 min. Following this step, the acid insoluble residue (AIR) was collected by filtration through pre-weighed glass fibre filter paper using a Buchner funnel. The filtrate was retained for acid soluble lignin (ASL) and carbohydrate (cellulose and hemicellulose) analysis. Cellulose and hemicellulose was determined by High Performance Liquid Chromatography (HPLC, Agilent 1100, Agilent technologies Australia Pty Ltd, VIC, Australia). The AIR was washed with approximately 50 mL Milli-Q water, oven dried at 100°C for 24 hours and then placed in a muffle furnace in crucible at 575°C for 24 hours for determination of ash content. The lignin content of the ASL fraction was estimated from absorbance over the wavelength range 190–300 nm using a UV-Visible spectrophotometer (Cary 50 Bio, Varian Inc., Agilent Santa Clara, CA, USA) following the method of Sluiter, et al.(2008a). Cellulose and hemicellulose derived sugars were identified using an Agilent 1100 HPLC equipped with a thermostated column compartment (operated at 60°C) and a refractive index detector (Agilent 1260 Infinity) maintained at 50°C. A monosaccharide (7.8 x 300 mm) column was used in conjunction with a Carbo-H guard cartridge pre-column (Rezex, Phenomenex, Torrance, CA, USA). Samples and standards were eluted with an isocratic mobile phase consisting of degassed dilute H_2SO_4 (0.005 N H_2SO_4 prepared in pure water). The flow rate was maintained at 0.6 mL min^{-1}. Glucose, xylose, arabinose and fructose standards of analytical grade (Sigma Aldrich; http://www.sigmaaldrich.com) were run.

3.2.6 Chemometrics

Spectra were processed using The Unscrambler software 10.3 v (CAMO, Oslo, Norway) to develop Non-linear Iterative Partial Least Squares (NIPALS) regression calibration models. Absorbance spectra were pre-processed using a Savitzky-Golay second order polynomial second derivative (over 9 data points (4 points on left and 4 points on right) for SWNIR and 55 points (27 points on right and 27 points on left) for FTNIR). The

Partial Least Square Regression (PLSR) models were assessed in terms of cross validation coefficient of determination (R^2), root mean square error of cross-validation (RMSECV), population mean ($\bar{X}$), standard deviation (SD), the number of principle components (PC's) and slope and bias. Full leave one out cross validation was employed. The calibration models were also tested on sample sets which were not included in the calibration to test the model performance, with results reported in terms of coefficient of determination (r^2), root mean square error of prediction (RMSEP), bias and slope.
A minimal model performance of ($R^2 = 0.76$) was considered as the lowest acceptable range for a model to be used for prediction, as this represents a standard deviation to RMSECV ratio of 2.0, assuming negligible bias, from the relationship:

$R^2 = 1 – (SEP/SD)^2$, where SEP is bias corrected RMSEP

The optimal wavelength region for model development was determined using a Matlab 12.b program using PLS Toolbox 7.5 (Wenatchee, WA: http://www.eigenvector.com/) as described by Guthrie et al 2005. This program exhaustively created models varying in start and end wavelength by 3 nm steps, with model performance compared in terms of RMSECV.

3.3 Results and Discussion

3.3.1 Reference (laboratory) method

The reference values for dry matter, total soluble solids, cellulose, hemicellulose and lignin of populations used for calibration and validation are listed in Table 3.1. The optically sampled volume of tissue inherent in the collection of spectra can only be approximately estimated and thus there will be sampling error associated with tissue sampled for destructive analysis of composition. Further, there is some repeatability error associated with the reference analysis. For Brix the repeatability of the refractometer assessment of juice is approximately 0.1 %, but error in sampling and juice extraction increases this error. For DM determination, errors are associated with weighing and effectiveness of water removal by the oven, with a typical RMSE of 0.4% (data not shown). Error in laboratory estimates of cellulose, hemicellulose and lignin has not been quantified.

Table 3.1: Summary statistics of dry matter (% DM w/w), total soluble solids (TSS) (° Brix) of fresh *A. tequilana* leaves and cellulose, hemicellulose, and lignin compositions (% w/w) of dried ground leaves of *A. tequilana* cultivars (Tcqu and L-19) from May 2012–August 2013.

Attribute	Site	sample no.	Population	Min	Max	Average	SD
TSS **(% w/v)**	Rockhampton	60	May-12	6.3	13.7	9.7	2.51
		60	Oct-12	8.6	17.3	12.2	2.32
		60	Nov-12	7.3	15	11.2	1.98
		60	Mar-13	5.8	12.4	9.3	1.86
		60	Aug-13	5.7	15.0	9.5	2.45
	Kalamia	180	Feb-13	3.8	15.0	8.7	2.13
		72	May-14	4.6	14.8	8.0	2.72
DM **(% w/dw)**	Rockhampton	60	May-12	10.8	17.6	13.9	2.34
		60	Oct-12	12.4	19.4	16.1	2.0
		60	Nov-12	12.8	19.8	16.3	2.02
		60	Mar-13	13.41	19.9	17.3	1.50
		60	Aug-13	11.5	20.2	16.1	1.92
	Kalamia	180	Feb-13	9.7	20.8	15.7	2.50
		72	May-14	9.4	20.8	14.4	3.45
Cellulose **(% w/dw)**	Rockhampton	80	May-12	29.8	38.6	34.7	2.67
	A. tequilana (Tcqu)	80	Nov-12	30.4	37.0	33.5	1.74
		80	Mar-13	26.9	35.7	31.2	2.76
		80	Aug-13	24.7	35.6	29.9	3.55
	A. tequilana (L-19)	24	May-12	25.5	31.8	29.7	2.77
		24	Nov-12	21.7	29.1	25.3	2.89
		24	Mar-13	23.9	27.1	25.1	1.25
		24	Aug-13	23.5	26.0	24.7	0.97
Hemicellulose **(% w/dw)**	*A. tequilana* (Tcqu)	80	May-12	13.3	16.4	15.0	0.94
		80	Nov-12	13.8	16.4	15.2	0.79
		80	Mar-13	10.3	13.5	11.8	1.00
		80	Aug-13	9.9	15.8	13.5	1.94
	A .tequilana (L-19)	24	May-12	9.3	13.7	11.8	1.7
		24	Nov-12	8.9	13.1	10.7	1.42
		24	Mar-13	11.9	14.0	12.8	0.72
		24	Aug-13	12.2	15.8	13.8	1.42
Lignin **(% w/dw)**	*A. tequilana* (Tcqu)	80	May-12	14.2	17.1	15.6	1.00
		80	Nov-12	15.6	19.8	18.1	1.45
		80	Mar-13	16	21.4	19.1	1.84
		80	Aug-13	16.5	21.1	18.6	1.28
	A .tequilana (L-19)	24	May-12	14.7	17.2	16.4	0.9
		24	Nov-12	17.3	20.8	18.9	1.36
		24	Mar-13	16.2	21.8	19.5	2.15
		24	Aug-13	15.2	19.1	17.3	1.54

3.3.2 Correlation between DM and TSS

Leaf DM and TSS was assessed in May 2012, Nov 2012, Mar 2013 and August 2013. The correlation coefficient of determination (R^2) between DM and TSS for the month of May 2012 was 0.89, while in later months it decreased to 0.74, 0.55 and 0.75, respectively. Across all populations, R^2 was only 0.49. Thus, the correlation decreased with leaf maturation, presumably due to increasing deposition of cell wall components.

3.3.3 Features of spectra of intact leaf

Absorbance and second derivative of absorbance spectra of the visible-SWNIR region (300–1100 nm) collected using the Nirvana unit contained prominent features at 400 and 680 nm associated with chlorophyll, a feature at 500 nm ascribed to carotenoids, and features in the SWNIR ascribed to the second and third overtone regions of O-H stretching and the third overtone of C-H stretching (Fig. 3.1). In the extended wavelength region of spectra collected by FTNIR and scanning grating units, features associated with the first (1690–1755 nm) and second (1127–1170 nm) overtones of C-H stretching, O-H combinations at 1940 nm and first overtone at 1440 nm were apparent (Fig. 3.1) (Workman 2014).

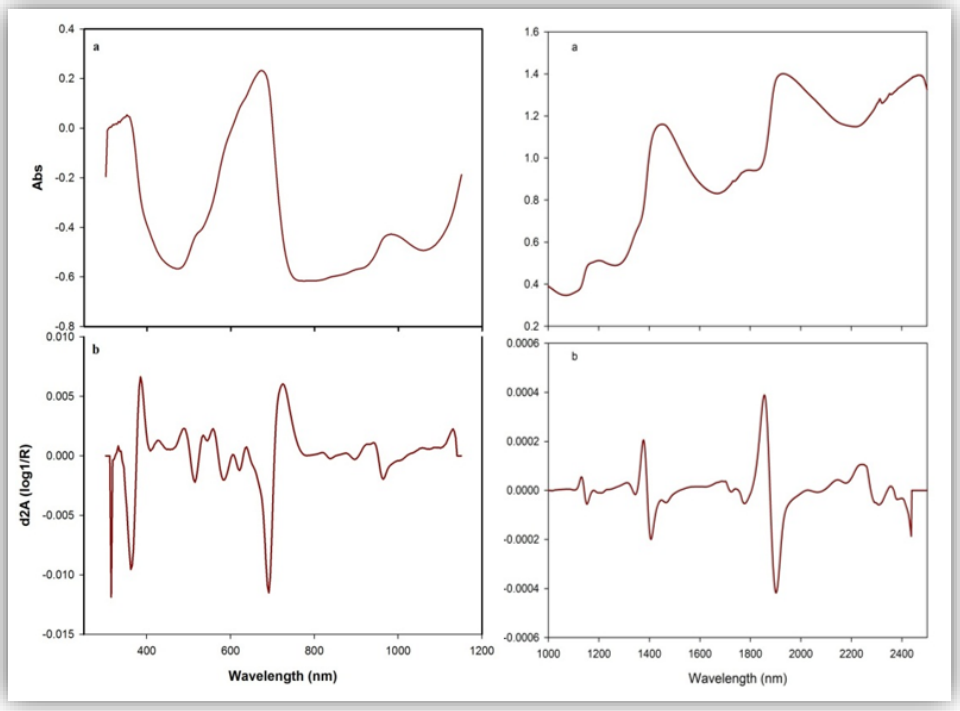


Figure 3.1: Visible-SWNIR spectra (300–1100 nm, average of n=552) (left panel) and FTNIR spectra (1000–2500 nm, average of n=432) (right panel) of fresh leaves of *A. tequilana*.

3.3.4 SWNIR—dry matter modelling for intact leaf

Partial least square regression models for dry matter were developed using the full visible SWNIR spectral range (300–1065 nm) and restricted ranges based on consideration of the level of noise in the b-coefficients of the model (Fig. 3 2). Restricting the wavelength range to 729–975 nm improved model statistics and reduced the number of PLS factors used, as did the use of second derivative pre-processing (Table 3.2). SNV pre-processing, alone or in combination with second derivative, did not improve model results. Using an exhaustive searching wavelength window approach, minimum RMSEC and RMSECV were achieved using the 782–969 nm window, followed by the 827–978 nm (Fig. 3.3), and although the result was similar to that achieved using 729–975 nm (Table 3.2). The poorest model statistics were associated with the population with the lowest SD.

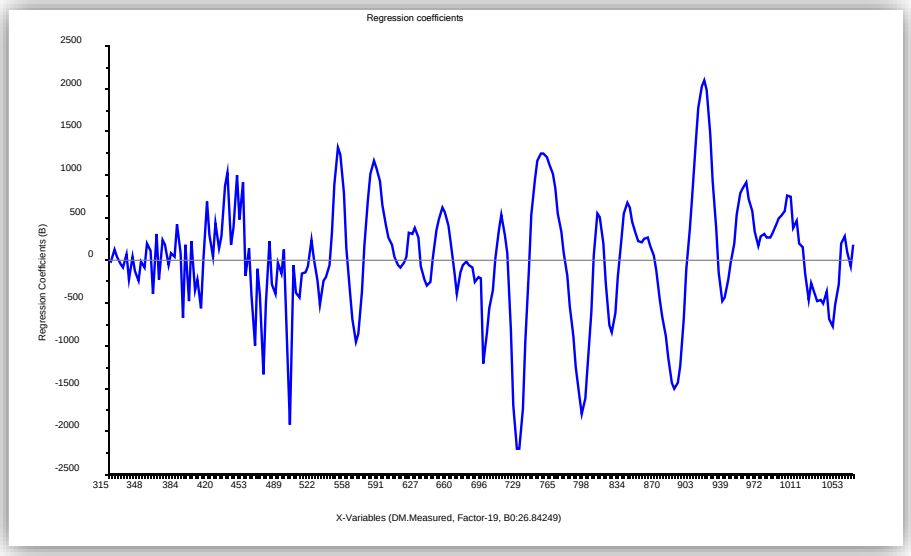


Figure 3.2: Regression coefficients a PLS model of % DM of *A. tequilana* using 300-1065 nm second derivative spectral data of Rockhampton and Kalamia populations (n=552).

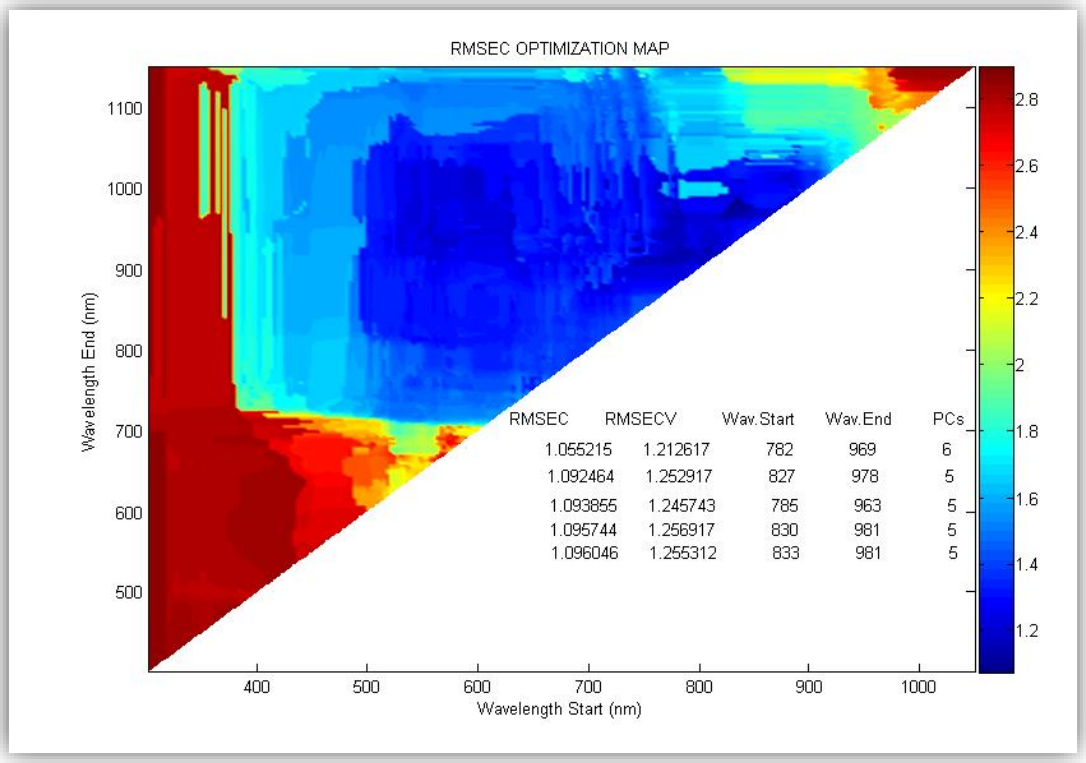


Figure 3.3: Optimisation of wavelength region used in PLSR model of dry matter estimation of fresh leaves of *A. tequilana* with presentation of RMSECV values on a colour scale index shown on the right for 300–1065 nm region (n=252). Wavelength region start wavelength on x-axis and end wavelength on y-axis.

Population SD on DM varied between 2.24 and 3.44% DM, with model statistics of R^2 and slope improving as SD increased (Table 3.2). For the population with the highest SD, model statistics of R^2 0.87, RMSECV 1.0% DM and slope 0.81 were achieved (Table 3.2). The PLS factor plot revealed some segregation of Rockhampton and Kalamia populations (Fig. 3.4).

Table 3.2: Summary statistics of PLS calibration models of percent dry matter (%DM) developed on spectra from the SWNIR regions of 300–1065 nm and 729–975 nm, of fresh leaf of *A. tequilana*. R^2 values >0.76 are shown in bold.

Sample /Range	PC	R^2	RMSECV	Slope
Kalamia 2013–2014 and Rockhampton 2012-2013, n=552, mean = 15.64, SD = 2.56				
300–1065 nm d^2A	7	0.46	1.89	0.48
729–975 nm d^2A	7	0.72	1.36	0.72
729–975 snv+d^2A	8	0.71	1.38	0.72
729–975 nm Abs	9	0.69	1.40	0.71
Kalamia 2013 and 2014, n=252, mean = 15.31, SD = 2.86				
729–975 nm d^2A	9	**0.82**	1.17	0.84
Kalamia 2013, n=180, mean = 15.61, SD = 2.48				
729–975 nm d^2A	9	**0.79**	1.13	0.81
Kalamia 2014, n=72, mean = 14.39, SD = 3.44				
729–975 nm d^2A	4	**0.92**	1.0	0.92
Rockhampton 2012–2013, n=300, mean = 15.91, SD = 2.44				
729–975 nm d^2A	8	0.64	1.35	0.66

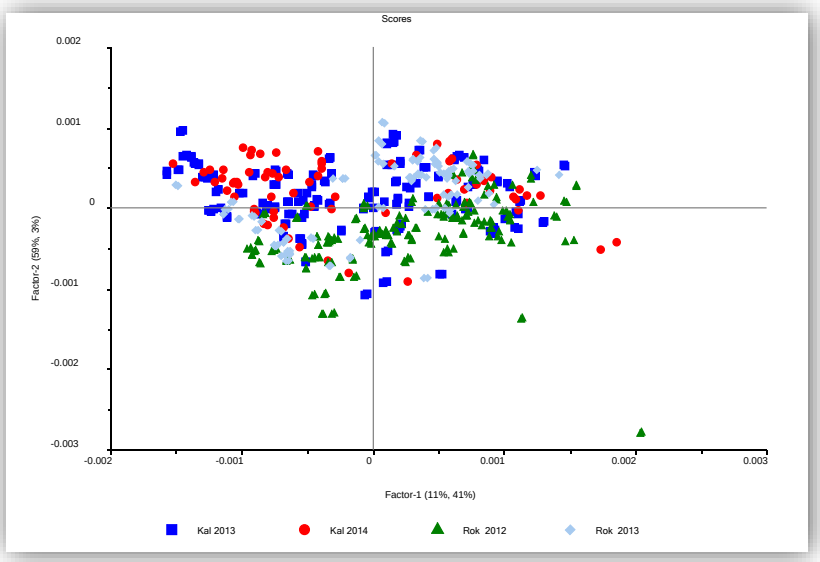


Figure 3.4: PLS factor plot for a % DM model based on SWNIR d2A spectra (729–975 nm) of samples from Rockhampton and Kalamia (n=552 samples).

Models developed using the 729–975 and 782–969 nm regions were used in prediction of populations not included in the calibration sets (Table 3.3). The Kalamia 2014 samples were predicted well (r^2 = 0.87) using a model based on Kalamia 2013 samples. However, prediction performance was poorer (r^2 = 0.49–0.50) when the Kalamia 2014 model was used for prediction of Kalamia 2013 samples, a result at least in part ascribed to the lower

SD of this group. For prediction involving populations from different growing locations, an r^2 of 0.64–0.66 was achieved. Similar results were achieved using either wavelength region (Table 3.3). In conjunction with the PLS factor plots, these prediction results indicate the need to include more sample variability, by inclusion of samples from further seasons, sites and growing conditions, to base a robust PLS model.

Table 3.3: Prediction of percent dry matter of using the PLS calibration models (d^2A) of fresh *A. tequilana* leaves grown within the same location (Kalamia, Qld) and different locations (Rockhampton and Kalamia, Qld). r^2 values >0.76 are shown in bold.

Populations and wavelength range	r^2	RMSEP	Bias	Slope
Kal 14 using Kal 13, n=72, mean = 14.39, SD = 3.44 % DM				
729–975nm	**0.87**	1.49	0.76	0.77
782–969 nm	**0.87**	1.50	0.92	0.81
Kal 13 using Kal14, n=180, mean = 15.61, SD = 2.48 % DM				
729–975 nm	0.50	2.31	-0.97	0.84
782–969 nm	0.49	2.32	-1.02	0.90
Kal (13 and 14) using Rok 12 and 13, n=252, mean = 15.31, SD = 2.85 % DM				
729–975 nm	0.66	1.84	-0.34	0.45
782–969 nm	0.64	1.94	-0.27	0.39

3.3.5 FTNIR—dry matter modelling for intact leaf

Partial least square regression models on dry matter were developed using the full FTNIR spectral range (1000–2500 nm) and a restricted range (1087–2035 nm) based on consideration of the level of noise in the b-coefficients of the model (data not shown). The use of the restricted wavelength range improved model statistics and reduced the number of PLS factors used (Table 3.4). Spectral pre-processing techniques (d^2A and d^2A + SNV) improved the model statistics to some extent (Table 3.4). For example, for a model based on second derivative absorbance spectra of the Kalamia 2013+2014 population, model cross validation (coefficient of determination) R^2 was improved from 0.58 to 0.69 with use of the restricted range (Table 3.4). Best model calibration statistics were achieved with the population of highest SD (Kalamia 2014) using second derivative spectra (R^2 = 0.81, RMSECV = 1.49% DM).

Table 3.4: Summary statistics of PLS calibration models of percent dry matter (% DM) developed on NIR region (1000–2500 nm) and (1087–2035 nm) log (1/R) spectra on fresh leaf of *A .tequilana* using FTNIR. Full cross validation results are reported. R^2 values >0.76 are shown in bold.

Sample /Range	Elements	Outlier	PC	R^2	RMSECV	Slope
Kalamia 2013 and 14 (mean 15.31, SD 2.86)						
1000–2500 nm (Abs)	252	0	11	0.58	1.87	0.62
1000–2500 nm (d^2A)	252	0	11	0.62	1.70	0.67
1000–2500 nm (SNV+d_2A)	251	1	11	0.64	1.73	0.74
1087–2035 nm (d^2A)	252	0	14	0.69	1.60	0.74
Kalamia 2013 (mean 15.67, SD 2.53)						
1000–2500 nm (d^2A)	180	0	13	0.49	1.82	0.57
1087–2035 nm (d^2A)	180	0	13	0.59	1.61	0.67
Kalamia 2014 (mean 14.39, SD 3.44)						
1000–2500 nm (d^2A)	72	0	9	**0.81**	1.54	0.85
1087–2035 nm (d^2A)	72	0	7	**0.81**	1.49	0.82
Rockhampton 2012 and 2013 (mean 15.68, SD 2.32)						
1000–2500 nm (d^2A)	177	3	12	0.58	1.48	0.65
1087–2035 nm (d^2A)	177	3	10	0.55	1.58	0.61

Models developed using the 1000–2500 and 1087–2035 nm regions were used in prediction of populations not included in the calibration sets, with equivalent results achieved using either range for the Kalamia populations (Table 3.5). The prediction of Kalamia 2014 samples using the Kalamia 2013 model gave a poorer result than that achieved using SWNIR (r^2 = 0.66 compared to 0.87). These results again indicate a need to include a greater range of samples (growing conditions) in the PLS calibration set to achieve a more robust prediction performance. The SWNIR instrument is recommended for this application over the FTNIR.

Table 3.5: Prediction of percent dry matter of fresh leaf of *A. tequilana* using the PLSR models developed in FTNIR (log 1/R) at different wavelength regions.

Predicted	PC	r^2	RMSEP	Bias	Slope
Kalamia 2014 using Kalamia 2013, n=72, mean = 13.39, SD = 3.44					
1000–2500 nm	13	0.66	2.18	0.73	0.54
1087–2035 nm	13	0.66	3.79	3.21	0.63
Rockhampton 2012–13 using Kalmia 2013–14, n=129 mean = 15.68, SD = 2.32					
1000–2500nm	11	0.53	2.63	2.37	0.64

3.3.6 SWNIR—TSS modelling for intact leaf

A similar approach was followed for analysis of the TSS data sets. The pre-processing of absorbance spectra using d^2A and SNV supported a small improvement in model statistics, with no notable differences between these treatments or their combination (data not shown), Similar results were obtained for several wavelength regions in the SWNIR, so for convenience the wavelength range utilised in the work on DM (729–975 nm) is reported here (Table 3.6). As DM and TSS are correlated, it is possible that a PLSR model based on spectra is not differentiating between these two attributes. However, even when the same wavelength range was used, the weighting of the b coefficients was different for the two models (data not shown). Therefore, further work was based on the 729–975 nm region, with d^2A pre-processing. As expected, the best PLSR model R^2 (0.81) was achieved for the population of highest SD (2.71% TSS), with RMSECV of 1.18% TSS (Table 3.6). A PLS factor plot demonstrated some separation of the Rockhampton 2012–2013 and Kalamia 2013 and 2014 populations (Fig. 3.5). This separation indicates that a single site or season model is likely to perform poorly in prediction of other sites or seasons.

Table 3.6: SWNIR – TSS: Summary statistics of PLS calibration models of percent total soluble solids (% TSS) developed using 729–975 nm with d^2A and d^2A+SNV spectra of fresh leaves of *A. tequilana*. Full cross validation results are reported. R^2 values >0.76 are bolded.

Instrument/Range	PC	R^2	RMSECV	Slope
Kalamia 2013–2014 and Rockhampton2012–2013, n=552, Mean = 9.52, SD = 2.5% TSS				
d^2A	9	0.69	1.46	0.69
SNV+d^2A	12	0.66	1.51	0.70
Kalamia 2013–2014, n=252, mean = 8.52, SD = 2.33% TSS				
d^2A	9	0.71	1.27	0.73
Kalamia 2013, n=180, mean = 8.73, SD = 2.13% TSS				
d^2A	9	0.67	1.22	0.71
Kalamia 2014, n=72, mean = 8.00, SD = 2.71% TSS				
d^2A	4	**0.81**	1.18	0.82
Rockhampton 2012–2013, n=300, mean = 10.36, SD = 2.49% TSS				
d^2A	10	0.66	1.46	0.69

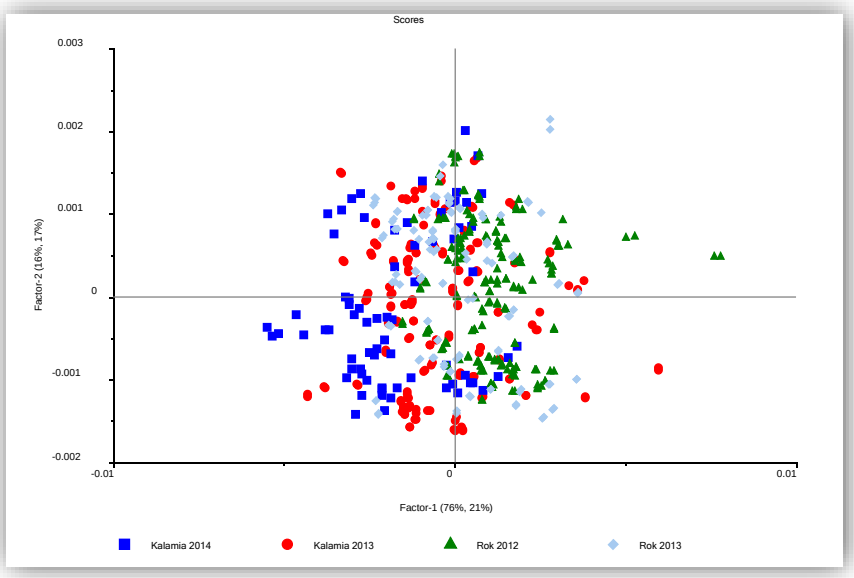


Figure 3.5: PLS factor plot from combined population TSS model developed using d^2A spectra over the range 729–975 nm (n=552).

Models were used in prediction of populations not included in the calibration sets (Table 3.7). The lowest RMSEP (1.2% TSS) and highest r^2 (0.69) was achieved when populations of the same planting location and year were involved as calibration and prediction sets. Across locations and years, the result was poorer, consistent with the need to include more sample variation into the calibration set to achieve a 'robust' model. The two site-two season based model should prove more robust in performance, but requires testing on new populations.

Table 3.7: Prediction of total soluble solids (TSS) using the PLS calibration models developed on absorbance S. Golay d^2A spectra (729–975 nm) of fresh *A. tequilana* leaves grown at Kalamia and Rockhampton, Queensland. Units of mean and SD are %TSS.

Predicted	r^2	RMSEP	Bias	Slope
Kalamia 13 using Kalamia 14 **n=180, mean = 8.73 SD = 2.13**	0.27	2.12	-0.05	0.54
Kalamia 14 using Kalamia 13 **n=72, mean = 8.02, SD = 2.72**	0.69	1.91	1.19	0.66
Rockhampton 12-13 using Kalamia 13–14 **n=300, mean = 10.36, SD = 2.49**	0.53	1.70	-0.22	0.54
Kalamia 13-14 using Rockhampton 12–13 **n=252, mean = 8.52, SD = 2.33**	0.34	2.31	-0.40	0.64
Rockhampton Oct 12 using May 12 **n=44, mean = 11.65, SD = 1.89**	0.69	1.70	-1.308	0.86
Rockhampton Aug 13 using May 12–Mar 13 **n=60, mean = 9.48, SD = 2.45**	0.64	1.99	1.35	0.67
Rockhampton Mar 13 using May 12–Nov 13 **n=55, mean = 9.4, SD = 1.87**	0.45	1.46	0.14	0.61

3.3.7 FTNIR—TSS modelling for intact leaf

Similarly, partial least square regression models on TSS were developed using the full FTNIR spectral range (1000–2500 nm) and a restricted range (1136–1851 and 1107–2150 nm) based on consideration of the level of noise in the b-coefficients of the model (data not shown). Slightly better results were obtained with use of the 1107–2150 nm range, and with the use of second derivative spectra (Table 3.8). Overall, results were similar to or slightly inferior to that obtained using SWNIR. The highest R^2 for a PLSR model based on FTNIR data was that of Kalamia 2014, with $R^2 = 0.72$, RMSECV 1.43 (Table 3.8).

Table 3.8: Summary statistics of PLS calibration models of percent total soluble solids (TSS) developed on NIR regions (1000–2500 and 1107–2150 nm) second derivative of log (1/R) spectra on fresh leaf of *A. tequilana* using FTNIR. Full cross validation results are reported.

Sample /Range	PC	R^2	RMSECV (%TSS)	Slope
Kalamia 2013–2014 and Rockhampton 2012–2013, n=432, mean = 9.12, SD = 2.41% TSS				
1100–2500 nm d^2A	11	0.56	1.60	0.59
1107–2150 nm d^2A	8	0.56	1.60	0.58
Kalamia 2013 and 2014, n=252, mean = 8.52, SD = 2.33% TSS				
1100–2500 nm d^2A	11	0.55	1.57	0.6
1107–2150 nm d^2A	11	0.59	1.5	0.64
Kalamia 2013, n=180, mean = 8.73, SD = 2.13% TSS				
1100–2500 nm d^2A	12	0.49	1.53	0.57
1107–2150 nm d^2A	11	0.55	1.43	0.60
Kalamia 2014, n=72, mean = 8.02, SD = 2.72% TSS				
1100–2500 nm d^2A	10	0.69	1.51	0.74
1107–2150 nm d^2A	6	0.72	1.43	0.73
Rockhampton 2012–2013, n=180, mean = 9.98, SD = 2.27% TSS				
1100–2500 nm d^2A	11	0.53	1.57	0.60
1107–2150 nm d^2A	8	0.55	1.52	0.58

Models developed using the 1107–2150 nm region were used in prediction of populations not included in the calibration sets (Table 3.9). Predictions were poor (r^2<0.56) in all cases (Table 3.9), and thus the SWNIR instrument is recommended over the FTNIR for this application.

Table 3.9: Prediction of TSS (measured in %TSS) of populations using the models developed using log1/R spectra (1107-2150 nm) collected using a FTNIR instrument.

Predicted	PC	r^2	RMSEP	Bias	Slope
Kalamia 2014 using Kalamia 2103, n=72, mean = 8.02, SD = 2.72% TSS					
	11	0.56	2.45	1.52	0.76
Kalamia 2013 using Kalamia 2014 n=180, mean = 8.73, SD = 2.13% TSS					
	6	0.36	2.65	-1.94	0.53
Rockhampton 2012–13 using Kalamia 2013–14, n=180, mean = 9.98, SD = 2.27% TSS					
	11	0.33	1.88	-0.21	0.37

3.3.8 FTNIR—compositional analysis modelling for dried ground leaf

PLSR models were developed using *A. tequilana* leaf powder reflectance spectra collected using two technologies, the FTNIR and scanning grating units, in context of cellulose, hemicellulose and lignin content. Prediction results were similar for the two instruments (Chapter 4, Table 4.1). Further detail is presented for the FTNIR data only. PLSR models were also developed using both pre- and post Soxhlet extracted powdered agave leaf samples for attributes of cellulose, hemicellulose and lignin content.
The models developed using Soxhlet extracted samples were better than those based on un-extracted samples (Chapter 4, Table 4.3), a result ascribed to the removal of soluble sugars, which are spectral similarities to the components of interest.

Overall, the models developed with S. Golay (d^2A) pre-processing were comparable to those based on raw absorbance (Table 3.10). The R^2 for cellulose (0.69–0.87), hemicellulose (0.58–0.88) and lignin (0.66–0.76) were obtained for different populations and cultivars (Table 3.10).

Table 3.10: Cross validation (LOO) statistics for PLS calibration models of cellulose, hemicellulose and lignin (% dw) of Soxhlet extracted dried *A. tequilana* leaf powder. Models were developed using FTNIR spectra over the wavelength region of 1000–2500 nm. R^2 values >0.76 are shown in bold.

Sample description	Attribute	PC	R^2	RMSECV	Bias	Slope
Rok 2012 and 2013, cellulose (n=104, mean = 30.88, SD = 4.04), hemicellulose (n=102, mean = 13.5, SD = 1.88) and lignin (n=102, mean = 17.81, 1.72)						
May 12–Aug 13/Abs	cellulose	7	0.72	2.04	0.03	0.76
May 12–Aug 13/ (d^2A)	cellulose	7	0.69	2.20	0.04	0.75
May 12–Aug 13/Abs	hemicellulose	12	0.61	1.21	0.00	0.67
May 12–Aug 13/(d^2A)	hemicellulose	9	0.58	1.23	0.00	0.66
May 12–Aug 13/Abs	lignin	18	**0.76**	0.83	0.03	0.80
May 12–Aug 13/(d^2A)	lignin	10	0.66	1.01	0.03	0.71
Rok 2012, cellulose (n=52 mean = 32.5, SD = 3.68), hemicellulose (n=50, mean = 14.23, SD = 1.92) and lignin (n=50, mean = 17.03, SD = 1.73)						
Cultivar Tcqu and L19 (d^2A)	cellulose	9	**0.77**	1.79	0.20	0.85
	hemicellulose	8	**0.76**	0.96	-0.02	0.80
	lignin	8	0.67	1.06	0.10	0.78
Rok 2013, cellulose (n=52 mean = 29.27, SD = 3.750), hemicellulose (n=52, mean = 12.82, SD = 1.56) and lignin (n=52, mean=18.56, SD = 1.33)						
Cultivar Tcqu and L19 (d^2A)	cellulose	14	**0.87**	1.35	-0.16	0.85
	hemicellulose	9	**0.83**	0.83	-0.01	0.84
	lignin	12	0.74	0.69	0.01	0.82

Sample description	Attribute	PC	R^2	RMSECV	Bias	Slope
Rok 2012 - 13 cellulose (n=78 mean = 32.31, SD = 3.27), hemicellulose (n=78, mean = 13.88, SD = 1.84) and lignin (n=78, mean = 17.75, SD = 1.75)						
Cultivar Tcqu (d^2A)	cellulose	12	**0.85**	1.31	0.06	0.87
	hemicellulose	12	**0.88**	0.60	0.06	0.93
	lignin	10	**0.76**	0.87	0.01	0.81

The attributes cellulose, hemicellulose and lignin were predicted in samples of populations independent of that used for calibration. The prediction r^2 for cellulose across cultivars was poor, varying between 0.64 and 0.36, but prediction across sampling periods achieved an r^2 0.76 and 0.55 and RMSEP 2.90 and 4.41% w/dw for the predicted sets of March 2013 and August 2013, respectively (data not shown). Hemicellulose was predicted better across cultivars (r^2 0.66, RMSEP 1.51% w/dw) than across different seasons or years. Lignin was predicted poorly across different years, seasons and between cultivars, with r^2 <0.21 (Table 3.11).

Table 3.11: PLSR model prediction statistics for cellulose, hemicellulose and lignin (% DW) in Soxhlet extracted dried and powdered agave leaf.

Predicted	Attribute	PC	r^2	RMSEP	Bias	Slope
Across years (cellulose, n=52, mean = 29.27, SD = 3.7; hemicellulose, n=50, mean = 14.22, SD = 1.92; lignin, n=50, mean = 17.03, SD = 1.73)						
Predict 2013 using 2012	cellulose	9	0.41	4.08	1.73	0.84
Predict 2012 using 2013	hemicellulose	12	0.24	2.96	2.27	0.003
Predict 2012 using 2013	lignin	13	0.05	3.48	1.92	0.36
Across cultivars (cellulose, n=24, mean = 26.08, SD = 2.41; hemicellulose, n=24, mean = 12.29, SD = 1.49; lignin, n=24, mean = 18.02, SD = 1.62)						
Predict L19 using Tcqu	cellulose	12	0.13	6.45	4.60	0.72
	hemicellulose	12	0.66	1.51	-0.40	0.003
	lignin	6	0.36	1.74	0.68	0.43

Past studies have indicated that NIR spectroscopy can be used to assess the cellulose, hemicellulose and lignin content of plant materials. For example, prediction statistics (r^2 0.962, RMSEP 0.46% w/w, n=52, SD = not available) have been reported for a model for cellulose in (pine, eucalyptus and poplar) wood samples, developed using FTNIR 1333–2326 nm spectra (He & Hu 2013).

In another study, cellulose content of 62 *E. globulus* samples were predicted using a model developed from 628 woodmeal samples with r^2 0.86 and RMSEP 1.50% w/w, SD not available) (Downes et al. 2012). Similarly, prediction statistics of r^2 0.93–0.96; RMSEP 1.03–0.57% w/w, SD = 0.14–1.72 (for n of only 15) have been reported for xylan (the major component of hemicellulose) content of corn stover and switch grass (Liu et al. 2010). Prediction statistics of $r^2 = 0.67$ was reported for lignin content of ground *Eucalyptus globolus* samples (Poke & Raymond 2006) and $r^2 = 0.86$, SEP=2.1, SD 1.6% w/w for lignin in rice straw samples (Jin & Chen 2007). Thus while the technology cannot be recommended for use with Agave at this stage, given applications in other plant residues, further work to develop a calibration set involving larger sample sizes across different seasons and years is recommended.

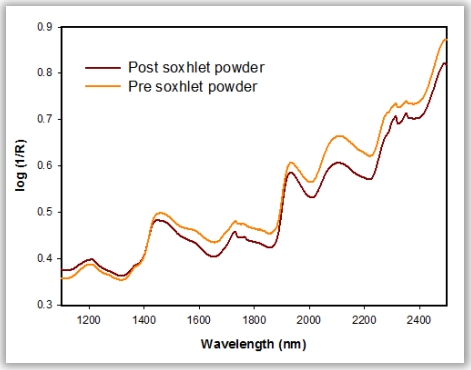
3.4 Conclusions

The estimation of crop harvest maturity is a common issue across many crops. For Agave-bioethanol production, leaf DM and TSS% are potential harvest maturity criteria, as is the relative proportion of cellulose, hemicellulose and lignin in dry matter. For assessment of DM and TSS of intact leaf in field, the SWNIR instrument gave better results than the FTNIR. This result is consistent with lower absorption at shorter wavelengths, enabling greater effective measurement depth. Agave leaf thickness is variable from base to tip, ranging from 15–20 mm at the base, 6–9 mm in the middle and 3–5 mm at the tip, and is coated with a thick cuticle and epidermis, the DM content of which will be quite different to the gross leaf, as measured in the reference procedure. To demonstrate the intended use of the technology for in-field measurements to guide the timing of the decision to harvest, tagged leaves were assessed non-destructively over 15 months using the portable instrument, with IR estimated DM values increasing from 15 to over17% w/w over a summer growing period (October to January).

High cellulose and low lignin is desirable for ethanol production. Leaf DM level was correlated (R^2 of 0.88) with the sum of leaf cellulose and hemicellulose content in the months of May 2012 and August 2013, while in November 2012 and March 2013 the relationship was weaker (R^2 of 0.56 and 0.62 respectively), with an overall relationship (May 2012–August 2013) of R^2=0.77 (data not shown). The correlation of DM and cellulose and hemicellulose content suggests that the decision on harvest timing could be guided by field measurement of DM. For estimation of cellulose, hemicellulose and lignin

content of dry matter, NIR assessment of dried ground material is recommended, with further work to extend the calibration set.

Chapter 4. Variation in cellulose, hemicellulose and lignin content of *Agave tequilana* leaf and their estimation using near infrared spectroscopy



Abstract

The cellulose, hemicellulose and lignin content of two cultivars of *A. tequilana*: Tcqu and L19 were assessed over a period of 15 months from May 2012 to August 2013 to determine change with respect to plant maturity and environmental conditions. A total of 104 leaf samples were dried, ground (150–530 µm particle) and scanned using two near infrared technologies: scanning grating and FTNIR. A standard National Renewable Energy Laboratory (NREL) procedure for leaf compositional analysis was used as a reference method. The powdered samples were scanned post and pre-Soxhlet extraction over the range of 1000–2500 nm. Slightly better results were obtained with post-Soxhlet samples using FTNIR. Predictions for cellulose content were better than for hemicellulose and lignin. The models developed using plant material from three sampling periods were used to predict percent cellulose content of plants of the fourth sampling period, which were not included in the model. Better prediction results were obtained for March 2013 and August 2013 groups (Rp 0.87 and 0.74 and RMSEP 2.90 and 4.41% w/w, respectively) than for other sampling periods (May 2012 and November 2012). Poorer results were obtained when a model was based on data from one year used in prediction of data from another year, indicating that data collected over a period of one year was not sufficient enough to develop a robust model for prediction.

4.1 Introduction

Information on chemical composition is required to optimise biofuel breeding and agronomic procedures to maximise leaf cellulose and non-structural carbohydrates, and

minimise lignin content in plant material, and also for selection of the most suitable pretreatment and fermentation methods for maximising bioethanol production (Li et al. 2012b). The use of *A. tequilana* leaves as feedstock for biofuel production is determined by the quality of this feedstock, assessed in terms of its content of structural (cellulose and hemicellulose) and non-structural carbohydrates. The composition of *Agave* leaf may vary with genotype, age and environmental conditions. For example, the chemical composition of *A. tequilana* of 1.25 and 2.5-year-old plants from Rockhampton (ROK) (23.32°S, 150.52°E) and Kalamia (KAL) (19.58°S, 147.41°E) were different, with cellulose varying from c.a. 35 to 39% w/w and hemicellulose (xylan+arabinan) from ca. 18 to 20% w/w (Rijal et al. 2016) (Chapter 5). The lignin content of the two cultivars was similar for the two sites (at 14.3 and 14.8%, respectively), as was soluble carbohydrate (glucose and fructose) content of untreated juice (3.3 and 3.0% w/v in ROK and KAL, respectively) (Rijal et al. 2016) (Chapter 5). The chemical composition of leaf is also variable between *Agave* species (e.g. *A. americana*, *A. sisalana* and *A. tequilana* cellulose 33.8, 32.1 and 33.7% w/w, hemicellulose 16.6, 15.6 and 12.8% w/w and K-lignin 8.2, 9.8 and 11.9% w/w, respectively) (Li et al. 2014).

Generally, compositional analysis of agave involves the use of high performance liquid chromatography (HPLC), dinitrosalicylic acid (DNS) method or nuclear magnetic resonance (NMR) (Iñiguez-Covarrubias et al. 2001; Li et al. 2014; Saucedo-Luna et al. 2011). These methods yield precise and accurate results but are laborious and time consuming, with hours of sample preparation and testing. A rapid, non-invasive technique with minimal sample preparation would be very useful to guide screening, breeding and agronomic programs. For example, F. Barton (2015, pers. comm.) described the use of a FTNIR unit (from Light Solutions Inc.) for in-field analysis of oil content in whole guayule plants, speeding the breeding program.

NIR spectroscopy has been applied to the determination of chemical composition and mechanical properties of wood and woody materials (Downes et al. 2011; Jones et al. 2006). A Scopus search for the period 1995 to 2015 based on the key words ‘NIRS and wood’ gave 94 results. For example, the technique has been applied to assessment of glucans (cellulose), xylan (hemicellulose) and lignin of rice straw (Jin & Chen 2007), corn stover (Ye et al. 2008), agricultural fibres (Kelley et al. 2004b), wood (Downes et al. 2011; Schimleck & Mora 2008) and herbaceous biomass (Guimarães et al. 2014;

Sanderson et al. 1996). In determination of glucose, xylose, galactose, mannose and lignin content of loblolly pine wood, a prediction of Rp = 0.56–0.80, RMSEP = 0.6–2.7 was achieved using the wavelength range 500–2400 nm and slightly better statistics (Rp = 0.54–0.84, RMSEP = 0.6–2.3) using the 650–1150 nm spectral range (Kelley et al. 2004a).

The choice of instrument is clearly important to the analytical result, with optimisation required in terms of wavelength range, wavelength resolution, signal to noise ratio, optical geometry etc. For example, while the FOSS scanning grating units (NIRS5000 and NIRS6500) are widely established in use for analysis of dried ground samples across feed and forage industries, these units have a wavelength resolution of only approximately 10 nm. Barton et al. (2005) demonstrated the value of the higher resolution of FTNIR over scanning grating technology in the detection of sticky cotton (free sugars on cellulose fibre). It is not clear from the literature whether wavelength resolution or signal to noise is the limiting factor for cellulose-hemicellulose-lignin determination.

In the current study, a FTNIR (Antaris, Thermo Inc., using a wavelength resolution of 8.0 cm^{-1}) and a scanning grating instrument (NIRS 6500, Foss P/L; wavelength resolution of 10 nm) were used for determination of the cellulose, hemicellulose and lignin chemical composition of agave leaves. As the presentation was repetitive with consideration of pre-processing method, wavelength range, principle component space and model robustness across locations and time for each attribute, only the results for cellulose are presented in this chapter, while those for hemicellulose and lignin are in Appendix C.

4.2 Materials and Methods

4.2.1 Plant material

Plant material from the experiment described in Chapter 3, Section 3.2.1 was used. Leaf samples of two cultivars of *A. tequilana* (Tcqu and L19) were collected during varying seasons and climatic conditions at Rockhampton from May 2012 to August 2013 at an interval of 4–6 months (4 harvests). The samples (n=104) were prepared as described in Chapter 3, then stored in air tight containers at room temperature prior to destructive leaf analyses of cellulose, hemicellulose and lignin.

4.2.2 Spectral acquisition

Sample spectra were acquired using two instruments, a FTNIR spectrometer (Nicolet Antaris Near IR Analyzer, Thermo, USA) and a scanning grating NIR spectrometer (NIR Systems 6500, Foss, VIC., Australia). Homogenous powdered samples (approx. 5 g per cup) were loaded into borosilicate glass sample cups fitted with a rotation accessory installed over an integrating sphere, and diffuse reflectance spectra was collected using the FTNIR spectrometer operated over the range 1100–2500 nm at a spectral resolution of 8.0 cm^{-1} with 64 scans averaged per sample (Fig. 4.1). A reference spectrum averaged from 64 scans was taken of the built-in white PTFE tile every 4 hours. For convenience of comparison of the two instruments, the wavelength scale of the FTNIR spectra was converted from cm^{-1} to nm.

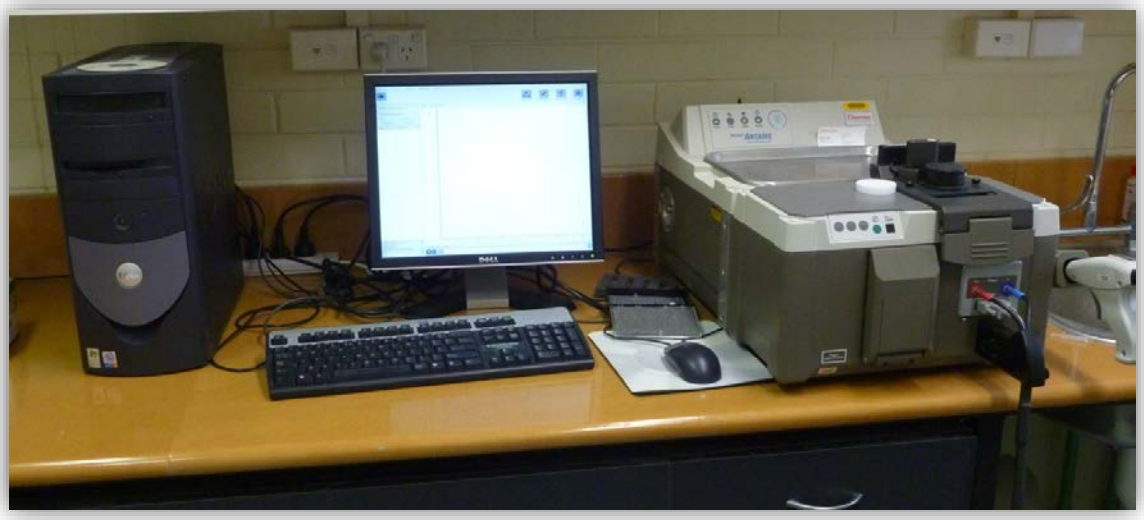


Figure 4.1: FTNIR instrumentation (Nicolet Antaris Near IR Analyzer, USA).

Sample material was then re-loaded into Foss quartz spinning cups and reflectance spectra recorded using the scanning grating unit, operated using a spinning cup accessory, averaging 32 scans per spectra (Fig. 4.2). As described in Section 3.2.4, Chapter 3, after scanning (i.e., pre-Soxhlet), sample material was subjected to Soxhlet extraction using Milli Q water for 12 hours (Sluiter et al. 2008a). Once dried (i.e., post-Soxhlet), samples were scanned again using the Antaris FTNIR and NIRS 6500 scanning grating units. The samples were then stored in air tight containers at room temperature prior to destructive leaf analyses of cellulose, hemicellulose and lignin. Although the total number of samples

were 104, the sample analysed varied from 80–104 in type and instrument used for analysis.

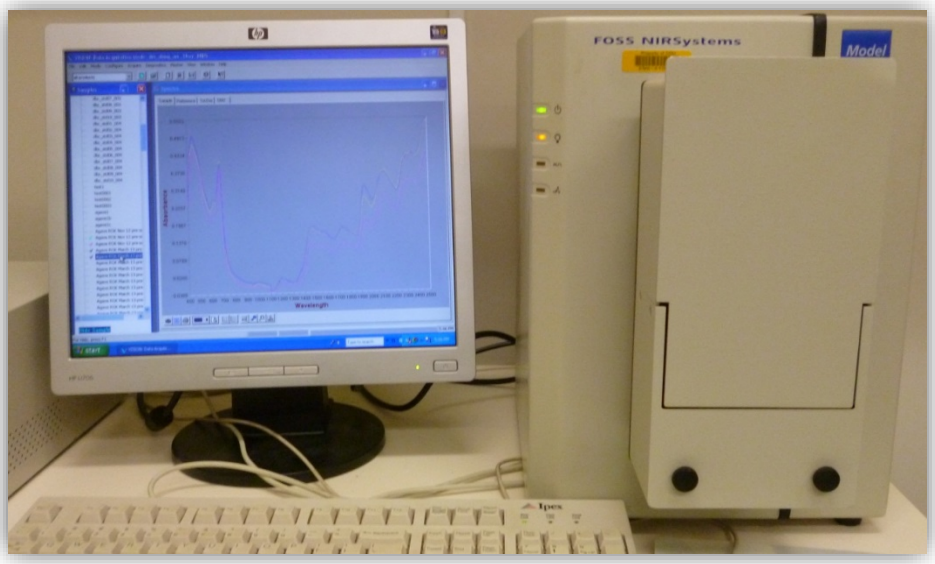


Figure 4.2: Scanning grating instrumentation (NIRS Systems 6500, FOSS).

4.2.3 Reference method

A total of 104 samples from Rockhampton were analysed for major chemical constituents, cellulose, hemicellulose and lignin, following the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al. 2008b) as described in Section 3.2.5, Chapter 3.

4.2.4 Chemometrics

Spectral data were processed using The Unscrambler software v.10.3 (CAMO, Oslo, Norway) to develop partial least squares regression (PLSR) calibration models. Several data pre-processing techniques were trialled. A Savitzky-Golay second order polynomial was used in calculation of second derivative. Several derivative window options were explored (data not shown), with reported results based on 25 data points (12 points each on left and right; i.e., a window over 25 nm) for the scanning grating unit and 55 points (27 points each on right and left; i.e., a window over 440 cm^{-1}) for the FTNIR unit. Multiplicative Scatter Correction (MSC) and Standard Normal Variate (SNV) were also used in order to reduce spectral distortion due to scattering (Agelet & Hurburgh 2010).

The PLSR models were assessed in terms of cross validation correlation coefficient (R_{CV}), root mean square error of cross-validation (RMSECV), population mean ($\overline{X}$), standard deviation (SD), the number of principle components (PC) and slope and bias. Two cross validation options were employed; full leave one out (LOO) cross validation and cross validation based on four populations, in both cases without removal of any outlier samples. Calibration models were also tested on sample sets that were not included in the calibration to test the model performance, with results reported in terms of coefficient of determination (R_P), root mean square error of prediction (RMSEP), bias and slope. A minimal model performance of R = 0.87 (R^2 = 0.75) was adopted, as this value represents a standard deviation to RMSECV ratio of 2.0, assuming negligible bias, from the relationship:

$R^2 = 1 – (SEP/SD)^2$, where SEP is bias corrected RMSEP

4.2.5 Statistical analysis

Analysis of Variance (ANOVA) (Genstat statistical package, Version 16.1, VSNI Ltd, UK) and Duncan's Multiple Range Test (DMRT) was used to compare cellulose, hemicellulose and lignin content at different maturity stages of the leaf, from May 2012 to August 2013. A value of $P \leq 0.05$ was considered as significant.

4.3 Results and Discussion

4.3.1 Leaf composition

Leaf cellulose, hemicellulose and lignin content varied significantly ($P<0.05$) in the two cultivars of *A. tequilana* (Tcqu and L19), from first sampling in May 2012 to the last in August 2013 (Fig. 4.3). Cellulose content (% w/w DW) declined significantly with maturity, decreasing from 34.7 in May 2012 to 29.9% w/w in August 2013 (Fig. 4.3 A). Hemicellulose composition of Tcqu also declined significantly ($P <0.01$) across this period. However, the lignin content increased significantly ($P <0.05$), from 15.6% w/w in May 2012 to 18.6% w/w in August 2013 (Fig. 4.3 A).

Similarly, cellulose content of L19 decreased significantly with maturity (from 29.3% in May 2012 to 24.7% w/w in August 2013) (Fig. 4.3 B), but an overall increase in hemicellulose was observed (from 11.8% in May 2012 to 13.8% w/w DW in August 2013) (Fig. 4.3 B). Similar to cultivar Tcqu, lignin content increased significantly

(P >0.05) from 16.4% in May 2012 to 19.5% w/w in March 2013, declining significantly (P >0.05) to 17.3% w/w in August 2013 (Fig. 4.3 B). The cellulose composition varied significantly (P<0.05) between Tcqu (32.3% w/w) and L19 (26.1% w/w). The mean hemicellulose content of Tcqu and L19 was 13.9 and 12.3% w/w, respectively. The lignin composition was similar in both cultivars (17.8 and 18.0% w/w in Tcqu and L19, respectively) (Fig. 4.3 A and B).

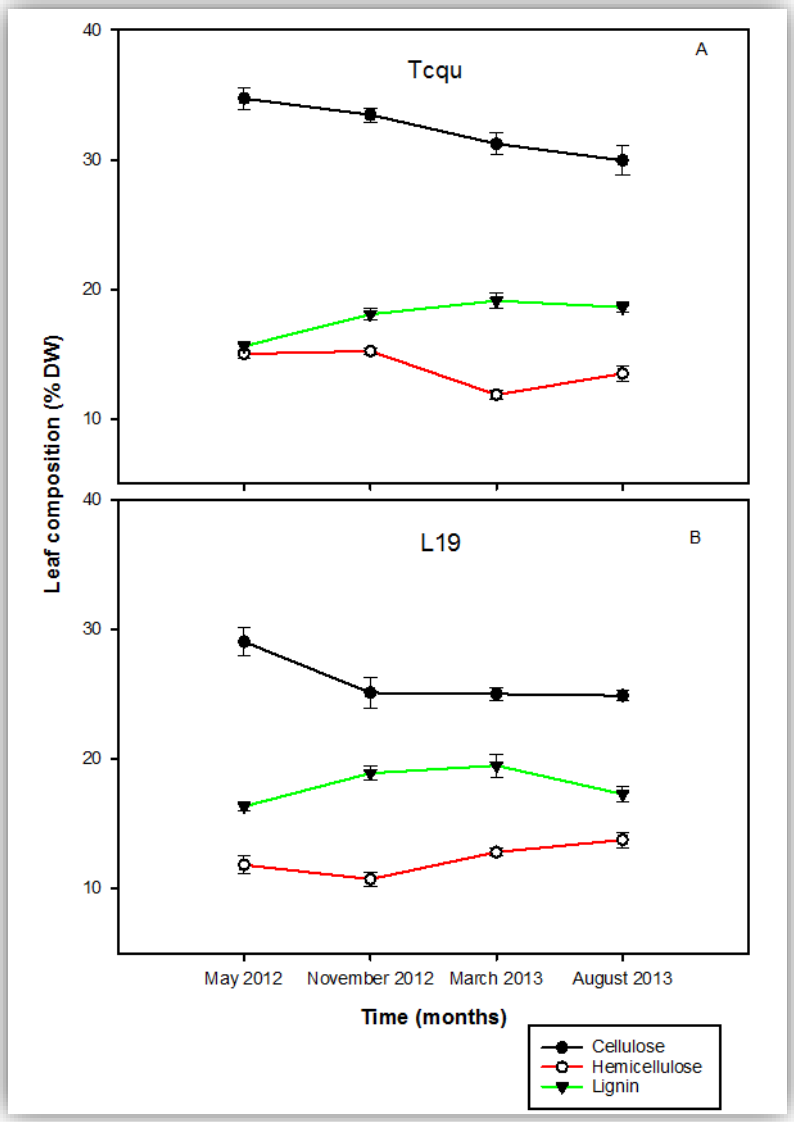


Figure 4.3: Content (% w/w DW) of cellulose, hemicellulose and lignin in leaves between May 2012 and August 2013 of two cultivars of *Agave tequilana*: (A) Tcqu, n=120 and (B) L19, n=36. Bars represent ± SE.

4.3.2 Spectral features

The spectra of FTNIR and scanning grating units were similar (Figs 4.4 and 4.5). Spectral features were associated with the 2nd overtone C-H and stretching vibrations of the CH_3 group between 1100–1150 nm, 1st overtone C-H combinations between 1190–1250 nm, 1st overtone of O-H and combination C-H peaks at around 1400–1650 nm, 1st overtone C-H around 1700–1770 nm, O-H combination band between 1950–2050 nm and N-H and O-H, and the C-H + C-H combination band between 2100–2300 nm (Figs 4.4 and 4.5).

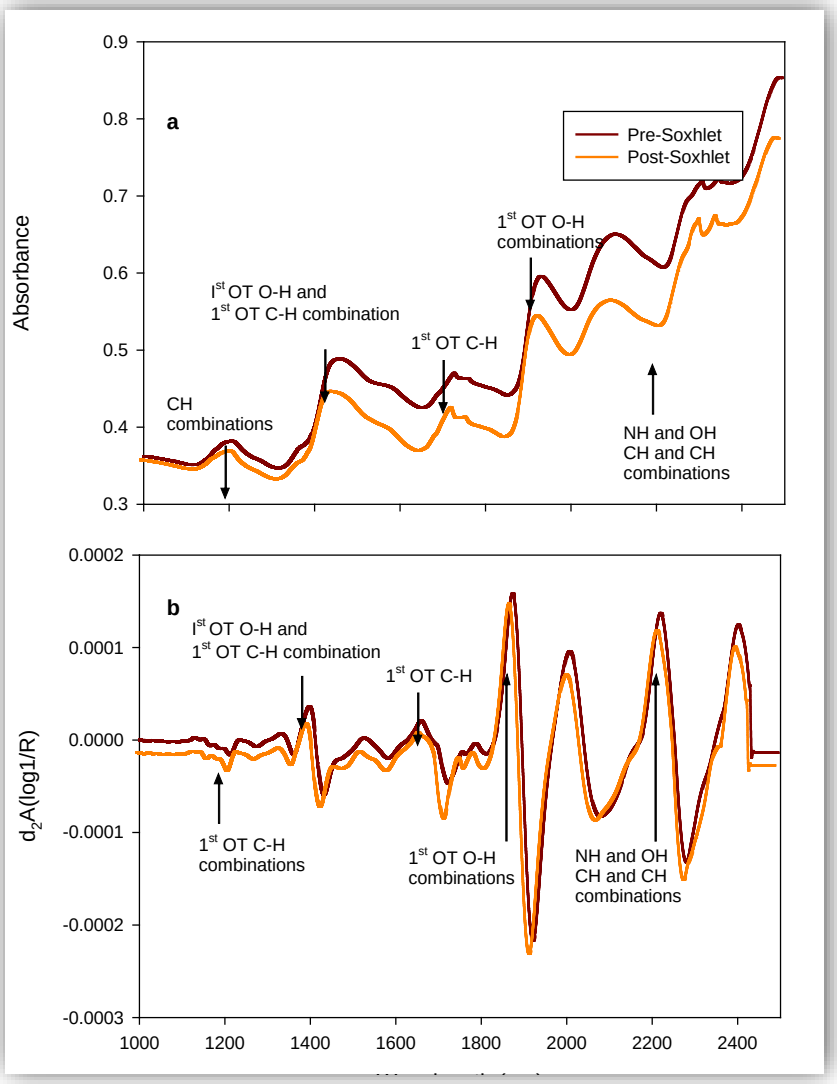


Figure 4.4: FTNIR (1000–2500 nm): Spectra of *A. tequilana* leaf powder as (a) average log 1/R spectra and (b) second derivative.

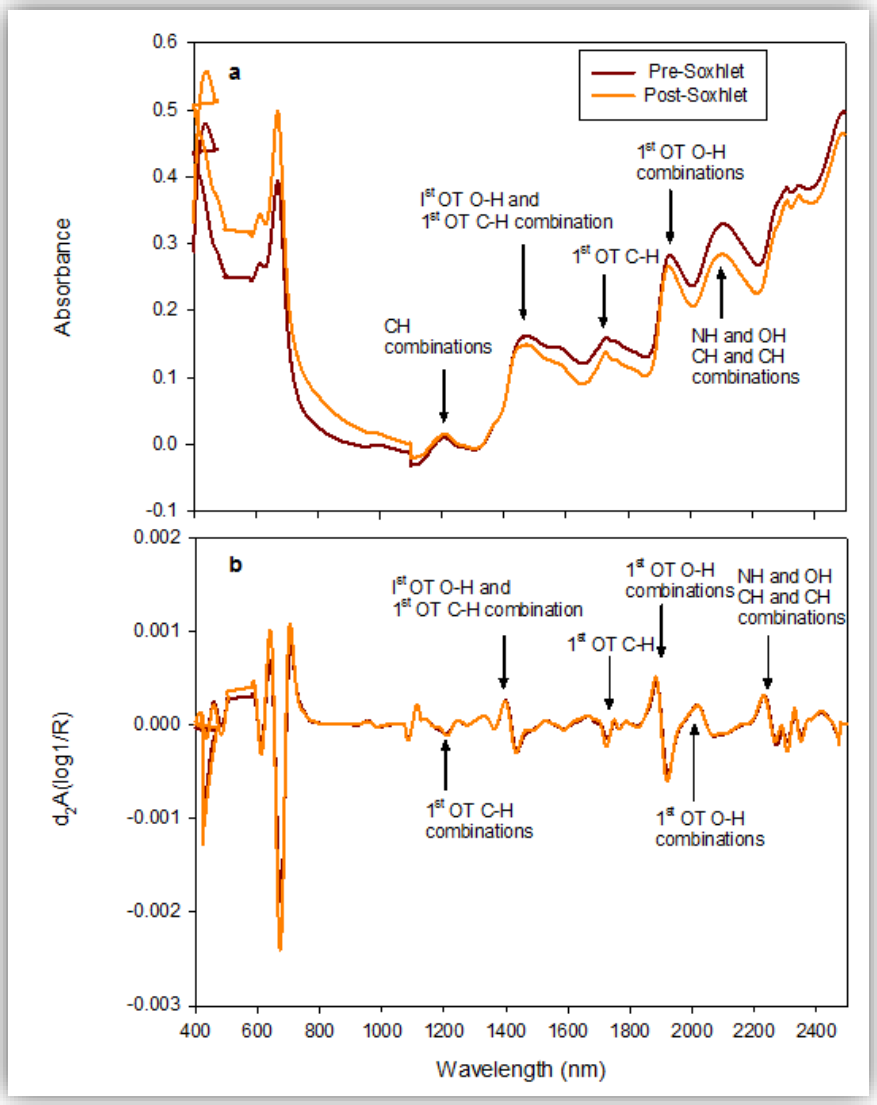


Figure 4.5: Scanning grating (400–2500 nm): (a) Average log 1/R spectra of *A. tequilana* leaf powder, and (b) second derivative spectra *A. tequilana* leaf powder, pre and post-Soxhlet extraction.

4.3.3 Component estimation using FTNIR and scanning grating technologies

4.3.3.1 Cross validation and test set validation

PLSR models were developed using reflectance spectra of leaf powder collected using two technologies, FTNIR and scanning grating, in context of cellulose, hemicellulose and lignin content. Calibration LOO cross validation R >0.95 were obtained using FTNIR spectra for all components (Table 4.1 A). Even better results can be achieved by removal of poorly performing samples (e.g., increase in Rcv from 0.77 to 0.92 with removal of two data points from n=80, Table 4.4). Many published studies would stop analysis at this point, providing an optimistic assessment of model performance. However, there were no

clear criteria for identification of outliers based on spectra alone, and therefore further analysis was based on inclusion of all samples. Further, to emulate practical application which involves prediction of samples from populations independent of the calibration set, cross validation was undertaken based on use of the four populations (one variety, at four times) as cross validation sets. Cross validation R decreased to <0.8 (Table 4.1 B). The much poorer result of the population based cross validation is indicative that the model does not contain adequate sample variation to predict future samples. To further demonstrate this, models developed from three sampling dates (May 12 to Mar 2013) were used in prediction of the fourth sample set (Aug 2013, Table 4.2).

4.3.3.2 Comparison of spectrometer technologies

Of the two instruments, the FTNIR had better cross validation statistics, using a model with fewer PCs, relative to the scanning grating using either leave one out cross validation or cross validation based on populations (Table 4.1).

In terms of prediction of an independent set, cellulose was predicted better (Rp = 0.71, RMSEP = 2.69) using FTNIR than scanning grating (Rp = -0.14, RMSEP 4.38), using the segmented CV model (Table 4.2). Hemicellulose was predicted better using scanning grating technology than FTNIR using a model based on full CV, but with segmented CV, FTNIR performance was better than that of the scanning grating (Table 4.2). In terms of lignin, the scanning grating unit had better predictive performance than FTNIR (Table 4.2). Overall, a better result was generally obtained with the FTNIR than the scanning grating. Further details are presented for the FTNIR data only.

Table 4.1: PLSR models statistics for spectral data pretreatments of reflectance spectra of 80 *A. tequilana* leaf samples, for cellulose (mean = 32.32, SD = 3.23% DW), hemicellulose (mean = 13.89, SD = 1.81% DW) and lignin (mean = 17.72 and SD = 1.73% DW) using the Antares FTNIR and NIRS 6500 scanning grating, over the range 1000–2500 and 400–2500 nm, respectively. R >0.87 in bold in (A) LOO CV (B) CV based on populations from different maturity stages (collected in different months/seasons).

(A) LOO-CV

	NIRS 6500				FTNIR			
Parameter/Pretreatment	PC	R	RMSECV	Slope	PC	R	RMSECV	Slope
Cellulose								
Abs	18	**0.94**	1.14	0.90	20	**0.97**	0.73	0.95
d^2A	5	0.77	2.06	0.64	20	**0.96**	0.94	0.94
MSC	14	**0.93**	1.22	0.88	19	**0.96**	0.88	0.94
Hemicellulose								
Abs	11	**0.92**	0.72	0.89	19	**0.98**	0.36	0.96
d2A	4	0.74	1.21	0.61	20	**0.97**	0.47	0.95
MSC	9	**0.91**	0.76	0.86	17	**0.97**	0.47	0.94
Lignin								
Abs	17	**0.93**	0.66	0.88	20	**0.97**	0.43	0.96
d^2A	9	0.84	0.95	0.74	20	**0.98**	0.34	0.97
MSC	15	**0.93**	0.66	0.90	19	**0.97**	0.45	0.96

(B) CV Based on populations

	NIRS 6500				FTNIR			
Parameter/Pretreatment	PC	R	RMSECV	Slope	PC	R	RMSECV	Slope
Cellulose								
Abs	7	0.70	2.54	0.72	7	0.69	2.43	0.61
d^2A	2	0.46	3.02	0.35	3	0.67	2.40	0.51
MSC	5	0.63	2.72	0.59	4	0.68	2.52	0.54
Hemicellulose								
Abs	18	0.46	1.78	0.39	1	-0.45	2.24	-0.18
d^2A	1	0.13	1.88	0.06	12	0.57	1.57	0.46
MSC	17	0.59	1.57	0.52	14	0.64	1.81	0.52
Lignin								
Abs	6	0.34	1.78	0.20	2	0.80	1.14	0.63
d^2A	5	0.35	1.75	0.22	2	0.49	1.81	0.39
MSC	8	0.63	1.52	0.62	2	0.63	1.57	0.57

Table 4.2: Model (developed on May 2012 to March 2013 data sets) used in prediction of cellulose, hemicellulose and lignin content of an independent set of samples (collected August 2013, n=20), for both FTNIR and scanning grating instruments. The prediction set mean and standard deviation on cellulose, hemicellulose and lignin was 29.95 ± 3.46, 13.5 ± 1.88 and 18.61 ± 1.24, respectively. Models developed using leave one out cross validation ('LOO') and based on populations ('seg') of different maturity stages collected at different months/seasons were trialled.

Instrument	Parameter	Model	PC	Rp	RMSEP	Bias	Slope
Scanning grating	Cellulose	LOO	20	0.71	3.14	2.01	0.42
		Seg	2	-0.14	4.38	2.28	-0.05
	Hemicellulose	LOO	17	0.67	2.84	-2.49	0.40
		Seg	1	-0.40	2.32	-0.39	0.18
	Lignin	LOO	17	0.71	2.03	0.52	1.16
		Seg	2	0.80	0.78	0.24	0.72
FTNIR	Cellulose	LOO	17	0.47	5.67	4.83	0.23
		Seg	7	0.71	2.69	0.41	0.75
	Hemicellulose	LOO	11	0.32	1.91	-0.49	0.22
		Seg	1	-0.10	2.55	-0.96	-0.07
	Lignin	LOO	16	0.29	1.74	1.08	0.25
		Seg	5	0.52	1.89	1.47	0.52

4.3.4 Effect of Soxhlet extraction on PLS models

Soxhlet extraction removes soluble materials such as sugars and organic acids from the sample. These materials possess O-H and C-H bonds and, as a result, will have similar spectra to the materials of interest in this study (celluloses, hemicelluloses and lignin). It was reasoned that the removal of such compounds would result in improved PLSR models for the attributes of interest. Reflectance spectra were visibly altered by the process of extraction, with small absorbance features around 1700 and 2300 nm becoming more noticeable, and an overall decrease in log 1/R at wavelengths above 1440 nm, consistent with decreased light scattering (Fig. 4.6).

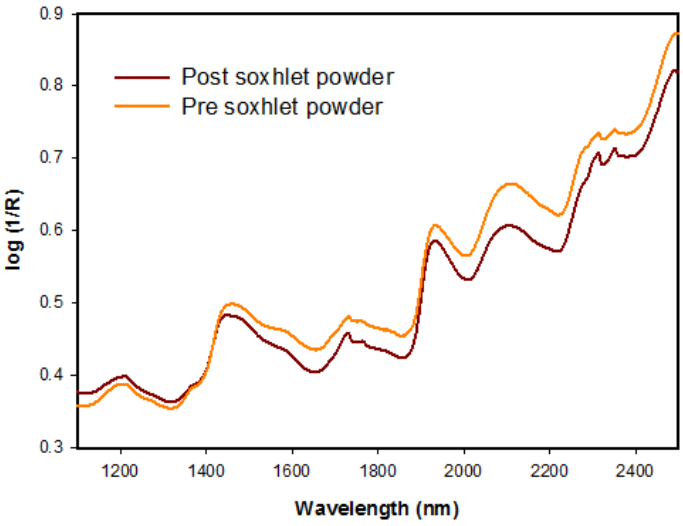


Figure 4.6: Average FTNIR log 1/R spectra (1000–2500 nm) of *A. tequilana* powder, pre-and post Soxhlet extraction (n=98).

In practice, post Soxhlet samples supported a PLSR model (1000–2500 nm) with similar Rcv of 0.85, but fewer PLS factors (e.g., 7 as compared to 11 for Abs data) compared to that involving pre-Soxhlet samples for a sample set containing data of two cultivars, Tcqu and L19 (n=98) (Table 4.3).

Therefore, further discussion on development of models for cellulose, hemicellulose and lignin content is constrained to use of post-Soxhlet FTNIR spectra.

Table 4.3: FTNIR Leave one out cross validation (LOO CV) statistics for PLS calibration models of percent cellulose (mean = 30.59, SD = 3.96) of dried *A. tequilana* leaf powder pre and post Soxhlet. Models were developed with 98 spectra using the wavelength regions of 1000–2500, 2000–2300 and 1880–2300 nm. The highest Rcv values for models ≥0.85 on % cellulose are shown in bold.

Wavelength Range		PC	Rcv	RMSECV	Bias	Slope
Pre-Soxhlet						
1000–2500nm	**Data pretreatment**					
May 12–Aug 13	Abs	11	**0.85**	2.11	0.00	0.76
May 12–Aug 13	S. Golay (d^2A)	6	0.80	2.38	-0.02	0.71
May 12–Aug 13	Abs-SNV	10	**0.85**	2.09	-0.06	0.78
May 12–Aug 13	Abs-MSC	10	**0.85**	2.11	-0.05	0.78
2000–2300 nm						
May 12–Aug 13	Abs	14	0.80	2.42	-0.05	0.72
May 12–Aug 13	S. Golay (d^2A)	14	0.79	2.43	0.03	0.72
1880–22900 nm						
May 12–Aug 13	Abs	11	0.84	2.15	-0.01	0.76
May 12–Aug 13	S. Golay (d^2A)	3	0.80	2.36	-0.02	0.63
Post-Soxhlet						
1000–2500nm						
May 12–Aug 13	Abs	7	**0.85**	2.04	0.03	0.76
May 12–Aug 13	S. Golay (d^2A)	7	0.83	2.20	0.04	0.75
May 12–Aug 13	Abs-SNV	11	0.83	2.21	0.00	0.76
May 12–Aug 13	Abs-MSC	11	0.82	2.24	-0.00	0.75
2000–2300 nm						
May 12–Aug 13	Abs	7	0.79	2.43	0.02	0.69
May 12–Aug 13	S. Golay (d^2A)	6	0.76	2.58	0.03	0.68
1880–2290 nm						
May 12–Aug 13	Abs	7	0.78	2.46	0.00	0.69
May 12–Aug 13	S. Golay (d^2A)	7	0.83	2.20	-0.02	0.68

4.3.5 Cellulose estimation—data pre-processing and wavelength range

Absorbance spectra were pre-processed using Savtikty Golay 2nd derivative, SNV and MSC. In general, the second derivative resulted in fewer PLS factors in the model, but model performance was not greatly improved (Table 4.3).

Ideally, a PLS model should be interpretable in terms of expected absorbance features. However, due to the overlapped nature of absorbance bands in the NIR, model weighting is often on the shoulders of attribute absorbance features. For example, PLS models for the estimation of cellulose and hemicellulose in loblolly pine wood were interpreted in

terms of the 1st O-H overtone, the combination C-H peak between 1400–1660 nm and a strong vibration identified between 2020–20250 nm regions assigned to cellulose hydroxyls (Kelley et al. 2004a). Information on lignin was identified in first and second overtones of aromatic and aliphatic carbon and hydrogen vibrations in the 1635–1835 and 1075–1250 nm regions (Kelley et al. 2004a).

PLS model b-coefficients were noisy (variable between adjacent wavelengths), particularly between 1000–1800 nm and above 2300 nm (Fig. 4.6 A). Avoiding these regions, models with smooth b-coefficients were developed with a spectral window between 1880–2290 nm or 2000–2300 nm (Fig. 4.7 B). However, there was no improvement in the model statistics (Rcv between 0.76–0.83, RMSECV 2.58–2.20, and number of PLS factors unchanged) for either pre or post-Soxhlet samples (Table 4.3).

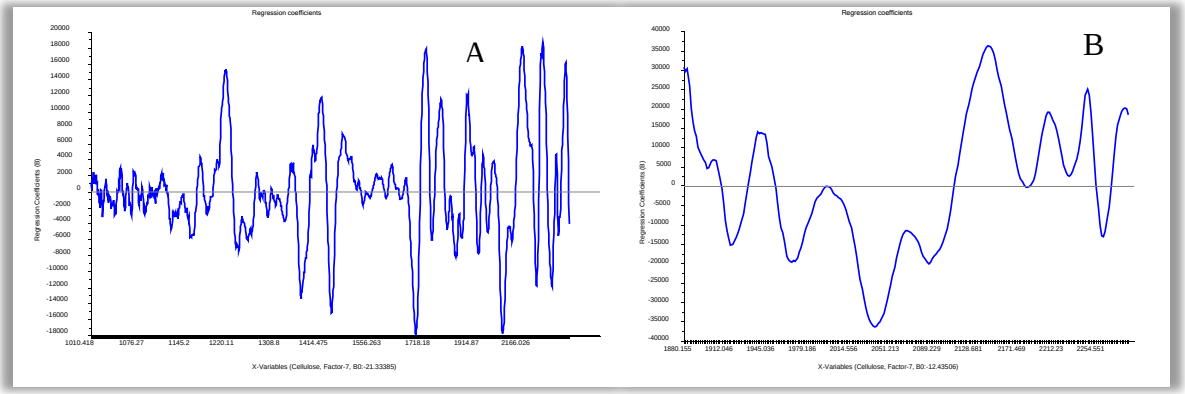


Figure 4.7: Regression coefficients of PLS models of % cellulose (DW basis) in *A. tequilana* leaf material, for models based on second derivative of log 1/R spectra using wavelength ranges of (A) 1000–2500 (left panel) and (B) 1880–2290 nm (right panel), (n=104).

4.3.6 Cellulose estimation—model robustness across cultivars and years

Models developed using combined populations (Tcqu and L19) supported comparatively better performance statistics than those based on a single population (Table 4.4). This result is in part due to the increased SD of the combined set. The variation in varieties and sampling events was distinct in PLS factor plot (Figs 4.8 A–C).

Table 4.4: FTNIR (1000–2500 nm): PLSR models developed using S. Golay (d^2A) at 1000–2500 nm of post-Soxhlet extracted leaf samples. Full leave one out cross validation results are reported on % DW cellulose.

Sample	Outlier	PC	Rcv	RMSECV	Slope
Tcqu (n=80, mean = 32.33, SD = 3.23)	0	5	0.77	2.06	0.64
Tcqu (n=78, mean = 32.31, SD = 3.27)	2	12	0.92	1.31	0.87
Combined 2012 (Tcqu+L19) (n=52, mean = 32.50, SD = 3.75)					
	0	9	0.88	1.79	0.85
Combined 2013 (Tcqu+L19) (n=52, mean = 29.27, SD = 3.68)					
	0	14	0.93	1.35	0.85

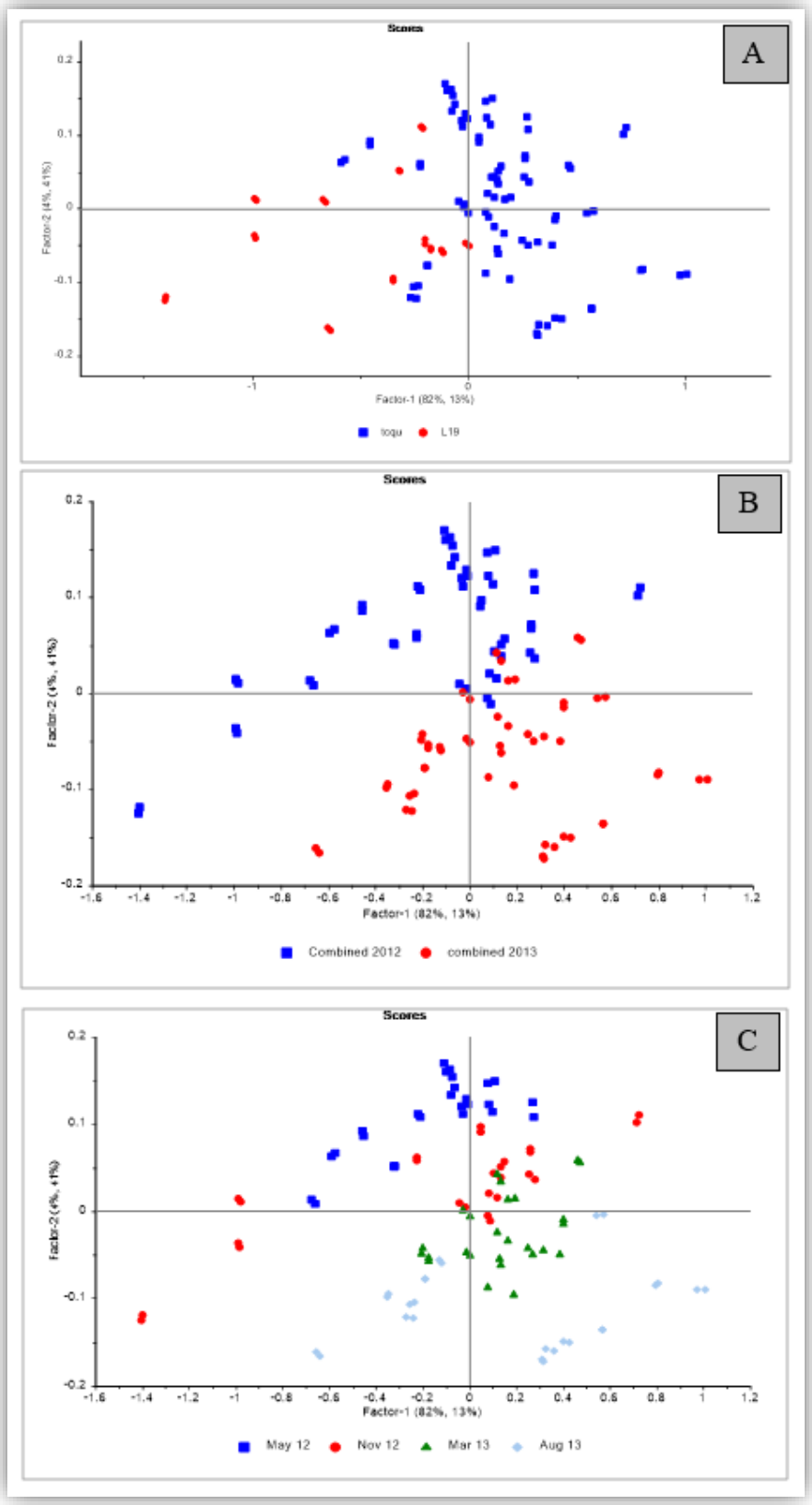


Figure 4.8: Plot of PLS factors 1 and 2 for a PLS model of cellulose (% DW) in *A. tequilana* leaf (n=104). Samples differentiated into (A) cultivars (Tcqu and L19), (B) years (2012 and 2013) and (C) sampling periods (May 12, Nov 12, Mar 13 and Aug 13).

4.3.7 Cellulose estimation—PLS model prediction performance

The models developed using three sampling periods (Table 4.5) were used to predict % cellulose content of the fourth sampling period (i.e., a set not included in the model) (Table 4.6). Populations March 2013 and August 2013 had better prediction (Rp 0.87 and 0.74 and RMSEP 2.90 and 4.41, respectively) as compared to other sampling periods (May 2012 and November 2012) (Table 4.6). The combined data of two the cultivars from 2012 (i.e., two populations) was used in prediction of 2013 data, with poorer prediction statistics (Rp = 0.64 and RMSEP 4.08) than when three populations were used. This result indicates that data collected over a period of one year was not sufficient to develop a robust model for prediction. It is recommended that the model should be prepared with data from at least two to three years of data, covering a wide range of seasonal and compositional variation.

Prediction of one cultivar using a model developed with the data of the other cultivar involved a low Rp = 0.36. This result could be due to distinct variation in the % cellulose content of the two different cultivars (Figs 4.3 A and B).

Table 4.5: FTNIR (1000–2500 nm): Combined PLSR models of % cellulose (w/w) developed with spectral pretreatment using S. Golay (d^2A) from May 2012–Aug 2013 (post Soxhlet). Full leave one out cross validation is reported. Population statistics of predicted group are shown in brackets.

Sample	Outlier	PC	Rcv	RMSECV	Slope
Nov 12, Mar 13 and Aug 13					
(n=78, mean = 30.02, SD = 3.90)	0	9	0.85	2.03	0.78
May 12, Mar 13 and Aug 13					
(n=76, mean = 30.60, SD = 4.09)	2	8	0.90	1.74	0.45
May 12, Nov 12 and Aug 13					
(n=76, mean = 31.19, SD = 4.18)	2	8	0.90	1.85	0.86
May 12, Nov 12 and Mar 13					
(n=76, mean = 31.56, SD = 3.92)	2	9	0.92	1.54	0.88

Table 4.6: Prediction statistics for % DW cellulose (post Soxhlet). Models were developed with spectral pretreatment using S. Golay (d^2A). Full leave one out cross validation is reported. R values >0.85 are shown in bold.

Instrument /Range	PC	Rp	RMSEP	Bias	Slope
FTNIR/1000–2500 nm					
Predict May 12 using Nov 12, Mar 13 and Aug 13					
(n=24, mean = 33.49, SD = 3.45)	9	0.66	3.10	-1.7	0.55
Predict Nov 12 using May 12, Mar 13 and Aug 13					
(n=26, mean = 31.54, SD = 3.97)	8	0.29	3.93	-0.8	0.15
Predict Mar 13 using May12, Nov 12 and Aug 13					
(n=26, mean = 29.80, SD = 3.55)	8	**0.87**	2.90	-2.0	1.05
Predict August 13 using May 12, Nov 12 and Mar 13					
(n=26, mean = 28.73, SD = 3.78)	9	0.74	4.41	3.4	0.78
Predict 2013 using 2012 (n=52, mean = 29.27, SD = 3.70)	9	0.64	4.08	1.73	0.84
Predict L19 using Tcqu (n=24, mean = 26.08, SD = 2.41)	12	0.36	6.45	4.60	0.72

Hemicellulose and lignin estimation and prediction results are presented in Appendix C.

4.4 Conclusions and Recommendations

In this exercise, non-destructive determination of cell wall components of *A. tequilana* with minimal sample preparation was trialled using two different techniques—FTNIR (Antares, Thermo) and scanning grating (Foss NIRS 6500). The FTNIR data yielded slightly better results. The FTNIR models developed on pre-and post Soxhlet extracted samples were also compared, and slightly better model statistics were achieved using post Soxhlet samples. Different wavelength regions were trialled for cellulose, hemicellulose and lignin based on smooth regression coefficients and distinct peaks at particular wavelength region. The best results were obtained using the full wavelength region of 1000–2500 nm. Cellulose was better predicted using this technology than the other two attributes (hemicellulose and lignin). This could be due to the relatively higher range of SD for cellulose as compared to those of hemicellulose and lignin.

Although good calibrations were obtained using LOO full cross validation, prediction results were poor to moderate for hemicellulose and lignin in this study (Appendix 3). PLS factor plots indicate that data collected over a period of one year was not sufficient to include samples representative of future sets, and thus insufficient for development of a robust prediction model. In order to have better prediction of the composition, the model

should be prepared with data from at least two to three years, covering a wide range of seasonal and compositional variation.

DECLARATION OF CO-AUTHORSHIP AND CONTRIBUTION

[This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears]

Title of Paper	Process options for conversion of *Agave tequilana* leaves into bioethanol.
Full bibliographic reference for Journal/Book in which the Paper appears	Rijal, D, Vancov, T, McIntosh, S, Ashwath, N & Stanley, GA 2016, 'Process options for conversion of Agave tequilana leaves into bioethanol', *Industrial Crops and Products*, vol. 84, pp. 263–272, DOI information: 10.1016/j.indcrop.2016.02.011.
Status	05 February 2016 Accepted and In Press 16 February 2016 Published Online

Nature of Candidate's Contribution
First Author: Conducting experiments, sampling, data collection, data analysis and writing the manuscript: 70%.
Nature of Co-Authors' Contributions
Co-author's contribution: 30% Dr Tony Vancov and Dr Shane McIntosh: Contributed in planning, experimental design, guiding the experiments in the laboratory and guidance in manuscript writing, Professor Grant Stanley and A/Prof. Nanjappa Ashwath contributed in procurement of funding for research, planning and guidance in manuscript writing. The experiment was conducted in the laboratory at DPI Wollongbar, NSW.
Candidate's Declaration
I declare that the publication above meets the requirements to be included in the thesis as outlined in the Publication of Research Higher Degree Work for Inclusion in the Thesis Procedures
Signature *Deepa Rijal*
Date *August 2016*

Chapter 5. Process options for conversion of *Agave tequilana* leaves into bioethanol[2]

Abstract

This paper reports on mild acid pretreatment options for the conversion of *Agave tequilana* leaves into composite sugars for ethanol fermentation. The effect of five different pretreatment conditions (time, temperature and acid concentrations) were assessed in terms of cellulose digestibility, hemicellulose solubilisation and lignin content in leaves of 1.5 years old *A. tequilana* plants from Rockhampton and 2.5 year plants from Kalamia. Dilute acid pretreatment and enzyme saccharification of *A. tequilana* leaf bagasse significantly improved total glucose recovery. A recovery of 273 mg g^{-1} (70% theoretical) was attained when the bagasse was pretreated with 2.0% H_2SO_4 for 60 min at 121°C and saccharified with 6% w/w CTec2. *Saccharomyces cerevisiae* efficiently fermented crude *A. tequilana* bagasse and juice hydrolysates within 13 h and 7 h respectively, yielding up to 38.6 g L^{-1} and 12.4 g L^{-1}. This corresponds to glucose to ethanol conversion rate of 68 and 61% for *A. tequilana* leaf bagasse and juice, respectively. With further developments, including fermentation of C5 sugars and inulinase saccharification of juices (release of fructose), this process could deliver greater yields, reinforcing its potential as a biofuel feedstock.

Keywords: *Agave tequilana*, Cellic® CTec2 saccharification, Dilute-acid pretreatment, Ethanol fermentation, Second generation biofuels.

[2] Note: This chapter has been published in *Industrial Crops and Products*, volume 84, 2016, pp. 263-272.

5.1 Introduction

The ever-increasing demand for transportation fuels combined with diminishing reserves of fossil fuels compels the production of energy from renewable sources. Biofuel produced from lignocellulosic feedstock is considered to be an exceptional solution owing to its favourable greenhouse gas (GHG) footprint status as a renewable resource and supply (McIntosh et al. 2012). Some *Agave* spp. could serve as potential sources of lignocellulosic biomass. *Agave* spp. are highly efficient in their use of water and require minimal supplementation with water or fertilisers (Holtum et al. 2010). Since they possess the crassulacean acid metabolic (CAM) photosynthetic pathway, they can be grown in arid and semi-arid regions that are unsuited to conventional agricultural crops or lignocellulosic feedstock such as poplar, *miscanthus* and switchgrass (Li et al. 2012b; Somerville et al. 2010b). Surprisingly, the agaves have higher average annual productivities ranging from 10–34 Mg ha^{-1} as compared to switchgrass (15 Mg ha^{-1}) and 11 Mg ha^{-1} for poplar wood (Somerville et al. 2010b).

There are numerous studies reporting the use of *A. tequilana* stem (piña) for production of potable alcohol (Tequila) but relatively few on biofuel production from agave bagasse (portion remaining after extracting fructose from the piña) (Caspeta et al. 2014; Hernández-Cortés et al. 2010; Hernández-Salas et al. 2009). The *A. tequilana* stem contain fructans as the storage carbohydrate, which constitute more than 60% of the total soluble carbohydrates (Mellado-Mojica & López 2012). The polyfructose solution obtained from the pulp of milled agave stem can also be hydrolysed with inulin enzymes to produce fructose syrup commonly used as sweeteners in food and beverage industries (Partida, Lopez & de Jesus Martinez Gomez 1998). However, the bagasse of both 'piña' and the leaves consists of complex structural carbohydrates and lignin, which require harsher treatments in order to release the lignocellulosic sugars (Li et al. 2012b; McIntosh et al. 2012; Sluiter et al. 2008b). The leaves of mature *A. tequilana* plants are suitable for bioethanol production because they contain up to 42% structural carbohydrates and only 12% lignin (Li et al. 2012b). In addition, up to 4.4% soluble sugars have been found to be present in the leaf juice (Li et al. 2014). Although the growth rate of *A. tequilana* is slow with a long cropping cycle taking up to 5–7 years for maturity (Escamilla-Treviño 2012), regular harvesting of leaves during this period presents an opportune feedstock for biofuel production.

Efficient utilisation of lignocellulosic biomass requires pretreatment to liberate cellulose from its lignin seal and disrupt its crystalline structure before effective enzymatic hydrolysis can take place. This is generally achieved through either chemical or physical methods or a combination of both. Reported pretreatment methods used in the hydrolysis of agave bagasse include dilute acid (HCl 1.2-2% v/v) (Hernández-Salas et al. 2009; Saucedo-Luna et al. 2011), dilute alkali (NaOH 2% w/v) (Hernández-Salas et al. 2009) and ionic liquid (1- ethyl -3 methylimidazolium acetate [C2mim][OAc]) (Perez-Pimienta et al. 2013) followed by enzymatic saccharification. A comparative acid-alkali pretreatment study of *A. atrovirens* bagasse showed that dilute acid pretreatment (1.2% HCl) was far less effective than its alkali counterpart (2% NaOH) in producing sugars after enzyme saccharification (Hernández-Salas et al. 2009). The dilute acid treatment yielded between 5–9.9% w/w reducing sugars from bagasse of agave piña and whole biomass (piña + leaves) while the alkaline treatment yielded 12–58% reducing sugar (Hernández-Salas et al. 2009). In contrast, Saucedo-Luna et al. (2011) demonstrated greater yields with the piña bagasse from *A. tequilana* using a dilute acid approach. They pretreated the bagasse with 1–3% w/w H_2SO_4 and, following enzymatic saccharisation, were able to recover 41 g L^{-1} fermentable sugar (73.6% theoretical). Similarly, *A. tequilana* stalk bagasse pretreated with a modified Ethanosolv method (combination of water, ethanol and H_2SO_4 at 10% w/v) led to the theoretical recovery of 91% of the total fermentable sugars (0.51 g g^{-1}) following saccharification with cellulase and beta-glucosidase (Caspeta et al. 2014). Also, agave bagasse pretreated with ionic liquid ([C2mim][OAc]) at 160°C with solid loading of 15% w/w resulted in 45.5% delignification and significant improvement in recovery of sugar, releasing 14 mg mL^{-1} from 7 mg mL^{-1} (two fold) as compared to untreated material (Perez-Pimienta et al. 2013).

In terms of converting agave bagasse to ethanol, a reported study using dilute acid pretreatment accompanied by enzyme saccharification at 10% w/w solid loading produced 18.3 g L^{-1} ethanol, equivalent to 0.18 g g^{-1} dry bagasse (Saucedo-Luna et al. 2011). In this study, the fermentation was carried out using the native yeast *Pichia carribica* (UM-5 strain), which could ferment both hexose and pentose sugars, with an overall theoretical ethanol yield of 56.8% w/w. However, in another study conducted by Hernández-Salas et al. (2009), alkaline pretreated/enzymatic saccharified agave bagasse yielded only 6.6 g L^{-1} ethanol from 56.4 g L^{-1} glucose (23% theoretical yield) following

fermentation with a non-recombinant strain of *Saccharomyces cerevisiae* (Hernández-Salas et al. 2009). Higher ethanol yields were reported by Caspeta et al. (2014), following enzymatic hydrolysis of the bagasse of *A. tequilana* stalk at high-solids loadings following dilute acid pretreatment. Employing an industrial strain of *S. cerevisiae*, a maximum ethanol yield of 0.25 g g^{-1} of dry agave bagasse, corresponding to 86% of maximum theoretical (0.29 g g^{-1}), was attained. Also, two different strains of *S. cerevisiae* were used on juice of *A. tequilana* leaves of 2–3 year-old plants, producing 11.4–13.8 g L^{-1} ethanol corresponding to 54–66% theoretical conversion (Corbin et al. 2015).

Reviews of the literature reveal only a small number of studies that may be construed to be associated with the use of *A. tequilana* leaves for biofuel production. These studies are limited to reports on their chemical composition, cellulose characterisation, with one attempt at pretreatment and enzymatic hydrolysis (Corbin et al. 2015; Li et al. 2012b; Li et al. 2014) and bioethanol production (Corbin et al. 2015). To the knowledge of the authors, this is the first study assessing the suitability of *A. tequilana* leaf bagasse derived from plants of different age groups (1.25 and 2.5 years from Rockhampton and Kalamia respectively) and localities with different environmental conditions (Bureau of Meteorology (BOM) 2016) as feedstock for bioethanol production (Table 5.1). Specifically, this paper examines and reports on the major chemical constituents of *A. tequilana* leaf biomass and details the use of dilute acid pretreatment and enzyme saccharification options of *A. tequilana* leaf bagasse. Dilute H_2SO_4 acid pretreatment was selected as it is an effective method for hydrolysing hemicellulose, disrupting the crystalline structure of cellulose enhancing enzyme saccharification (Lee et. al 1999). Moreover, it is a method of choice for a number of lignocellulosic to ethanol operations as high hydrolysis yields have been reported with dilute H_2SO_4 pretreatment (Mosier et al. 2005). The fermentation potential of recovered sugars was also examined using an ethanol-fermenting *S. cerevisiae* strain.

Table 5.1: Geographical location, environmental conditions and age of the plant used in the study (Bureau of Meteorology, 2016).

State/country	Geographical location	Average annual rainfall (mm)	Average annual temperature (Min–Max °C)	Age of the plant (Years)
Queensland, Australia	Rockhampton, 23.32°S,150.52°E	811.2	16.7–28.4	1.25
Queensland, Australia	Kalamia, 19.58°S,147.41°E	1076.5	17.9–29.1	2.5

5.2 Materials and Methods

5.2.1 Materials

The chemicals used in these experiments were of analytical grade, obtained from Sigma chemicals Co. (St. Louis MO). Cellulase (Cellic® CTec2) was supplied by Novozymes (Bagsvaerd, Denmark).

5.2.2 Methods

Agave tequilana leaves were tested for their composition at different maturity stages. Five different pretreatment options involving combinations of various temperatures, time and acid concentration were assessed to determine the role of various pretreatment conditions in solubilisation of sugar and production of degradation or inhibitory compounds. Enzyme saccharification of pretreated material was also assessed for changes in sugar composition in response to pretreatment parameters. Finally, the hydrolysates obtained from enzyme saccharification were tested for bioethanol production.

5.2.2.1 *Agave tequilana* leaf processing

Agave tequilana leaf samples were obtained from field trials at Rockhampton (ROK) and Kalamia (KAL), Queensland, Australia. The trials at Rockhampton were established in September 2010 and those at Kalamia were started in June 2009. Mature and fully expanded leaves were sampled randomly through each plot using a long handled pruning device. Sampled leaves were washed with distilled water and cut into approximately 2 cm pieces. These were mixed and a subsample of approximately 1.5 kg fresh weight was dried in an oven at 60 °C for 96 h prior to compositional analysis. The dried samples were ground in a laboratory grinder (Mikro Feinmuhle Culatti, Janke and Kunkel GmbH and Co, Staufen, Germany) and sieved. The fraction between 150–530 µm was collected and stored in an airtight container prior to determination of structural carbohydrates and lignin.

The remaining portion of the fresh leaf pieces were blended in a mixer grinder (Bajaj-GX7, IN) to macerate the pieces, and put through a juicer (Sunbeam Café Series-JE 8600, Sunbeam Australia) to separate the juice from the bagasse. The bagasse was further squeezed to obtain the remainder of the juice which was then stored at -20°C

for further analysis by HPLC. The residual bagasse was washed with hot distilled water, squeezed and then oven dried at 60°C for 48 h. The dried bagasse was ground using a rotary mill (Retsch ZM 1000) and sieved. Fractions between 530–1100 µm were retained for subsequent pretreatment experiments.

5.2.3 Pretreatment

Five pretreatment options were selected with different combinations of H_2SO_4 acid concentration (1, 1.5, 2 and 4% v/v), time (30, 60, and 90 min) and temperature (115, 120 and 130°C) to test their combined effects on sugar recovery (Table 5.2). *Agave tequilana* bagasse sample loadings were 5% w/v in all pretreatments. Pretreatments were performed in triplicate using a Labec AA20 autoclave (Labec, Australia).

Table 5.2: Pretreatment parameters of acid concentration, time and temperature.

	Sample Code	H_2SO_4 concentration (% v/v)	Time (min)	Temp (°C)
1	A	1	30	115
2	B	1.5	60	115
3	C	2	60	120
4	D	2	90	130
5	E	4	60	120

Following each pretreatment regime, hydrolysates were separated into solid and liquid fractions using a Buchner funnel. The liquid fractions (pre-hydrolysate) were retained for sugar analysis by HPLC (analytical section). The solid fraction remaining in the Buchner funnel was further washed with 200–600 mL hot Milli-Q water (100 mL each time) until the pH reached between 4 and 5. Washed solid fractions were stored at -20°C until further analysis.

5.2.4 Enzymatic saccharification

Enzymatic saccharification of solid substrates was performed in 50 mM citrate buffer pH 5.0 containing 0.02% sodium azide. Four different enzyme doses were prepared in duplicates in a total of eight reactions for each pretreatment condition. Cellic® CTec2 (137 FPU/ml) (Novozymes) was used in the enzyme saccharification trials at concentrations of 3, 6, 10, and 15% w/w (g enz /100 g cellulose). The enzyme dose was based on the initial cellulose content of the material. In this case, cellulose content was

39% for the KAL sample, and the FPU equivalents in the enzyme saccharifications were 1.4, 2.8, 4.6 and 6.9 per g of cellulose.

After the addition of enzymes, the vials were mixed in a rotary incubator at 50 rpm and 50°C for a total of 96 h, with sampling at 24 h intervals for HPLC analysis. Samples were centrifuged immediately after removal from the incubator and stored at -20°C until further analysis. The amount of glucose and xylose recovered by enzyme saccharification was expressed as a percentage of theoretical value based on compositional analysis of glucan and xylan content (section 5.2.6 Analytical methods) of *A. tequilana* leaves.

5.2.5 Fermentation

5.2.5.1 Preparation of fermentation hydrolysates from *A. tequilana* leaf bagasse

The best pretreatment and enzymatic saccharification conditions were selected for fermentation trial. The pretreatment condition of 2% H_2SO_4, 121°C for 60 min was selected in combination with a cellulase load of 15% (g enz/g solids) to obtain maximum sugar recovery. The starting substrate for pretreatment was 90 g. Fifteen grams of bagasse was mixed with 300 ml of 2% w/v H_2SO_4 in each of six 500 ml Schott Duran bottles consisting of 5% w/v, allowed to stand for 30 min and then autoclaved for 60 min at 121°C. After pretreatment, the mixture was separated by filtration using glass fibre filter paper through a Buchner funnel. The liquid hydrolysate was collected and total volume recorded and sampled for HPLC. The recovered solids were washed with hot reverse osmosis (RO) water until a pH of approximately 5 was achieved. The washed solids were sub-sampled (1g) in duplicate and dried at 100°C to determine the DM content. The substrate had a DM content of 11.25% for KAL and 11.3% for ROK pretreated bagasse.

For enzyme saccharification, 123 g of KAL pretreated bagasse (wet) and 104 g of ROK pretreated bagasse (wet) was mixed with 200 mL citrate buffer in three 1 L sterilised Erlenmeyer flasks. Cellic® CTec2 was added at 15% w/w and flasks were incubated at 50°C and agitated at 40 rpm for 72 h. At completion, sugar hydrolysates were separated from residual solids by vacuum filtration using a Büchner funnel and Whatman® glass microfiber GF/A filters and stored at -20°C until fermentation.

5.2.5.2 Fermentation of *A. tequilana* hydrolysates

Active, dry *S. cerevisiae* (Thermosacc® Dry, Lallemand, WI, USA) was used in accordance with the manufactures instructions for shake-flask fermentation trials. Sugar hydrolysates were prepared from water-washed recovered fibre as outlined above (section 5.2.5.1). Given the relatively low glucose concentrations (*ca.* 26 g L^{-1}), glucose was added to a final concentration of about 110 g L^{-1} in the fermentation media. For leaf juice fermentations, the sugar hydrolysate was prepared as outlined in *A. tequilana* leaf processing (section 2.2.1). The fermentation media consisted of filter sterilised hydrolysate (0.22 µm, nylon filter, Millipore, MA, USA) containing 2 g L^{-1} KH_2PO_4, 1 g L^{-1} $(NH_4)_2SO_4$ and 1 g L^{-1} $MgSO_4$ and were inoculated with dried yeast at the rate of 10 g L^{-1} to initiate fermentation. Fermentations were conducted in 200 mL Schott Duran® bottles with a working volume of 150 mL and were incubated at 30°C with agitation (50 rpm). Samples were taken at regular time intervals (hourly for 13 h and at 24 h), centrifuged at 8000 *g* for 5 min, syringe filtered with a 0.45 µm Minisart® (Sartorius, Germany), and stored at -20°C for further analysis. Sugars and ethanol were quantified by HPLC.

The maximum ethanol volumetric productivity (g L^{-1} h^{-1}) was calculated from $\Delta p/\Delta t$ where Δp is the change in the ethanol concentrations over the time period Δt. The theoretical ethanol yields (Y_E) from glucose were calculated according to the following equation:

$$\textbf{\% Theoretical ethanol yield } Y_E = [E] / (0.51 \times [G]) \times 100 \quad (1)$$

Where [E] is the final ethanol concentration and [G] is the initial glucose concentration.

5.2.6 Analytical methods

Samples (10 g DW) of *A. tequilana* leaves from ROK and KAL were collected for Soxhlet extraction. The samples were subjected to H_2O extraction for 12 h and ethanol extraction for 18 h, and the extractives retained for HPLC analysis. Extractive-free solid samples were air dried and stored in airtight containers for compositional analysis (Sluiter et al. 2008a).

Specific carbohydrate and lignin contents of untreated and treated materials were determined following concentrated acid hydrolysis as described by NREL (2008b).

The carbohydrate, furan, ethanol and carboxylic acid compositions were determined using high performance liquid chromatography (HPLC). The HPLC separation system was equipped with a solvent delivery system (Controller 600 Waters, Millford, MA) consisting of an auto sampler (717, Waters) and a refractive index detector (410 differential refractometer, Waters) operated using Waters Empower® software. Carbohydrates were analysed using a RHM-monosaccharide (7.8 x 300 mm, Rezex) column fitted with a pre-column, Carbo-H guard cartridge (Rezex). A temperature of 60 °C was maintained in the column. Test samples and standards were eluted with an isocratic mobile phase consisting of degassed dilute H_2SO_4 (0.005 N H_2SO_4 prepared in Milli-Q water). The flow rate was maintained at 0.6 mL/min. The refractive index detector was maintained at 50°C (McIntosh et al. 2012; Sluiter et al. 2008b).

Peaks detected by the refractive index detector were identified by retention times and quantified by comparison with analytical standards analysed within each batch. Similarly, water and ethanol extractives were also analysed by HPLC according to the method described by Sluiter et al. (2008a). The amount of free sugars contained in the juice was also measured using HPLC following the same method to quantify structural carbohydrates.

Nitrogen content was analysed using the CN Analyser (TruMac, LECO, St. Joseph, MI). Dry homogenised sample (200 mg) was placed in open ceramic boats. EDTA containing 40.87% carbon and 9.54% nitrogen was used as a standard. Crude protein was analysed using the following equation, and a nitrogen factor (NF) of 6.25.

% Protein = % N x 6.25

5.2.7 Statistical analysis

The results were expressed as mean ± standard error (SE) of means of three replications for compositional analysis. The results of compositional analysis were also analysed using Analysis of Variance (ANOVA) using factors as cellulose, hemicellulose, lignin and crude protein. The analysis was performed using the Genstat statistical package, Version 16.1 (VSNI Ltd, UK). A value of $P \leq 0.05$ was considered as significant.
The results of pretreatment were expressed as mean ± standard error (SE) of three replications and that of enzyme saccharification were expressed as mean ± SE of two replications. Duncan's Multiple Range Test (DMRT) was used to compare the means of

different treatments. The result of fermentation was expressed as means of two separate experiments done in duplicates.

5.3 Results and Discussion

5.3.1 Compositional analysis

5.3.1.1 Composition of *A. tequilana* leaf

The compositions of ROK and KAL samples differed significantly in cellulose and hemicellulose content but were similar in lignin content (Table 5.3). The cellulose (glucan) content of *A. tequilana* varied significantly between different locations, 34.8% and 38.9% w/w from Rockhampton and Kalamia ($P < 0.01$), respectively. Similarly, the hemicellulose (xylan + arabinan) concentration also varied significantly between different locations ($P < 0.05$) and was 18% and 19.7% for ROK and KAL, respectively. However, the total lignin content composed of Acid Soluble Lignin (ASL) and Acid Insoluble Lignin (AIL) were not significantly different between localities ($P > 0.05$), at 14.8% and 14.3%, respectively, for ROK and KAL (Table 5.3). The ash content was negligible.

Table 5.3: Structural carbohydrates and lignin composition of *Agave tequilana* leaf (% DW) from two sites, Rockhampton (ROK) and Kalamia (KAL). Data represents the average value of experiments done in triplicate ± SE (n=3).

	Composition % Present study			
Component	**ROK**	**KAL**	**Li et al. (2012)**	**Corbin et al. (2015)**
Glucan (Cellulose)	34.81 ± 0.35	38.85 ± 0.35	30	49.5 ± 1.9
Xylan	16.49 ± 0.28	18.31 ± 0.12	7	11.4 ± 1.0
Arabinan	1.5 ± 0.04	1.38 ± 0.05	-	0.3 ± 0.1
Hemicellulose	17.98 ± 0.30	19.69 ± 14	-	15.8 ± 1.3
Acid soluble lignin	4.88 ± 0.10	4.67 ± 0.11	-	3.6 ± 0.3
Acid insoluble lignin	9.89 ± 0.38	9.65 ± 0.51	11.9	9.1 ± 1.4
Total lignin	14.77 ± 0.17	14.32 ± 0.31	-	12.7±1.7
Protein	8.35 ± 0.08	7.02 ± 0.02	5.6	5.8 ± 0.7
Water extractives[a]	12.48 ± 0.49	9.69 ± 0.28	14.2	-
Ethanol extractives[a]	3.89 ± 0.20	3.31 ± 0.10	3.2	-
Ash	ND*	ND*	6.4	5.5 ±1.1

ND* = Not detected, [a] Data reported are mean values of two replicates

The chemical composition of *Agave* is influenced by many factors such as species, geographical location, climate and stage of harvest or age of the plant. The glucan levels

for ROK and KAL were significantly higher than values reported by Li et al. (2012b), who showed that the leaves of *A. tequilana* contained approximately 42% structural carbohydrates, comprising 30% glucan, 7% xylan, 2% galactan and 2% arabinan in 4-5 year-old plants. The composition of K-lignin (Acid Insoluble Lignin) was found to be 9.9% for ROK and 9.7% for KAL samples, which is lower than 11.9% and 12.7 % reported by Li et al. (2012b) and Corbin et al. (2015), respectively. Moreover, the xylan content of current study samples were almost two fold higher and 60% greater than those reported by Li et al. (2012b) and Corbin et al. (2015) respectively. Likewise, crude protein levels were 8.35% and 7.02% for ROK and KAL samples, respectively (Table 5.3), which are higher than reported by Li et al. (2012b) and Corbin et al. (2015) (5.6–5.8%). We attribute plant maturity (our samples were between 1.25–2.5 years at harvest compared to the 4–5 year-old reported by Li et al. (2012b), cultivar and/or location for xylan and protein content discrepancies. Although water extractives were found to be lower than those reported by Li et al. (2012b) (12.5 and 9.7% for ROK and KAL samples, respectively), ethanol extractives were similar (Table 5.3). Although samples of *A. tequilana* (KAL) analysed in our study were from the same location to those used by Corbin et al. (2015), we attribute marked differences in composition to leaf maturity, cultivar analysed and time of harvest (wet/dry season). On the basis of higher carbohydrate content and potential ethanol yields, all subsequent investigations (pretreatment and enzyme saccharification) were conducted on KAL leaf bagasse samples.

5.3.1.2 Composition of *Agave tequilana* juice

The total sugar present in untreated and pretreated (water/60 min/121°C; 1% H_2SO_4 /60 min/121°C) agave juice are shown in Table 5.4. The total monosaccharide content of untreated juice of *A. tequilana* of ROK and KAL samples were 33.5 and 30.5 g L^{-1}, respectively, which were comparable to those reported by Corbin et al. (2015). The glucose concentration increased by 7% in the KAL sample with 1% acid and autoclaving at 121°C for 60 min. The fructose content was similar ($P > 0.05$) with or without pretreatment in the KAL sample (2.5-year-old plant). However, the fructose content was 26–27% higher ($P < 0.05$) in the ROK sample (1.25-year-old) pretreated with 1% H_2SO_4 at 121°C for 60 min and water at 121°C for 60 min as compared to untreated. This is presumably due to hydrolysis of oligosaccharides by thermal treatment (González-Cruz et al. 2011). The total sugar content (glucose and fructose) were maximised after

pretreatment with 1% H_2SO_4 at 121 C for 60 min yielding 39.6 and 31.0 g L^{-1} for ROK and KAL samples, respectively (Table 5.4). The discrepancy of the fructose content of the ROK and KAL samples after pretreatment could be due to the presence of more inulin, and/or other fructan polysaccharides in ROK sample, which were easily hydrolysed to monomeric sugar following water and mild acid pretreatment as compared to KAL sample (Ávila-Fernández et al. 2011).

Table 5.4: Sugar composition of *A. tequilana* leaf juice (g L^{-1}). Data represents mean values of two replicates.

	Pretreatment	Glucose	Fructose	Total monosaccharides	P-value
ROK	untreated	18.3 ± 0.1	15.2 ± 0.1	33.5	<0.001
	water/60 min/121°C	17.7 ± 0.1	20.9 ± 0.1	38.6	
	1% H_2SO_4/60 min/121°C	18.6 ± 0.3	20.9 ± 0.2	39.6	
KAL	untreated	16.3 ± 0.1	14.0 ± 0.1	30.3	0.003
	water/60 min/121°C	16.7 ± 0.1	13.2 ± 0.2	29.9	
	1% H_2SO_4/60 min/121°C	17.7 ± 0.2	13.3 ± 0.1	31.0	

5.3.2 Pretreatment of *A. tequilana* leaf bagasse

Using dilute acid at elevated temperature is an effective means of hydrolysing hemicellulose and liberating soluble mono/oligomers into the resulting liquors. Cellulase activity and hydrolysis efficiency usually reflects the degree of removal of hemicellulose (Sun & Cheng 2002). To evaluate the effectiveness of dilute acid pretreatment and to define the relationship between hemicellulose solubilisation and pretreatment parameters of time, temperature and acid concentration, xylose levels in pre-hydrolysate liquors from KAL bagasse were quantified (Fig. 5.1). Pretreatment A (1% H_2SO_4/30 min/115°C) was insufficient to solubilise and release the total hemicellulose content as simple sugars (Fig. 5.1). Xylose content was significantly different ($P < 0.05$) across the pretreatments with maximum yields recovered in treatment D (117.9 mg g^{-1}). Higher temperatures had greater effect on solubilisation of hemicellulose compared to acid strength or time. This is evident by incremental release of xylose levels in pretreatment conditions A and B (1–1.5 % acid at 115°C) vs C and E (2-4% acid at 120°C) vs D (2.0% acid at 130°C). The presence of glucose in pre-hydrolysate liquors suggests that disruption of the cellulose fraction has occurred with glucose yields correlating to increased acid concentration and temperature settings. Glucose release significantly increased ($P < 0.05$) in pretreatments D

and E (Fig. 5.1), with a maximum release observed in D at 37.63 mg g^{-1}. Arabinose content did not vary significantly across the pretreatments.

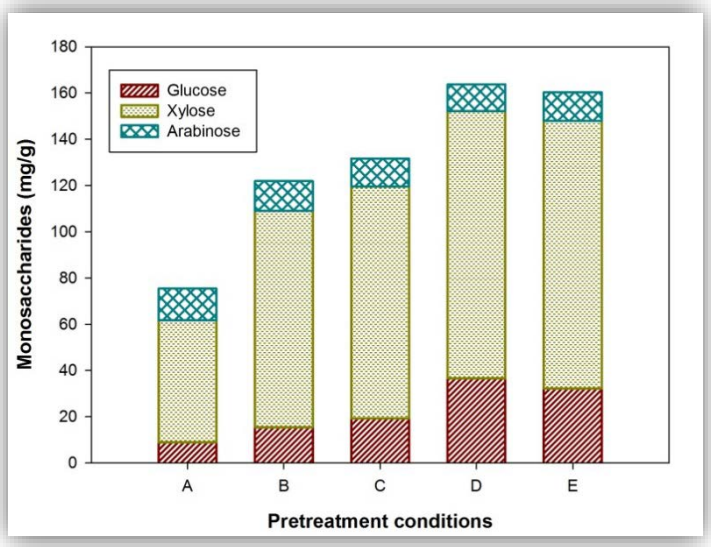


Figure 5.1: Glucose, xylose and arabinose yields (mg g^{-1} original dry matter) obtained in KAL pre-hydrolysates (5% w/v solid load) under different pretreatment conditions: A = 1% H_2SO_4/60 min/115°C; B = 1.5% H_2SO_4/60 min/120°C; C = 2% H_2SO_4/60 min/120°C; D = 2% H_2SO_4/90 min/130°C; and E = 4% H_2SO_4/60 min/120°C. Data represents average values of experiments done in triplicate.

Similar trends in sugar release are reported in several other pretreatment studies on sugarcane (Benjamin, Cheng & Görgens 2013; Laopaiboon et al. 2010; Neureiter et al. 2002) and agave bagasse (Hernández-Salas et al. 2009). For example, ionic liquid pretreatment on agave bagasse showed that increasing the temperature from 120 to 160°C was associated with higher yields of glucose and xylose levels (Perez-Pimienta et al. 2013). Likewise, pretreatment of agave leaf bagasse (*A. tequilana*, *A. salmiana* and *A. americana*) at higher temperatures resulted in greater sugar yield as compared to pretreatments with longer reaction time (Li et al. 2014). However, higher pretreatment temperatures also resulted in a concomitant rise/release in compounds such as acetate, furfural and 5-hydroxymethyl 2-furaldehyde (HMF) (Fig. 5.2).

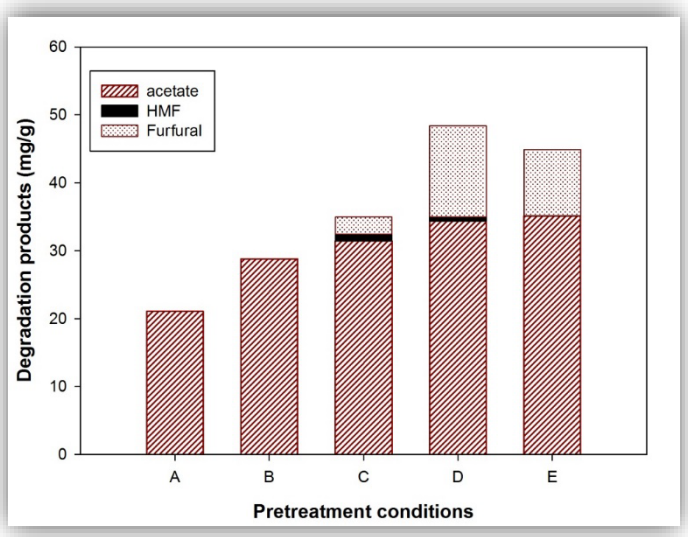


Figure 5.2: Acetate, furfural (2-furaldehyde) and HMF obtained from pretreatment of KAL leaf bagasse under the following conditions: A = 1% H_2SO_4/60 min/115°C; B = 1.5% H_2SO_4/60 min/120°C; C = 2% H_2SO_4/60 min/120°C; D = 2% H_2SO_4/90 min/130°C; and E = 4% H_2SO_4/60 min/120°C. Data represents average values of experiments done in triplicate.

Most pretreatments produce or release compounds such as furfural and HMF that negatively impact on other downstream processes such as enzymatic saccharification and fermentation. Acetate is a common by-product, which is released from lignocellulosic material as a result of de-acetylation of hemicelluloses during hydrolysis (Palmqvist & Hahn-Hägerdal 2000). Elevation of temperature and acid strength were associated with increased liberation of acetate (Fig. 5.2). The acetate content was highest in pretreatment E and significantly different ($P < 0.05$) from pretreatments A and B. However, there was no significant difference in acetate content of pretreatments C, D and E. Formation of acetate was also directly proportional to reaction time. Similarly, incremental increases in the formation of furfural at elevated temperature and acid concentration were evident. Furfural production was the highest in pretreatment D and varied significantly ($P < 0.05$) across the pretreatments (Fig. 5.2). Although HMF was not detected in pretreatments A, B and E, its content varied significantly ($P < 0.05$) in pretreatments C and D. Furfural and HMF are produced from the degradation of pentose and hexose sugars, respectively (Almeida et al. 2007; Dunlop 1948; Palmqvist & Hahn-Hägerdal 2000; Ulbricht, Northup & Thomas 1984).

Low temperatures were inadequate in terms of disrupting the lignocellulosic matrix and solubilising hemicellulose (Fig. 5.2). In essence, higher temperatures provide better disruption of cellulose and greater solubilisation of hemicellulose and maximised pentose

sugar recovery, but at the same time produce more degradation and inhibitory compounds (Palmqvist & Hahn-Hägerdal 2000). Likewise, high acid concentration and temperature combinations produced more inhibitory compounds than low acid concentration. Therefore, the pretreatment conditions with mild acid at moderate temperature (i.e., pretreatment C) can be considered the most favourable for generating pentose sugar streams from *A. tequilana* bagasse for fermentations. This recommendation is similar to that of Saucedo-Luna et al. (2011) who found the optimal condition for acid hydrolysis of *A. tequilana* stem bagasse to be 2% H_2SO_4 for 15 min at 147°C, recovering 24.9 g L^{-1} fermentable sugar (equivalent to 36% saccharifiable material), with the formation of less than 1 g L^{-1} degradation products such as furfural and HMF. Similar findings have been reported for wheat stubble, eucalypt thinnings and sugarcane bagasse (McIntosh et al. 2012; Neureiter et al. 2002; Vancov & McIntosh 2011).

5.3.3 Enzymatic saccharification of pretreated bagasse

Enzyme saccharification is a key biochemical process, which has a major effect on overall cost in biofuel production (Humbrid et al. 2011). Therefore, enzyme saccharification processing is designed according to the feedstock type and pretreatment regime to achieve near maximum sugar yields; i.e., release of glucose from the cellulose fraction (Klinke, Thomsen & Ahring 2004; Merino & Cherry 2007). Iso-dosing trials at four different enzyme concentrations (3%, 6%, 10% and 15% of Cellic® CTec2) were undertaken to evaluate the effectiveness of the pretreatment regime and determine the appropriate enzyme dose for maximising glucose recovery.

Glucose recovery was significantly different in all digests except for pretreatment B vs D, and E and C vs E, with the following order of highest to lowest recovery: pretreatment C >E >D >B and A (Fig. 5.3). There was no significant difference in the glucose recovery between 48 and 72 h ($P > 0.05$), but the recovery was significantly lower at 24 h as compared to 96 h. Pretreatment C enzyme digests did not differ significantly ($P > 0.05$) in glucose release with enzyme loads of 6%, 10% and 15% during 48 to 96 h; however, at an enzyme load of 3% glucose yields significantly declined (Fig. 5.3 C-I). The glucose recovered after 48 hours with 6% enzyme load was 253.8 mg g^{-1} (65.3% theoretical) whilst the maximum (267.9 mg g^{-1}, 69% theoretical) was released following 96 h of digestion at a 15% enzyme load (Fig. 5.3 C-I).

Pretreatment and enzyme digestion studies of agaves are seldom reported in the literature. Hernández-Salas et al. (2009) reported on the poor release of reducing sugars from HCl (1.2% v/v) pretreated *A. atrovirens* pinecone (metzal-5%) and pinecone with leaves (metzonette-10%). However, when these bagasse samples were treated with 2% NaOH and hydrolysed by different enzyme preparations, Cellucast and Viscozyme were found to release high reducing sugar yields of up to 58% and 36% from metzal and metzonette, respectively. In contrast, the study conducted by Saucedo-Luna et al. (2011) recovered 36% theoretical sugars following dilute sulfuric acid treatment of *A. tequilana* bagasse, which increased to 61.5% after enzyme saccharification (10% cellulose load) for 72 h.

A maximum glucose yield of 69% achieved in this study appears slightly better than those reported by Saucedo-Luna et al. (2011), although it required marginally higher enzyme loads (15%) and longer durations (96 h). However, from Fig. 5.3 C-I, it is evident that a reduction in both enzyme load (6%) and duration (48 h) resulted in similar glucose recoveries (65.3%). In the study conducted by Saucedo-Luna et al. (2011), the enzymatic hydrolysis of stem bagasse of *A. atrovirens* pretreated with 1-3 % H_2SO_4 and hydrolysed using a commercial enzyme mixture consisting of cellulase-β-glucosidase mixture recovered 61.5 % fermentable sugars in 72 hours. Similarly, in a study conducted by Hernandez –Sala et al. (2009), alkaline-enzymatic hydrolysis using (2% w/v) NaOH and (20% w/w) viscozyme (multienzyme complex preparation of arabinase, β-glucanase, hemicellulase, cellulase and xylanase) produced a reducing sugar of 58% in 4 hours. Although widely used, quantifying sugar content via reducing sugar estimations is comparatively inaccurate to that of HPLC analysis, as reducing sugar assays tend to overestimate the true concentrations (Nguyen & Player 1997). Therefore, glucose recovery of 63% achieved within 24 h with a 6% cellulase load suggest that H_2SO_4 pretreatment in this study is superior to those described by Saucedo-Luna et al. (2011) and Hernández-Salas et al. (2009).

Saccharification data (Fig. 5.3 -II) also revealed that xylose was still present post pretreatment and was inversely proportional to pretreatment severity (temperature and acid levels). The order from highest to lowest is pretreatment A >B >C >E and D.
In pretreatment A with 15% enzyme load, a maximum of 29.9 mg g^{-1} (15.7% theoretical) xylose was released after 96 h (Fig. 5.3 A-II). In enzyme digests with pretreatment C, xylose recovery with 6% enzyme at 48 h was only 6.2 mg g^{-1} (3.4% theoretical) and was

consistent at 72 and 96 h. Increasing the enzyme load to 15% enzyme produced slightly more xylose (9.6 mg g^{-1} corresponding to 5.2% theoretical). Similar trends were evident for remaining digests (pretreatments B, D and E). Arabinose release was not detected post enzymatic hydrolysis and is consistent with studies reported for other biomass residues such as wheat stubble, sorghum, cotton gin-trash and feedlot cattle manure (McIntosh et al. 2014; Vancov & McIntosh 2011; Vancov & McIntosh 2012; Vancov et al. 2015). Release of residual hemicellulose sugars in the remaining pretreated material is attributed to the CTec2 cellulase blend, which is tailored for lignocellulosic hydrolysis, containing a combination of cellulases, xylanases and ancillary glycosyl hydrolyases (Ju et al. 2014).

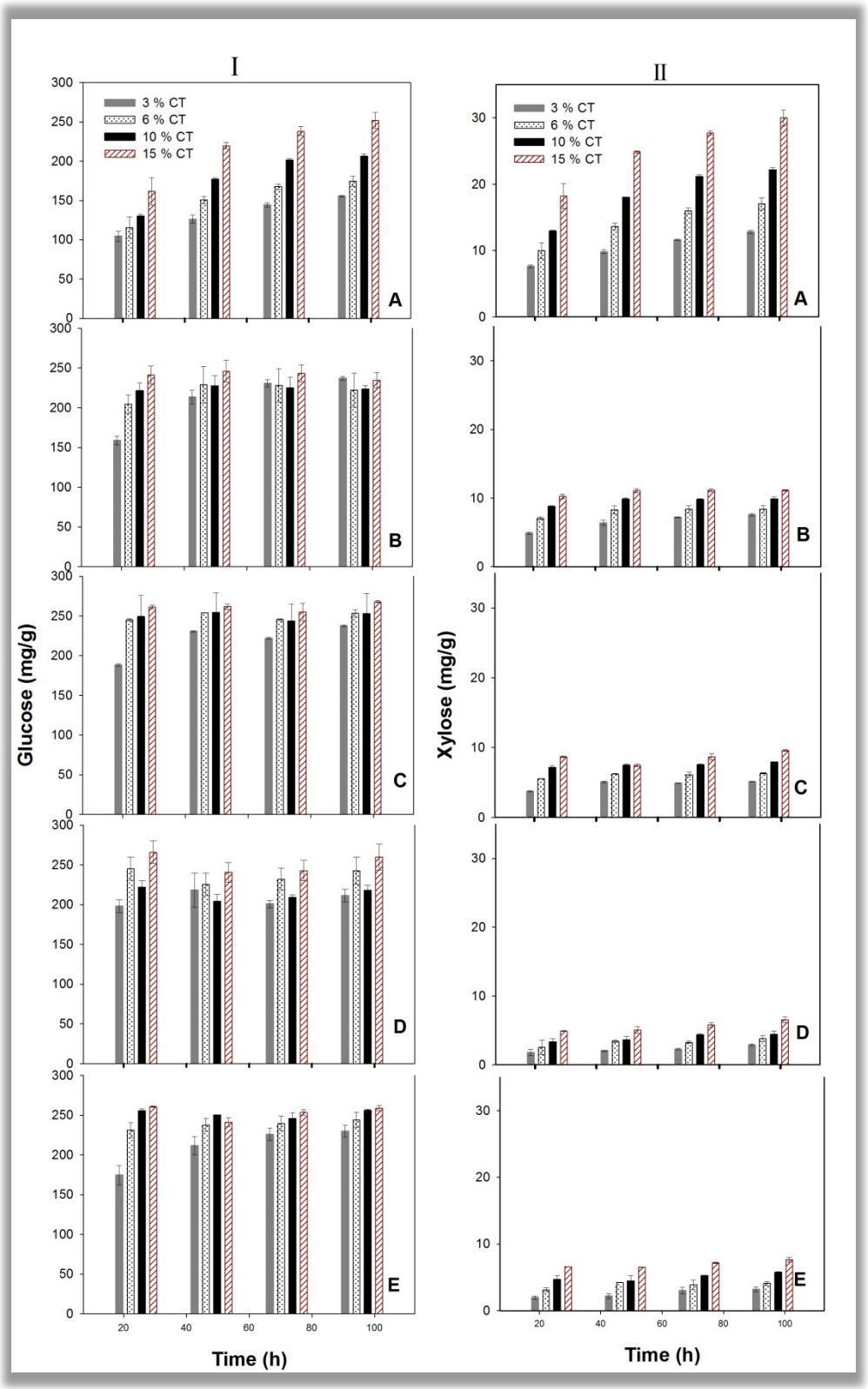


Figure 5.3: Glucose (I) and xylose (II) release from pretreated *A. tequilana* (Kalamia) bagasse as a function of enzyme dose. Conditions of pretreatments are: A = 1% H_2SO_4/60 min/115°C; B = 1.5% H_2SO_4/60 min/120°C; C = 2% H_2SO_4/60 min/120°C; D = 2% H_2SO_4/90 min/130°C; E = 4% H_2SO_4/60 min/120°C. The respective Cellic® CTec2 (CT) doses were 3%, 6%, 10% and 15% per 100 g of initial cellulose. Bars represent ± SE, (n=2).

5.3.4 Combined glucose yield subjected to different enzyme doses

The combined total glucose yield from pretreatment conditions subjected to 6% (medium) and 15% (high) enzyme dose are presented in Fig. 5.4. The total glucose yield was lowest in treatment A (Fig. 5.4-I), yielding only 41% theoretical glucose recovery followed by treatments B, D and E (62, 67 and 69% theoretical glucose, respectively). In this study, the maximum combined sugar yield was obtained in treatment C with moderate severity with 273 mg g^{-1} (*ca.* 70% theoretical). This result is similar to that reported by Saucedo-Luna et al. (2011), who pretreated *A. tequilana* stem bagasse with 2% H_2SO_4 followed by 6% cellulase-glucosidase enzyme digestion and produced up to 80% fermentable sugars. Differences in agave species and origin, growth conditions, maturity, source of agave substrate (leaf vs stem) and cellulase blends presumably accounts for the discrepancies in sugar recoveries between this study and those reported by Saucedo-Luna et al. (2011).

The milder pretreatments A and B acquiesce only 67 and 64% of the theoretical yield (Fig. 5.4-II). The maximum total glucose yield (76% theoretical) was achieved from material pretreated in 2% H_2SO_4 for 90 min at 130°C (treatment D), although similar recoveries of 74 and 75% were achieved with treatments C and E. In essence, doubling the cellulase load and the duration of saccharification has delivered minimal gains in glucose release.

Total sugar yield increased as pretreatment severity intensified, with temperature having greater impact over acid strength and time. This result is consistent with other crop residues such as sorghum and wheat stubble, which also benefited by harsher process conditions (Vancov & McIntosh 2011; Vancov & McIntosh 2012). In these studies, temperature had a greater impact on sugar yield, followed by acid strength and reaction times. Total sugar yield from sorghum straw improved by approx. 33% when enzyme dose (cellulase and β-glucosidase) was raised 6-fold. Although the saccharification was run for 63 hours, 83% theoretical recovery was obtained within 14 hours (Vancov & McIntosh 2012).

Similar results were obtained in our study where total glucose yields increased incrementally with enzyme dose (6 to 15%), irrespective of pretreatment conditions (Fig. 5.4 I and II). Overall, moderate pretreatment conditions with 2% H_2SO_4 at 120°C for 60 min (pretreatment C) also produced similar results, producing up to 74% theoretical yield. To minimise cost without significantly compromising yield, enzymatic

hydrolysis using 6% CTec2 for 48 h is recommended for a yield of 70% theoretical glucose with minimal degradation products (Figs 5.2 and 5.4- I).

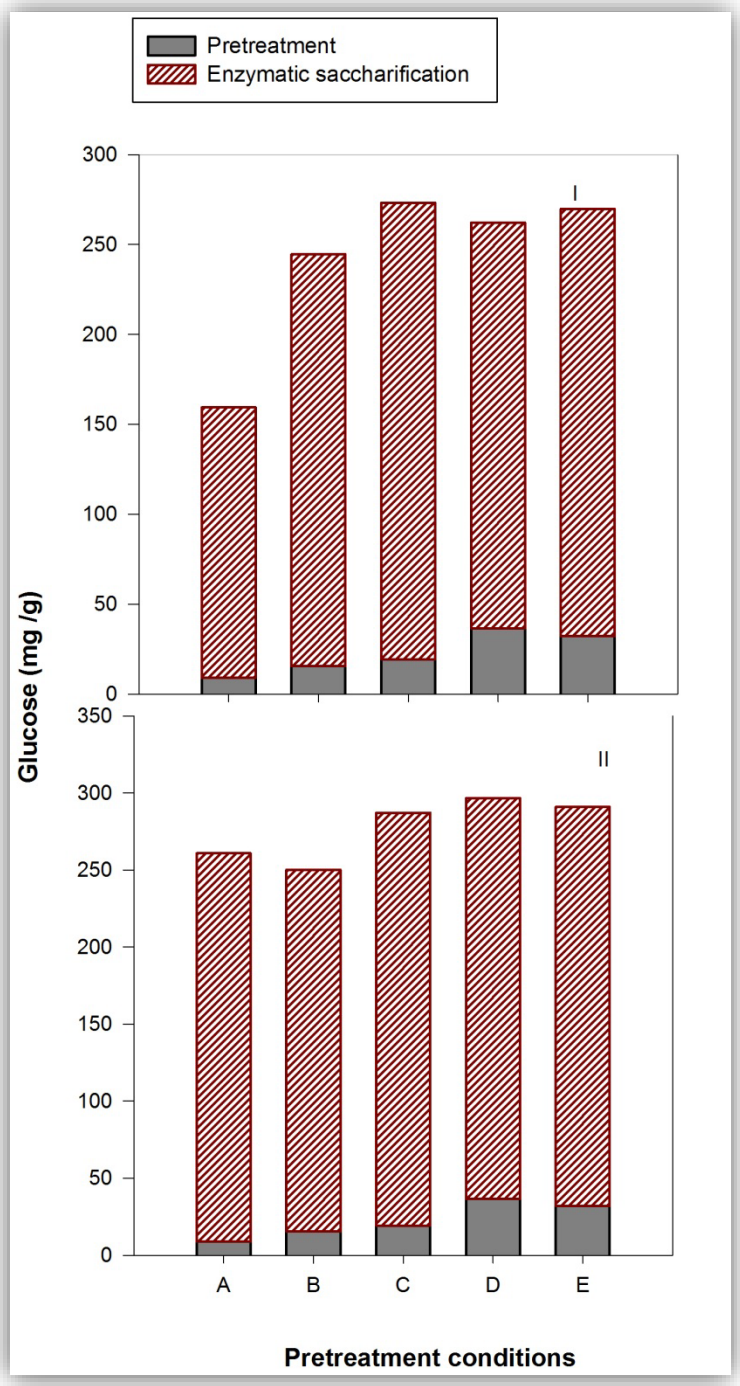


Figure 5.4: Total glucose yield from different pretreatment and enzyme saccharification regimes at 48 h (I) and 96 h (II). Conditions A = 1% H_2SO_4/60 min/115°C; B = 1.5% H_2SO_4/60 min/120°C; C = 2% H_2SO_4/60 min/120°C; D = 2% H_2SO_4/90 min/130°C; and E = 4% H_2SO_4/60 min/120°C. Cellic® CTec2 at 6 wt. %, was used for up to 48 hours and 15 wt. % was used for up to 96 hours.

5.3.5 Fermentation of *A. tequilana* sugar hydrolysates from leaf bagasse and juice.

5.3.5.1 *A. tequilana* leaf bagasse

The ethanol fermentation potential of sugar hydrolysates resulting from processed agave bagasse were evaluated using the commercial *S. cerevisiae* strain Thermosacc® Dry. Although this strain does not ferment xylose, the utility of Thermosacc® Dry in C6 sugar lignocellulosic hydrolysate fermentations has previously been demonstrated (Vancov et al. 2015). In order to minimize toxicity during fermentation, pretreatment and enzyme saccharification conditions which yielded high glucose but low xylose levels were used to produce the hydrolysate for fermentation. That is, agave leaf bagasse was pretreated at 5% load for 60 min with 2.0% H_2SO_4 at 121°C, separated and washed with water to remove toxic inhibitors prior to enzyme saccharification with CTec2. Total glucose recovered from pretreated and enzyme saccharified ROK and KAL were 25.4 and 26.8 g L^{-1} respectively. With the intention of simulating industrial fermentation conditions, glucose was added to a final concentration of about 110 g L^{-1} in the fermentation hydrolysate. In fermentations conducted on leaf juice of *A. fourcroydes*, hydrolysates were also supplemented with molasses to attain 12° Brix (Cáceres-Farfán et al. 2008).

Under glucose-fortified conditions, the fermentations were completed within 13 h yielding 38.3 and 38.6 g L^{-1} ethanol for KAL and ROK hydrolysates, respectively. The overall maximum ethanol volumetric productivity for KAL and ROK samples were similar at 2.9 g L^{-1} h^{-1} (Figs 5.5 A and B). Analysis of variance failed to show any significant difference ($P < 0.05$) in glucose consumption and ethanol production over time between hydrolysates derived from plants from the two sites, despite differences in plant maturity and growth conditions at ROK and KAL. The ethanol yields from both KAL and ROK hydrolysates correspond to *ca.* 68% theoretical, and are higher than those reported for *A. atrovirens* and *A. tequilana* (Hernández-Salas et al. 2009; Saucedo-Luna et al. 2011).

Hernández-Salas et al. (2009) reported ethanol yields of 6.5 g L^{-1} from 19.45 g L^{-1} reducing sugar (65.5%) following fermentation of *A. atrovirens* stem and leaf bagasse hydrolysates pretreated with dilute HCl. However, with alkaline pretreatment and enzymatic hydrolysis, ethanol yields declined to 43.3% theoretical. In another study,

A. tequilana hydrolysates obtained from final optimised acid plus excess enzymatic (100 FPU/g of total solids) process treatment generated 8.99 g ethanol/50 g of bagasse (*ca.* 57% theoretical) when fermented with the xylose-utilising yeast *P. carribica* (UM-5) (Saucedo-Luna et al. 2011).

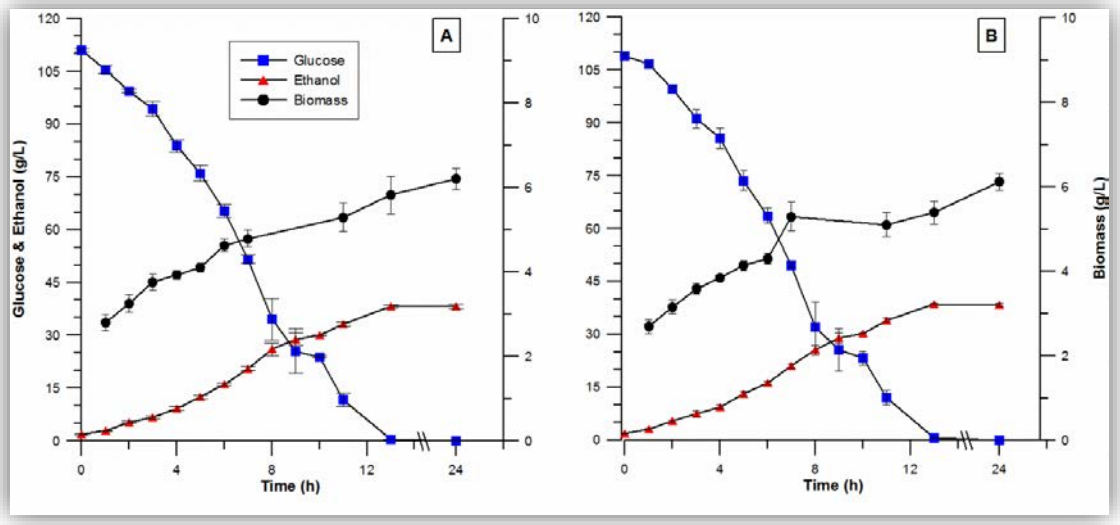


Figure 5.5: Fermentation profile of *S. cerevisiae* (Thermosacc® Dry) in KAL (A) and ROK (B) hydrolysate of *A. tequilana* leaf bagasse prepared by pretreating 5% solids (w/v) with 2% H_2SO_4, for 60 min at 121°C followed by enzyme saccharification (50°C, pH 5 for 72 h) with Cellic CTec2 (15%). Data represents averages of two separate experiments; bars indicate SE.

Based on glucose recovery data from the optimised pretreatment conditions reported in this study (*ca.* 76% theoretical) and assuming 68% conversion to ethanol, calculated fermentation yields for ROK and KAL are 117 and 131 L, respectively, of ethanol per metric tonne of agave leaf bagasse. Assuming similar conversion efficiency for xylose (122 and 134 kg t^{-1} release for ROK and KAL, respectively), an additional 54 and 59 L of ethanol (*ca.* total of 171 and 190 L) per metric tonne of ROK and KAL bagasse, respectively, may be achieved. Improving ethanol yields via C5/C6 co-fermentation with recombinant xylose fermenting yeast is subject to further studies.

5.3.5.2 *Agave tequilana* leaf juice

The juice of *A. tequilana* leaves consist of a range of different sugars in various forms and includes glucose, fructose (as inulin), sucrose and other sugar oligomers (Corbin et al. 2015; Li et al. 2012b). Ethanol fermentations were conducted in shake flasks with ROK and KAL juice hydrolysates, which contained approximately 17 g L^{-1} glucose and 22 g L^{-}

[1] fructose. Profiles of the shake-flask fermentations are presented in Figs 5.6 A and B and show that both sugars are fermented within 7 h.

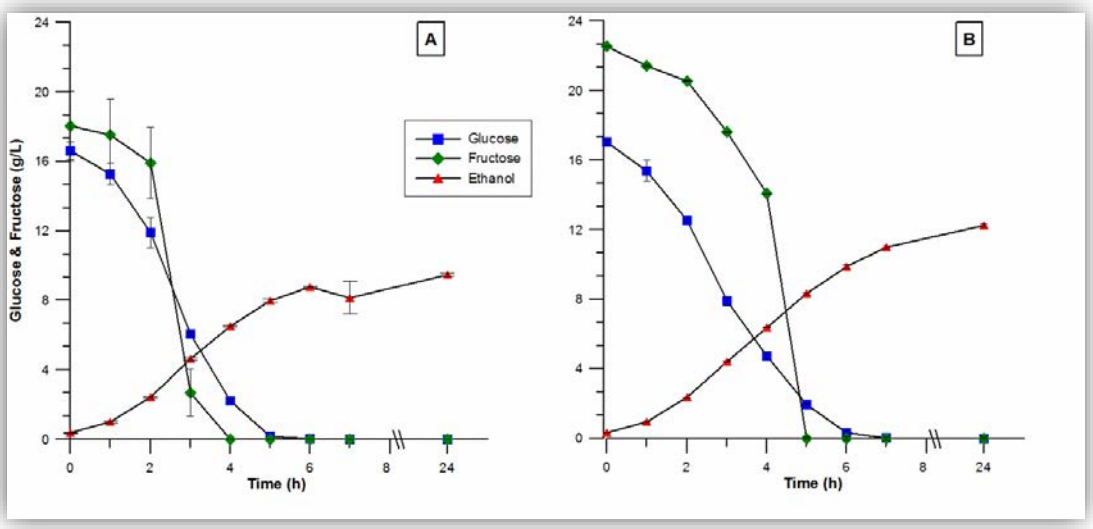


Figure 5.6: Fermentation profile of *S. cerevisiae* (Thermosacc® Dry) of *A. tequilana* KAL (A) and ROK (B) leaf juice. Data represents averages of two separate experiments; bars indicate SE.

The rapid metabolism of glucose in both juice hydrolysates was completed within 5 h; however, the metabolism of fructose required an addition 2 h to reach completion. Under these circumstances, fermentations were completed within 7 h, yielding 12.44 and 9.6 g L^{-1} ethanol for ROK and KAL juices, respectively, with overall maximum ethanol volumetric productivities of 1.77 and 1.37 g L^{-1} h^{-1}. This corresponds to ethanol yields of *ca.* 61 and 48 % theoretical for ROK and KAL, respectively. The discrepancy in ethanol yield between the samples is attributed mainly to the presence of inhibitory compounds such as saponins (Monterrosas-Brisson et al. 2013; Yokosuka & Mimaki 2009). Saponins are generally found in increasing amounts with plant maturity (Pinos-Rodríguez et al. 2009). Kalamia fermentation juice hydrolysate was extracted from 2.5-year-old plants, compared to 1.25 years for ROK.

The results of this study are consistent with those reported on fermentations of thermal acid-treated *A. fourcroydes* juice (Villegas-Silva et al. 2014). Villegas-Silva et al. (2014) initially reported that *Kluveromyces marxianus* produced up to 13.7 g L^{-1} ethanol from treated *A. fourcroydes* juice (50% theoretical), and then showed that the yield could be substantially increased (80%) by liberating fructose from inulin via enzyme (inulinase) saccharification. Although not determined in the present study, the juice of *A. tequilana*

consists of other sugars such as inulin and oligofructans (Li et al. 2012b), which upon inulinase saccharification would lead to higher sugar and ethanol yields. Nevertheless, the outcomes of this study clearly demonstrate that the juice from *A. tequilana* leaves can be fermented directly without additional treatments and should be genuinely considered as a feedstock for ethanol production.

5.4 Conclusion

During the course of this work, agave bagasse treated with 2% H_2SO_4, 60 min at 120°C was deemed as the best pretreatment option in terms of relatively high sugar recovery with minimal release of inhibitory compounds. Out of four different enzyme doses, a 6 % load was sufficient to recover maximum glucose under these pretreatment conditions. *Agave tequilana* leaves are a potentially useful biomass feedstock for production of second generation biofuels. They are amendable to mild acid pretreatment and enzyme saccharification with total sugar conversions approaching 76% theoretical. Fermentation trials with ensuing C6 hydrolysates validated the practicality of converting agave bagasse and juice into ethanol (68 and 61% efficiency, respectively). With further developments (i.e., fermentation of C5 sugars and or inulinase digestion), this process could deliver greater yields.

Chapter 6. Summary and future directions

6.1 Summary

Targets for ethanol use in transport fuel have been set by the Queensland and NSW governments, leading to the consideration of alternative sources for feedstocks for biofuel production. The characteristics of *Agave* species, such as water use efficiency and being a non-food crop, and its historical use in the production of alcoholic beverages such as Tequila and Mezcal make this genus worthy of considering as a highly potential biomass feedstock. Traditionally, only stem has been used in the production of Tequila or Mezcal. However, the leaves, which account for 38–55% of the above ground biomass, can also be used as the potential sources of bioethanol feedstock, as these leaves contain high cellulose and low lignin.

With *Agave* cropping being novel in Australia, its commercial viability is yet to be determined. The following questions were raised in the context of the suitability of *Agave* as a biofuel feedstock:

1. What species/cultivar of Agave is most appropriate for biofuel production in Australia?
2. What rate of biomass accumulation can be achieved?
3. What agronomic practices should be used for Agave cultivation, growth and harvest?
4. Does the leaf composition change with plant maturity?
5. Is there an optimal harvest time? Can the leaf composition be rapidly assessed?
6. What processes might be recommended for bioethanol production from Agave?

The answers to these issues will address the technical and commercial feasibility of *Agave* as a feedstock in Australia.

Chapter 2 described the growth potential of *F. foetida*, ten species of *Agave* and six cultivars of *A. tequilana* in the CQ region. From this study, five species, viz., *F. foetida*, *A. tequilana*, *A. decipiens*, *A. americana* and *A. sisalana*, were identified as the fastest growing species, based on number of leaves unfolding and on the AGB production comparable to other commonly used feedstocks.

Of the six cultivars of *A. tequilana* tested, the three fastest growing cultivars, in terms of maximum number of leaves unfolding and biomass accumulation, were Tcqu, 17 and L19. The AGB production of 12.9 and 14 t ha^{-1} yr^{-1} were recorded from two geographically separate sites (based on the harvest at the end of 5 years of growth). Higher yields, of up to 25 t ha^{-1} yr^{-1}, are reported from Mexico, indicating that either climatic conditions or the agronomic conditions used were limiting these cultivars to express their maximal yield potential.

Of 11 genotypes of *Agave* tested, *F. foetida*, *A. tequilana*, *A. decipiens*, *A. sisalana* and *A.americana* are recommended for biofuel production based on their biomass accumulation. Among these, *A. tequilana* is the most studied species, as it has been cultivated for decades in Mexico for production of Tequila or mezcal. Furthermore, *A. tequilana* has lower saponin content compared to other *Agave* species (Mielenz et al. 2015). Thus, the use of *A. tequilana* is recommended in Australia for rapid establishment of an *Agave*-based ethanol industry, with the additional advantage of using leaves in cellulosic second generation ethanol production.

Saponins have antifungal and antimicrobial properties. As a result, they will inhibit the fermentation process. This inhibitory effect can be minimised by adding inulinase enzyme during the hydrolysis of pretreated agave bagasse. Inulinase is reported to contain a saponinase-like function with hydrolysis of saponins serving as a detoxification agent (Bernards et al. 2011; Mielenz et al. 2015). However, given that the cost of enzyme is approximately 16% of the total cost of ethanol production (Voegele 2013), it would be more appropriate in the longer term to select *Agave* genotypes that have low saponin content, preferably amongst the lines of the fast growing species identified in this study.

The ability of all species/varieties to withstand saturated conditions (~556 mm rainfall in a month) and high wind (including a category 3 cyclone) demonstrated the capacity of these species to handle hostile environmental conditions. Plants of all genotypes were disease-free, except the cultivars E9 and L9 of *A. tequilana*, which were affected by *F. oxysporium* (leaf curl disease) (Pers comm. Dr Noel Sammon, 2013).

Agave tequilana plants that were grown with the use of either offshoots or seedlings were comparable in terms of their biomass accumulation with those propagated through tissue culture. As a low cost technique, propagation via the use of offshoots is recommended, provided the soils from which the stocks are harvested are free from diseases. For rapid multiplication, tissue culture methods can be relied upon for propagation.

In Chapters 3 and 4, the leaves of *A. tequilana* cultivars Tcqu and L19 were assessed for DM content, TSS and tissue compositions (cellulose, hemicellulose and lignin) at intervals of 4–6 months over a period of 15 months from May 2012 to August 2013. In this process, NREL laboratory methods were used, in addition to a non-destructive method of NIR spectroscopy. Based on these studies, the use of a field-portable short wave NIR spectroscope is recommended for in-field estimation of DM and TSS of intact leaves. Dry matter content is an index of total structural and non-structural carbohydrate content, and this attribute is relevant to making decisions on the right time to harvest leaves to maximise ethanol production. Two different spectrometer technologies, viz., the FTNIR and scanning grating, were compared for assessment of cellulose, hemicellulose and lignin content of dried leaf powder. Of these, the FTNIR technology is recommended.

To prove predictive performance of an instrument, a robust calibration model should be developed using plant populations that have been raised in different locations and grown with differing agronomic conditions at varying stages of their physiological maturity.

Finally, Chapter 5 described the potential of processing *A. tequilana* leaves for bioethanol production from two sites: Rockhampton and Kalamia. There was a significant difference ($P < 0.05$) between the two sites for cellulose (34.8–38.9% w/w) and hemicellulose (xylan + arabinan) (18–19.7% w/w) compositions, but no apparent differences were noted between the sites for lignin (14.3–14.8% w/w) and soluble carbohydrate compositions (3 and 3.3% w/v) ($P > 0.05$) (Rijal et al. 2016).

Pretreatment was necessary for the release of hexose and pentose sugars present in the agave leaf matrix. Dilute acid pretreatment was optimised for maximal monosaccharide yield, and ensured that minimum degradation products (e.g., HMF and furfural) were produced during the process. Optimised pretreatment for agave bagasse consisted of 2% v/v H_2SO_4 for 60 min at 121°C, followed by enzyme hydrolysis. Determination of the appropriate dose of enzyme is another key factor in the production of ethanol. Of four enzyme doses of Cellic Ctec2 investigated, a 6% w/v load gave maximum recovery of glucose under optimised pretreatment conditions. These results could serve as a foundation for the development of future pilot-scale demonstrations.

The fermentation of *A. tequilana* leaf bagasse and juice using an industrial strain of *S. cerevisiae* achieved glucose to ethanol conversion efficiency of 68 and 61% respectively. Based on this study, ethanol yields of up to 180 L t^{-1} DW of agave leaves can be obtained. This corresponds to an ethanol yield of 1269 L ha^{-1} yr^{-1} (based on 4.8 years of growth) using only the leaves. If the above ground whole plant biomass is considered, up to 3447 L ha^{-1} yr^{-1} (based on 4.8 years of growth) could be produced. This yield is comparable to corn, sugarcane bagasse and poplar-based ethanol (see Table 1.1, Chapter 1).

For conversion of *Agave* biomass to ethanol, greater yields could be obtained by using a strain of yeast that can ferment both C5 and C6 sugars. Furthermore, the sugar recovery could be further increased by using the enzyme inulinase, which has the potential to release more sugars from oligosaccharides present in the leaves.

6.2 Considerations for Establishment of an *Agave* Industry

6.2.1 Genotype selection

In this thesis, *A. tequilana* was selected for compositional analysis and processing for bioethanol production. Further research is warranted on the testing of fast growing species identified in this study, including *F. foetida*, *A. decipiens*, *A. sisalana* and *A. americana*, with an emphasis placed on selecting species or provenances that contain low saponin and high cellulose content. The saponin content was not identified in the current study due to time limitation.

6.2.2 Recommendation on *Agave* agronomy

Sugarcane production involves a central processing mill and is based on the system where the sugarcane price is determined by both the cane yield and sugar content of the cane (e.g., commercial cane sugar content; CCS). The timing of sugarcane harvest is therefore determined by the CCS value and the biomass. A similar system for *Agave* is recommended, with harvest timing guided by the use of a hand-held SWNIR spectroscopy instrument for estimation of leaf dry matter content. The FTNIR technology is recommended for use within processing plants to assess biomass composition in terms of cellulose, hemicellulose and lignin, to optimise production of bioethanol.

In Tequila production, *A. tequilana* plants are grown to the stage just prior to the emergence of inflorescence (up to 7 years). This prolonged growth is envisaged in order to infuse a certain flavour in the resulting Tequila. Leaf pruning is also practiced, as farmers believe that pruning increases the size of piña, although this belief is not supported by published literature (Nobel 2003). By pruning leaves, the photosynthetically active radiation available to the remaining leaves can be increased, thus leading to an increased nocturnal acid accumulation (Nobel 2003). It is claimed that removal of 8% and 20% of the leaf area can increase the nocturnal acid accumulation by 9% and 21%, respectively (Nobel 2003). On the other hand, in *A. salmiana*, pruning the leaf area by 40% decreased the number of new leaves unfolding by 30%, as compared to the unpruned control, thereby decreasing the overall productivity (Nobel 2003).

For bioethanol production, leaves from lower whorls of the plant could be harvested after 2 to 3 years of planting, as cellulose content of the leaves is higher at this stage (Chapters 4 and 5). Stems need only be harvested at around 4–7 years to achieve maximum biomass accumulation rates per annum. In the *Agave* genotypes grown for this study, approximately 40–45% of the leaves, mainly from lower whorls, were dead at the time of harvest (Chapter 2). Thus, harvesting these leaves before they dry out and or decay could recover more biomass. Conversely, harvesting of lower leaves could contribute to low rates of leaf unfolding or reduced rates of carbohydrate accumulation in the stem. Nevertheless, it is likely that a certain rate of leaf removal is unlikely to affect the above processes, and this pruning rate needs to be determined from *A. tequilana* grown in different agroclimatic conditions. In Mexico, planting and harvesting involve manual labour. This will not be economical in the Australian context for bioethanol production.

Mechanised harvesters must be devised for collecting both the leaves and the stems from the field and AusAgave P/L is currently pursuing this research (pers. comm. Don Chambers, 2016). Mechanical harvesting of the whole plant is relatively straightforward, but selective leaf harvesting should avoid damage to the mother plant. Mechanical cutting of the leaves at 10–15 cm above the leaf bases can be complicated, as the tractors need to be modified and the planting distances optimised. A planting spacing of at least 3 m between the rows and 2 m between the plants seem to be appropriate for *A. tequilana*, both to maximise its biomass accumulation and to allow mechanical harvesting.

In sugarcane, the wounded stalk is susceptible to microbial infection; thus, the sugarcane must be processed within 16 hours of harvest (Canegrowers 2016). *Agave* feedstock contains only a small percentage (3–4% w/v) of readily fermentable soluble sugars in the leaf, as compared to sugarcane (10–14% w/v) and thus it should not be as susceptive to fungal infection following harvest. This would be an advantage in using agave feedstock. The agave stems consists of 20–28% w/w (FW) soluble carbohydrates, but they are present mostly in the form of fructans and inulin, hydrolysed to simple sugars only when the feedstock is cooked in ovens. Therefore, rapid processing may not be required for agave feedstock for bioethanol production. For Tequila production in Mexico, the piña are transported to the factories as soon as they are harvested to avoid weight loss, as the farmers are paid according to the fresh weight of the piña and not based on inulin content (Cedeño Cruz & Alvarez-Jacobs 1999) rather than because of concerns with respect to fungal growth.

6.2.3 Land requirement for *Agave* production

Identification of appropriate growing areas is required to capitalise on the information generated in this study. Agaves can be grown without irrigation on an average annual rainfall of 400–800 mm, average maximum day temperatures between 25–35°C and minimum night-time temperatures between 5–15°C, preferably on well drained volcanic soils (Luvisol, Lithosols or Cambisols) that are slightly acidic (Nobel et al. 1998). The ability of these plants to tolerate extreme climatic conditions (maximum of 50°C and minimum -4°C) (Nobel 2003) provides an opportunity for its establishment on marginal cropping land in Australia. It is recommended that *Agave* be grown in areas adjacent to sugarcane production zones, along the eastern coast of Australia, particularly in the vicinity of sugar mills between Grafton in northern NSW to Mossman in far north

Queensland (Holtum et al. 2010; Canegrowers 2016). This would ensure that high plant productivity is maintained and sugar mills that remain idle for six months of the year (ACFA 2016) are utilised for ethanol production from *Agave*. This will also minimise transportation costs and avoid the high infrastructure costs that are needed for initial set up of the bioethanol industry.

Alternatively, degraded landscapes such as decommissioned mine sites that are located in the above mentioned agroclimatic zones and soils that are conducive to *Agave* growth may be planted with selected fast growing *Agave* species. If substantial areas of *Agave* can be planted, it might warrant the establishment of new processing plants. Such a scenario is unlikely to occur unless the crude oil prices reach the order of $100 a barrel. This approach, however, requires a long-term commitment to entrepreneurs, as significant costs are needed to set up such an industry. This approach also requires the support of national and state governments such as the Queensland and NSW governments (Fair trading NSW 2015; Queensland Government 2016).

The 3% and 6% v/v ethanol mandate of the Queensland and NSW governments, based on a consumption rate of 18.7 BL of petrol in 2012–2013, will require a total of 1683 ML of ethanol. Only 265 ML of ethanol was produced Australia wide in 2014 (BREE 2014). In this study, an agave leaf production rate of 7.1 t ha^{-1} yr^{-1} and an ethanol conversion efficiency of 180 L t^{-1} from leaf was recorded. Assuming the use of both whole leaf and stem biomass, at the average conversion efficiency of 374 L t^{-1} for stem and 180 L t^{-1} for leaf at a total productivity of 12.85 t ha^{-1} yr^{-1}, the land area required will be 488,251 ha. In comparison, sugarcane is currently cultivated on 380,000 ha of land (ASMC 2016a). The above estimation suggests that *Agave* alone cannot provide the entire supply of bioethanol. However, if *Agave* can be grown in the areas adjacent to sugarcane belts in areas equivalent to 20% of the area of the sugarcane belt (76,000 ha), then 262 ML of bioethanol can be produced. This will then contribute to 16% of the requirement of the government mandate. This is feasible under the current crude oil price and this option becomes more attractive if the crude oil price increases due to depletion of current oil resources.

The total production capacity from the three existing ethanol-producing plants in Australia (using sugarcane molasses, red sorghum and waste wheat starch as feedstock) is 440 ML (BREE 2014). In order to supply a gap of 1243 ML, and assuming the full

production capacity of 440 ML by the three existing ethanol plants, 360,603 ha of land would be required under *Agave* production. In comparison, given the productivity of 92 t ha^{-1} sugarcane with 2.65 t ha^{-1} molasses and 26.32 t ha^{-1} bagasse, and an ethanol conversion rate of 280 and 340 L t^{-1} from molasses and bagasse (O'Hara 2011), 128,266 ha of sugarcane production area would be required to meet this demand using sugarcane by-products or 39,738 ha of land would be required if the total sugarcane crop was used.

Ideally, as a second generation biofuel crop, *Agave* should be planted in areas that do not currently support food crops. This is possible due to its high water use efficiency (WUE). Certainly, there is an economic advantage, as the cost for suitable agricultural land to grow sugarcane is \$15,000–20,000 ha^{-1} (ARRE 2016), while that of less productive grazing and non-irrigated cropping land in Queensland is approximately \$277 ha^{-1} (Harcourts 2016) and \$985 ha^{-1} (Dent & Ward 2014), respectively.

6.2.4 Estimated cost of ethanol production

Clear estimates of the costs of *Agave* biomass production and those of ethanol production will be required to attract investment in this enterprise. A base for such considerations is provided below.

The cost of production of *Agave* biomass in Australia on marginal land is estimated at \$28,166 ha^{-1} extrapolated from the data obtained from the first *Agave* trial at Ayr, Queensland (Table 6.1). In this calculation, supplemental irrigation is budgeted only in the first year for establishment, with the growth in the following years based on rainfall. These costs reflect crop establishment in the first year, maintenance in years 2 to 4, and the harvesting cost in year 5 (Table 6.1) (Nunez, Rodriguez & Khanna 2010). Labour costs are assumed to be minimised with the use of mechanical devices for weeding and harvesting.

Table 6.1: Annual cost of producing *A. tequilana* in Australia assuming 4000 plants ha^{-1} and harvest five years from planting based on *Agave* trial, Kalamia, Queensland. Recalculated from Subedi (2013).

Activities	Cost Aus $/hectare				
	Yr. 1	Yr. 2	Yr. 3	Yr. 4	Yr. 5
Land cost (marginal land)	985	0	0	0	0
Land preparation	2,306.5	0	0	0	0
Irrigation	5,500	0	0	0	0
Plants	1,844	0	0	0	0
Planting	2,599	0	0	0	0
Replanting	0	0	391	0	0
Transport of plants and inputs	200	0	50	0	0
Maintenance (labour e.g., weeding, pruning)	1,865	1,633	2,932	2,143	2,143
Fertilizer and chemicals	726	0	0	700	0
Harvesting	0	0	0	0	1,200
Transport of crop to mill	0	0	0	0	949
Total per year (A$ ha^{-1})	16,025.5	1,633	3,373	2,843	4,292

Total cost ha^{-1} in five years with irrigation in the first year = $ 28,166.5

Table 6.2: Unit cost of processing the feedstock for ethanol production at the factory gate, modified from (Nunez, Rodriguez & Khanna 2010; Subedi 2013)

Items	$/L
Capital cost/Interest (initial project investment and interest over 10 years of operation)	0.07
Chemicals (enzymes/yeast and process chemicals)	0.022
Energy/Utility (electricity, water)	0.086
Operations/Maintenance (production, administration, maintenance, maintenance supplies, accounting, legal, office and laboratory, travel and miscellaneous)	0.034
Total	**0.212**

The cost of processing ethanol is estimated to be \$0.212 L^{-1} (Table 6.2). Based on the biomass yield of the current study, total ethanol yield at harvest in five years will be 17,236–25,784 L ha^{-1} at the rate of 180 and 374 L t^{-1} from leaf and stem, respectively, and

401 L t^{-1} for the whole biomass (Table 6.3). To produce 17,236–25,784 L ha^{-1} ethanol, the cost of processing will be \$3,564–5,466 ha^{-1} at the rate of \$0.212 L^{-1} for ethanol processing (Tables 6.2 and 6.4). With the total cost of feedstock production at \$28,166 ha^{-1} (Table 6.1), in order to produce 64.3 t ha^{-1} of biomass, the unit cost of feedstock will be \$438 t^{-1} (Table 6.4). Given an expected recovery of 17,236-25,784 L ha^{-1}(Table 6.3), the total cost of production of ethanol at the factory gate will be between \$1.85 L^{-1} for lower rate of ethanol yield as identified in this study to \$1.30 L^{-1} based on a higher ethanol yield as estimated by Corbin et al. (2015).

The average cost of ethanol derived from *A. tequilana* of \$1.58 L^{-1} at the factory gate is much higher than that estimated by Subedi (2013) and Nunez, Rodriguez and Khanna (2010) for *Agave* derived ethanol, which are \$0.51 L^{-1} and \$0.90 L^{-1}, respectively, as well as that derived from sugarcane (\$0.70 L^{-1}) (O'Hara 2011).

Table 6.3: Estimated ethanol yield (L ha^{-1}) at the end of 5 years of growth

Option	Plant part	Estimated biomass (t ha^{-1})	Ethanol conversion (L t^{-1})	Estimated ethanol (L ha^{-1})	Comments
1	Stem only	29.0	374*–401#	10,846–11,629	
2	Whole plant	64.3	180^ & 374*	17,236	Current study using stem and leaf
3	Whole plant	64.3	401#	25,784	Based on Corbin's estimated ethanol yield for the whole plant

Note:

Option 1 includes harvesting stem only

Option 2 and 3 includes harvesting stem and leaf as a whole plant

*Value calculated based on average value of 417 L t^{-1}(Davis, Dohleman & Long 2011) and 330 L t^{-1} (Nunez, Rodriguez & Khanna 2010) for stem

^ value based on current study for leaves

value based on Corbin et al. (2015)

Table 6.4: Potential cost for *Agave* based ethanol

Item	Unit	Estimated yield/cost
Feedstock produced in 5 years	t ha^{-1}	64.3
Total cost of producing feedstock (Table 1)	\$ ha^{-1}	28,166
Unit Cost of feedstock	\$ t^{-1}	438.05
Estimated ethanol production in 5 years (Table 3)	L ha^{-1}	^17,236–25,784#
Cost of processing for ethanol production (Table 2)	\$ L^{-1}	0.212
Total cost of processing biomass to ethanol	\$ ha^{-1}	^3564–5466#
Total cost of ethanol production (feedstock cost + processing cost)	\$ ha^{-1}	^31,820–33,632#
Total cost of ethanol at the factory gate	\$ L^{-1}	^1.85–1.30#
Average cost of ethanol at the factory gate	\$ L^{-1}	1.58

Note:

value based on ethanol conversion rate (401 L t^{-1}) of Corbin et al. (2015)

^ value based on ethanol conversion of current study for leaves (180 L t^{-1}) and average ethanol conversion for stem (374 L t^{-1}) calculated from Davis, Dohleman & Long (2011) and Nunez, Rodriguez & Khanna (2010).

6.2.5 Estimated gross return

Considering the average value of ethanol production of \$1.58 L^{-1}(Table 6.4), total revenue of \$27,232 to \$40,738 ha^{-1} can be expected by producing 17,236–25,784 L ha^{-1} of ethanol. With the total cost of production for feedstock at \$28,166 ha^{-1}, for low ethanol yield there will be a net loss of \$934, and for higher ethanol yield there will be a gross margin of \$12,572 ha^{-1} over 5 years. Therefore, from the results of the present study, ethanol production from *Agave* becomes uneconomical if ethanol yields are low.
The revenue estimated by Subedi (2013) was \$23,336.14 ha^{-1} yr^{-1} (calculated using the estimated ethanol production rate of 45,619 L ha^{-1} yr^{-1}, ethanol conversion rate of 600 L t^{-1} and the price of ethanol at \$0.51 L^{-1} at the factory gate). Thus, ethanol production from *Agave* estimated by Subedi (2013) appears to be ambitious, and the revenue obtained from it is 65% higher than that at the higher end of ethanol yield estimated in this study. One potential area for improvement is to reduce the cost of feedstock production as it currently accounts for 86% of the total cost. The results of the bench scale data of the current study is based on relatively low rates of ethanol production per unit biomass. However, the efficiency of pretreatment, enzymatic hydrolysis and fermentation of *Agave* biomass could be further improved and optimised. In order to undertake a pilot scale study, for example, the initial dry matter content of the lignocellulosic biomass should be >20% w/w and the sugar concentration of the

hydrolysate after pretreatment and enzymatic hydrolysis should be ≥8% w/w to achieve a final titre of ethanol >4% w/w, a benchmark for efficient and economical distillation (Koppram et al. 2014; Larsen et al. 2008).

6.2.6 Agave–a new industry?

The establishment of a new industry, involving production of a new crop and associated processing capability is a daunting task, typically taking up to 50 years even when economic conditions are favourable (Wood, Chudleigh & Bond, 1994, cited in Fletcher and Kregor (1998)). The body of work presented in this thesis suggests that an agave-ethanol industry has potential, but further research is needed to improve productivity and to reduce the cost of biomass production in an Australian context, and to improve conversion efficiency of the *Agave* feedstock.

Future research in Agave feedstock should focus on:

1. Consideration of other *Agave* species for biomass accumulation and testing of their composition.
2. Development of agronomic practices for Agave in an Australian context to improve yield with the view to generating low-cost feedstock (cost-benefit analysis)
3. Development of mechanical harvesting procedures.
4. Extending the calibration set for cellulose, hemicellulose and lignin composition of agave leaf powder using FTNIR, incorporating samples from different varieties, maturity stages and locations from at least 3–4 years of growth.
5. Use of inulinase enzyme to solubilise inulin in the leaves and stems, and
6. Set up of a pilot scale demonstration, including the use of C5 and C6 utilising yeasts to maximise ethanol production.

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Appendix A: *Agave* classification and species number

Subgenus	Group or Section	No of species	Examples
LITTEA	Amolae	8	*A. attenuata* Salm., *A. pedunculifera* Trel., *A. vilmoriniana* Berger
	Chloritepalae	3	*A. ellemeetiana* Jacobi
	Filiferae	8	*A. filifera* Salm., *A. multifilifera* Gentry
	Marginatae	21	A. lechuguilla Torr., *A. xylonacantha* Salm.
	Parviflorae	4	*A. parviflora* Torr. Subsp. *parviflora*, *A. scotti* Engelm., *A. toumeyana* Trel.
	Polycephalae	5	*A. celsii* Hook
	Striatae	3	*A. dasyliriodes* Jacobi & Bouche, *A. stricta* Zucc.
	Urceolatae	2	*A. utahensis* var. *nevadensis* Engelm.
AGAVE	Americanae	6	*A. americana* L., *A. lurida* Aiton, *A. scraba* Salm. Dyck
	Campaniflorae	3	*A. promontorii* Trel.
	Crenatae	6	*A. bovicornuta* Gentry
	Deserticolae	10	*A. cerulata* Trel., *A. deserti* Engelm., *A. sobria* Brandeg
	Ditepalae	10	*A. murpheyi* F Gibson, *A. palmeri* Engelm.
	Hiemiflorae	12	*A. atrovirens* Karw. ex. Salm, *A. lagunae* Trel
	Marmoratae	4	*A. zebra* Gentry
	Parryanae	6	*A. parryi* Engelm. *A. parryi* var. *huachucensis* (Baker) Little ex Benson
	Rigidae	12	*A. angustifolia* Haw., *A. fourcroydes* Lem., *A. macrocantha* Zucc., *A. rhodacantha* Trel., *A. tequilana* Weber
	Salminae	5	*A. salmiana* Otto ex Salm ssp. *crassispina* (Trel.) Gentry
	Sisalaneae	6	*A. sisalana* Perrine, A. *weberi* Cels ex Poisson
	Umbelliforae	2	*A. shawii* Engelm.

Source: Gentry (1982) as cited by Nobel (2003)

Appendix B: *Agave* planting plan at Rockhampton.

Row	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	A. ang M	A. ang M	A. ang M	A. ang M	A. ang M	F. foe	F. foe	F. foe	F. foe	F. foe	B1(spare)	B2(spare)	B3(spare)	X	X
1	Tcqu	Tcqu	Tcqu	Tcqu	Tcqu	L3	L3	L3	L3	L3	F. foe	F. foe	F. foe	F. foe	F. foe
2	E9	E9	E9	E9	E9	17	17	17	17	17	A. deci	A. deci	A. deci	A. deci	A. deci
3	L19	L19	L19	L19	L19	L9	L9	L9	L9	L9	A. att	A. att	A. att	A. att	A. att
4	L9	L9	L9	L9	L9	L19	L19	L19	L19	L19	E9	E9	E9	E9	E9
5	L3	L3	L3	L3	L3	17	17	17	17	17	ABM	ABM	ABM	ABM	ABM
6	Tcqu	Tcqu	Tcqu	Tcqu	Tcqu	A att	A att	A att	A att	A att	A. deci	A. deci	A. deci	A. deci	A. deci
7	Tcqu	Tcqu	Tcqu	Tcqu	Tcqu	17	17	17	17	17	A. deci	A. deci	A. deci	A. deci	A. deci
8	L19	L19	L19	L19	L19	A des V	A des V	A des V	A des V	A des V	L9	L9	L9	L9	L9
9	E9	E9	E9	E9	E9	A. ang	A. ang	A. ang	A. ang	A. ang	L3	L3	L3	L3	L3
10	A. sis	A. sis	A. sis	A. sis	A. sis	Tcqu	Tcqu	Tcqu	Tcqu	Tcqu	L19	L19	L19	L19	L19
11	A. deci	A. deci	A. deci	A. deci	A. deci	L3	L3	L3	L3	L3	L9	L9	L9	L9	L9
12	A. ame V	A. ame V	A. ame V	A. ame V	A. ame V	E9	E9	E9	E9	E9	A.des V	A.des V	A.des V	A.des V	A.des V
13	L3	L3	L3	L3	L3	Tcqu	Tcqu	Tcqu	Tcqu	Tcqu	A. ang M	A. ang M	A. ang M	A. ang M	A. ang M
14	L19	L19	L19	L19	L19	E9	E9	E9	E9	E9	A des	A des	A des	A des	A des
15	A. ame	A. ame	A. ame	A. ame	A. ame	L9	L9	L9	L9	L9	17	17	17	17	17

Note: E9 = *A. tequilana* cultivar E9, L9 = *A. tequilana* cultivar L9, L19 = *A. tequilana* cultivar L19, L3 = *A. tequilana* cultivar L3, 17 = *A. tequilana cultivar* 17, Tcqu = *A. tequilana* cultivar Tcqu, A. ang M = *A. angustifolia* 'Marginata', A. ang = *A. angustifolia*, A. deci = *A. decipiens*, A. ame = *A. americana*, A.ame V = *A. americana* 'Variegata', ABM = unknown *Agave* sp. 1, A. des = *A. desmetiana*), A. des V = *A. desmetiana* 'Variegata' and F. foe = *Frucraea foetida*, A. att = *A. attenuata*

Appendix C: NIRS and Hemicellulose and Lignin

An extension of Chapter 4

A3.1 Hemicellulose Estimation—Data Pre-processing and Wavelength Range

The absorbance spectra on percent hemicellulose were pre-processed using Savitzky Golay 2nd derivative, SNV and MSC. Of all the pre-processing routines trialled, the Savitzky Golay second derivative supported the best models, with fewer PLS factors used in the model (Table A3.1).

The b-coefficients of this model were noisy (Fig. A3.1A). Models with smooth β-coefficients were developed with a spectral window of between 1976–2349 nm and 1940–2430 nm (Fig. A3.1 B). However, the model statistics were not improved (Table A3.1) and, therefore, the full spectral range was used.

Table A3.1: Leave one out cross validation (LOO CV) statistics for PLS calibration models of percent hemicellulose of 104 (Mean = 13.52, SD = 1.86) spectra of dried *A. tequilana* leaf powder. Models were developed from May 2012 to Aug 2013 using wavelength regions at 1000–2500 nm, 1976–2349 nm and 1940–2430 nm.

Instrument /Range	Outlier	PC	Rcv	RMSECV	Slope
FTNIR /1000-2500nm					
Abs	0	12	0.75	1.25	0.66
Abs	2	12	0.77	1.21	0.67
Abs-d^{2}A (S. Golay)	2	9	0.76	1.23	0.66
Abs-SNV	2	17	0.85	0.69	0.78
Abs-MSC	2	17	0.82	1.10	0.74
FTNIR/1976-2349 nm					
Abs	2	12	0.63	1.46	0.46
Abs-d^{2}A (S. Golay)	2	2	0.52	1.60	0.29
FTNIR/1940-2430 nm					
Abs	2	7	0.67	1.40	0.50
Abs-d^{2}A (S. Golay)	2	2	0.54	1.58	0.31

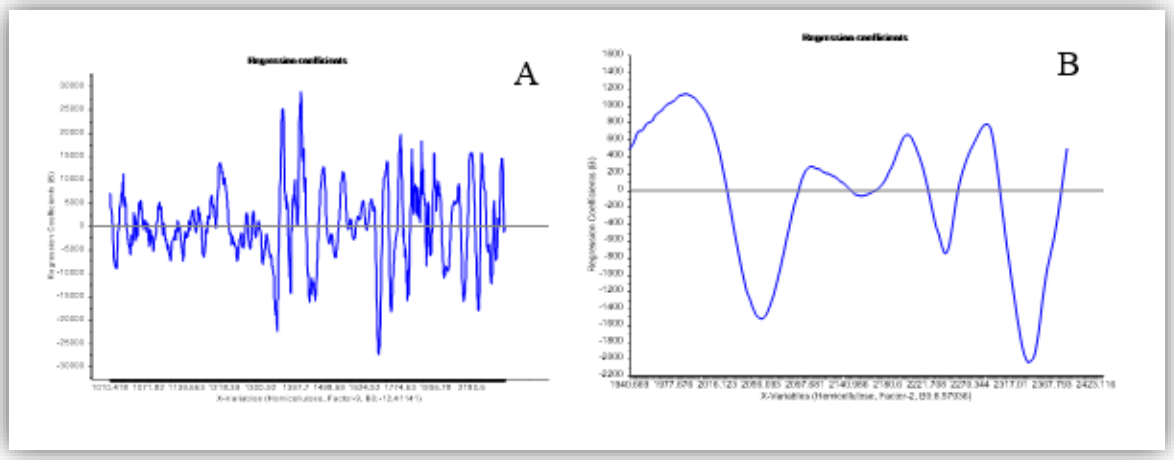


Figure A3.1: PLS regression coefficients of % hemicellulose (DW basis) in *A. tequilana* leaf material, for models based on second derivative of log 1/R spectra using wavelength ranges of (A) 1000–2500 (left panel) and (B) 1940–2230 nm (right panel), (n=104).

A3.2 Hemicellulose Estimation—Model Robustness across Cultivars and Years

Statistics for models developed for single cultivars (Tcqu or L19) were comparatively better than the combined models including both cultivars and years (e.g., cultivar Tcqu model, Rcv = 0.94, RMSECV = 0.60) (Table A3.2). The poor model statistics of the combined model could be due to the variation in % hemicellulose content of Tcqu and L19 as the mean hemicellulose content for Tcqu was 13.9, as compared to 12.3 for L19 (Figs 4.3 A and B, Chapter 4). Models were also developed using two cultivars across two seasons (2012 and 2013, Table A3.3). The model statistics of combined models grouped in 2012 and 2013 were better with Rcv between 0.87–0.91 and RMSECV between 0.83–0.96. This variation is evident in the principal component (PC) plot (Figs A3.2 A–C).

Table A3.2: PLSR models developed with spectral pretreatment using S. Golay (d^2A) on hemicellulose content at 1000–2500 nm (post-Soxhlet). Full leave one out cross validations are reported.

Sample	Outlier	PC	Rcv	RMSECV	Slope
Tcqu (2012 and 2013)					
(n=78, mean = 13.88, SD = 1.84)	2	12	**0.94**	0.60	0.93
Combined 2012 (Tcqu and L19)					
(n=50, mean = 14.23, SD = 1.92)	2	8	**0.87**	0.96	0.80
Combined 2013 (Tcqu and L19)					
(n=52, mean = 12.82, SD = 1.56)	0	9	**0.91**	0.83	0.84

Table A3.3: PLSR models developed with spectral pretreatment using S. Golay (d^2A) on % hemicellulose (post Soxhlet) May 2012–Aug 2013. Full leave one out cross validations are reported.

Sample	Outlier	PC	Rcv	RMSECV	Slope
Nov 12, Mar 13 and Aug 13					
(n=78, mean = 13.27, SD = 1.87)	0	9	0.75	1.25	0.64
May 12, Mar 13 and Aug 13					
(n=76, mean = 13.27, SD = 1.75)	0	10	**0.88**	0.85	0.83
May 12, Nov 12 and Aug 13					
(n=76, mean = 14.00, SD = 1.86)	0	10	0.76	1.25	0.69
May 12, Nov 12 and Mar 13					
(n=76, mean = 13.49, SD = 1.95)	0	11	**0.87**	0.96	0.81

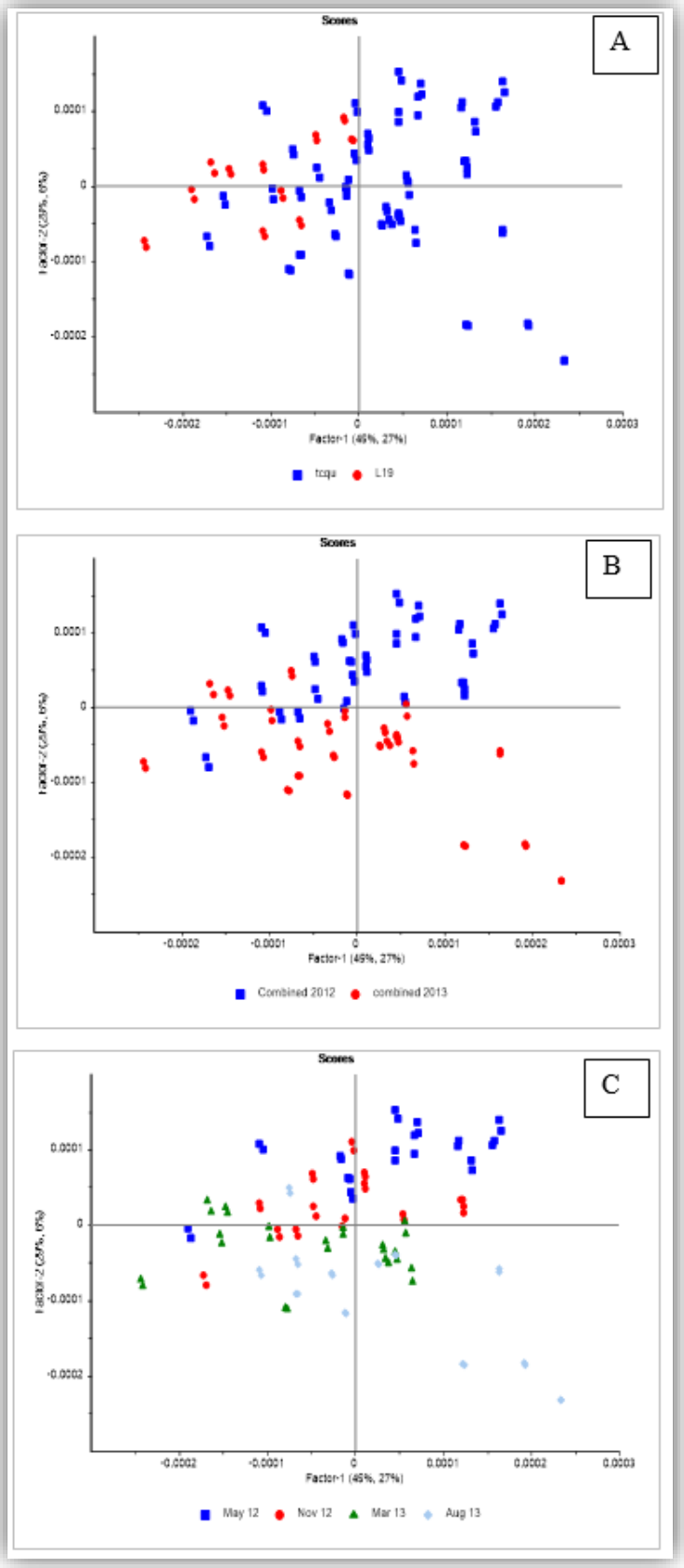


Figure A3.2: Plot of PLS factors 1 and 2 for a PLS model of hemicellulose (% DW) in *A. tequilana* leaf (n=104). Samples differentiated into (A) cultivars (Tcqu and L19), (B) years (2012 and 2013) and (C) sampling periods (May 12, Nov 12, Mar 13 and Aug 13).

A3.3 Hemicellulose Estimation—PLS Model Prediction Performance

Model predictive performance on samples from populations not represented in the calibration set was tested across populations varying in plant variety and season of harvest. The best prediction result was achieved from a model developed on samples of one cultivar but all harvest events in prediction of samples of another cultivar (Rp = 0.81 and RMSEP = 1.51), in comparison to predictions across harvest events (Table A3.4).

The cross cultivar prediction result for hemicellulose was poorer, but close to the results obtained by Liu et al. (2010) for xylan (% w/w, the major component of hemicellulose) of corn stover and switch grass (R = 0.93–0.96; RMSEP = 1.03–0.57 and n=15).

Table A3.4: Prediction statistics of % DW hemicellulose developed with spectral pretreatment using S. Golay (d^2A) at 1000–2500 nm using FTNIR. Leave one out cross validation models are used.

Sample predicted	Outlier	PC	Rp	RMSEP	Bias	Slope
L19 using Tcqu						
(n=24, mean = 12.29, SD = 1.49)	0	12	0.81	1.51	-0.40	0.003
2012 using 2013						
(n=50, mean = 14.22, SD = 1.92)	0	12	0.49	2.96	2.27	0.003
May 12 using Nov 12, Mar 13 and Aug 13						
(n=24, mean = 14.27, SD = 1.75)	2	8	0.45	1.86	0.32	0.46
Nov 12 using May 12, Mar 13 and Aug 13						
(n=26, mean = 14.19, SD = 2.11)	0	10	-0.45	3.54	-1.74	-0.33
Mar 13 using May 12, Nov 12 and Aug 13						
(n=26, mean = 12.07, SD = 0.98)	0	10	-0.14	2.22	1.15	-0.22
August 13 using May 12, Nov 12 and Mar 13						
(n=26, mean = 13.56, SD = 1.68)	0	11	0.21	2.12	-0.45	0.21

A3.4 Lignin Estimation—Data Pre-processing and Wavelength Range

The absorbance spectra on % lignin were pre-processed using Savtizky Golay 2nd derivative, SNV and MSC. Models based on Savitzky Golay second derivative treated spectra utilised fewer PLS factors (Table A3.5).

The b-coefficients across the full wavelength range model were noisy (Fig. A3.3 A). Models with smooth b-coefficients were developed with a spectral window of between

1410–2208 nm and 1490–1740 nm (Fig. A3.3 B). However, relative to use of full wavelength region, model statistics were decreased (Rcv = 0.72–0.76, RMSECV = 1.12–1.19), although the PLS factors were also reduced (Table A3.5).

Table A3.5: Leave one out cross validation statistics for PLS calibration models of percent lignin of *A. tequilana* leaf powder (post-Soxhlet). Models were developed with 104 spectra at different wavelength regions 1000–2500 nm, 1410–2208 nm and 1490–1740 nm.

Instrument/Range	Outlier	PC	Rcv	RMSECV	Slope
FTNIR 1000–2500nm					
May 12–Aug 13 /Abs					
(n=104, mean = 17.79, SD = 1.71)	0	17	0.84	0.94	0.78
May 12–Aug 13 /Abs					
(n=102, mean = 17.81, SD = 1.72)	2	18	**0.87**	0.83	0.80
May 12–Aug 13 /d^2A					
(n=102, mean = 17.81, SD = 1.72)	2	10	0.81	1.01	0.71
May 12–Aug 13 /SNV					
(n=102, mean = 17.81, SD = 1.72)	2	15	0.84	0.94	0.76
May 12–Aug 13 / MSC					
(n=102, mean = 17.81, SD = 1.72)	2	15	0.84	0.94	0.76
FTNIR/1410–2208 nm					
May12– Aug 13/Abs					
(n=102, mean = 17.81, SD = 1.72)	2	13	0.83	0.98	0.75
May12–Aug 13 /d^2A					
(n=102, mean = 17.81, SD = 1.72)	2	8	0.76	1.12	0.62
FTNIR/1490–1740 nm					
May12–Aug 13/Abs					
(n=102, mean = 17.81, SD = 1.72)	2	8	0.76	1.12	0.64
May12–Aug 13 /d^2A					
(n=102, mean = 17.81, SD = 1.72)	2	6	0.72	1.19	0.55

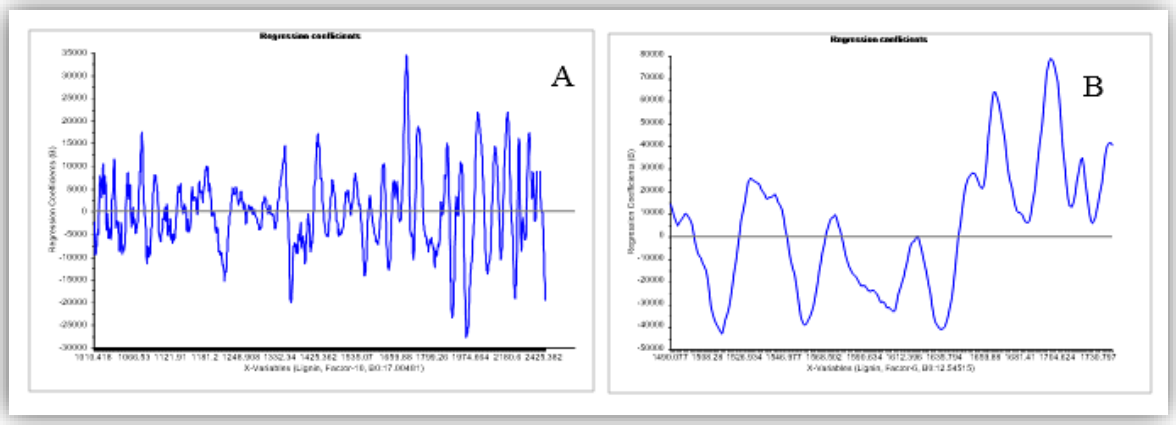


Figure A3.3: PLS regression coefficients of % lignin (DW basis) in *A .tequilana* leaf material, for models based on second derivative of log 1/R spectra using wavelength ranges of (A) 1000–2500 (left panel) and (B) 1490–1740 nm (right panel), (n=102).

A3.5 Lignin Estimation—Model Performance by Cultivar and Years

Similar to other attributes cellulose and hemicellulose, the individual models grouped into cultivars (Tcqu and L19) were comparatively better than the combined models including both cultivars and years. Cultivar Tcqu was the best of all the models (Rcv = 0.87, RMSECV = 0.0.87) (Table A3.6) as in cellulose and hemicellulose. The poor combined model statistics of % lignin could be due to variation in lignin composition of two cultivars (Fig. A3.4 A) during each sampling period from May 12–August 13 (Figs 4.3 A and B, Chapter 4). There is distinct variation between May 12 and November 12 samples. However, some of Nov 12, most of March 13 and August 13 samples are clustered into one group in the PC plot (Fig. A3.4 C). The models were also developed using two cultivars across two years of 2012 and 2013 (Table A3.6). The model statistics of combined models grouped in 2013 were better (Rcv = 0.86 and RMSECV = 0.69) than those of 2012 (Rcv = 0.80 and RMSECV = 1.06) and the separation of 2012 and 2013 samples in the PC plot is distinct (Figs A3.4 B–C).

Table A3.6: FTNIR (1000–2500 nm): Combined PLSR models developed with spectral pretreatment using S. Golay (d^2A) of *A. tequilana* leaf samples post-Soxhlet extraction. Full leave one out cross validation are reported on % DW lignin.

Cultivar/year	Outlier	PC	Rcv	RMSECV	Slope
Tcqu/d^2A					
(n=78, mean = 17.75, SD = 1.75)	2	10	**0.87**	0.87	0.81
Combined 2012/d^2A					
(n=50, mean = 17.03, SD = 1.73)	0	8	0.80	1.06	0.78
Combined 2013/d^2A					
(n=52, mean = 18.56, SD = 1.33)	0	12	**0.86**	0.69	0.82

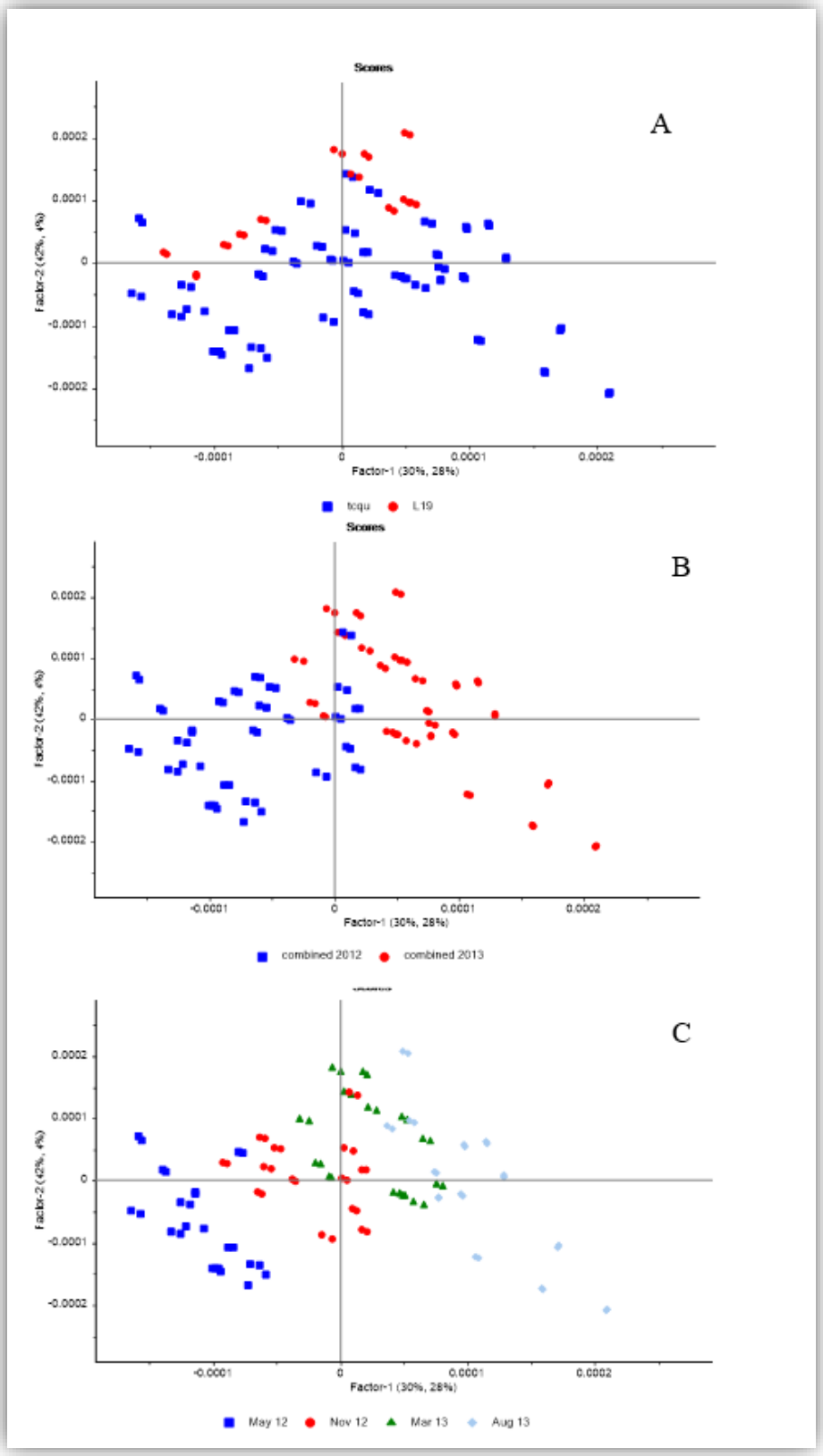


Figure A3.4: Plot of PLS factors 1 and 2 for a PLS model of lignin (% DW) in *A. tequilana* leaf (n=104). Samples differentiated into (A) cultivars (Tcqu and L19), (B) years (2012 and 2013) and (C) sampling periods (May 12, Nov 12, Mar 13 and Aug 13).

A3.6 Lignin Estimation—Prediction Performance of the PLS Models

Three of four sampling periods are used to develop models (Table A3.7) to predict the fourth sampling period, which was not included in the model (Table A3.8). August 2013 had the best prediction (Rp = 0.75 and RMSEP = 3.21) of the rest of the sampling periods. The poor prediction of the other sampling periods could be due to significant variation ($P < 0.05$) in percent lignin content of both cultivars in first sampling period (May 12) to that of third and the fourth (March 13 and August 13).

Cultivar Tcqu was also used to predict the other cultivar L19 with <0.50 prediction statistics (Rp = 0.46 and RMSEP = 1.74). The prediction was poor although the mean % lignin content of L19 and Tcqu were very similar (L19 = 17.8% and Tcqu = 18%). However, the % lignin content of both cultivars varied significantly from May 2012 to August 2013. Similarly, the models developed using two cultivars of 2013 could not predict 2012 well, due to distinct variation of % lignin content in the two years. This could also be due to significant variation ($P < 0.05$) in percent lignin content of both cultivars in the first sampling period (May 12) to that of third and the fourth (March 13 and August 13). For lignin, models developed using absorbance spectra gave better prediction statistics as compared to d_2A. Since there are no reports of prediction of chemical composition of *Agave* using NIR, results obtained for other biomass feedstocks such as hardwood, rice straw, corn stover and switchgrass are compared.

By comparison, Poke and Raymond (2006) report prediction of % lignin composition of 14 ground samples of solid wood of *E .globulus* in which poor correlation coefficients (R^2 = 0.54) were obtained while Jin and Chen (2007) report good prediction of Klason lignin composition of 9 rice straw samples between the wavelength region of (4100-7700 cm^{-1}) 1333–2439 nm (R^2 = 0.86; SEP = 2.1, SD = 1.6). This result is comparatively better than the current study but this could be due to small sample size for prediction as compared to ours.

Table A3.7: FTNIR (1000–2500 nm): Combined PLSR models of % lignin from May 2012–Aug 2013 (post-Soxhlet). Full leave one out cross validation is reported.

Instrument/Range	PC	Rcv	RMSECV	Slope
Nov 12, Mar 13 and Aug 13/Abs				
(n=78, mean = 18.46, SD = 1.34)	15	0.82	0.77	0.72
Nov 12, Mar 13 and Aug 13/d^2A				
(n=78, mean = 18.46, SD = 1.34)	8	0.64	1.03	0.48
May 12, Mar 13 and Aug 13/Abs				
(n=76, mean = 17.65, SD = 1.81)	15	0.93	0.66	0.91
May 12, Mar 13 and Aug 13/d^2A				
(n=76, mean = 17.65, SD = 1.81)	11	0.91	0.77	0.88
May 12, Nov 12 and Aug 13/Abs				
(n=76, mean = 17.47, SD = 1.69)	16	0.90	0.73	0.84
May 12, Nov 12 and Aug 13/d^2A				
(n=76, mean = 17.47, SD = 1.69)	11	0.85	0.90	0.76
May 12, Nov 12 and Mar 13/Abs				
(n=76, mean = 17.64, SD = 1.82)	17	0.89	0.82	0.84
May 12, Nov 12 and Mar 13/d^2A				
(n=76, mean = 17.64, SD = 1.82)	17	0.81	1.10	0.74

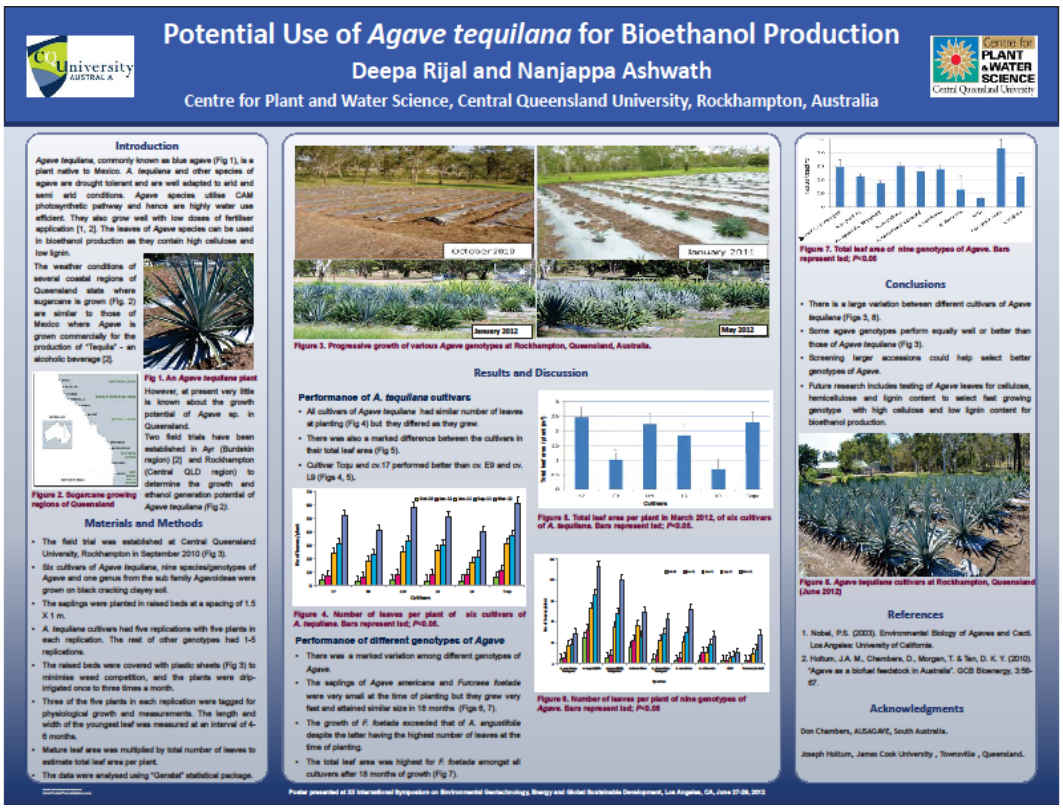
Table A3.8: Prediction statistics of % lignin (post-Soxhlet) at different sampling periods. Full leave one out and segmented (sampling period = 4 segments) cross validation are reported.

Instrument/Range FTNIR/1000–2500 nm	CV	PC	Rp	RMSEP	Bias	Slope
L19 using Tcqu (d^2A)						
(n=24, mean = 18.02, SD = 1.62)	Seg	6	0.46	1.74	0.68	0.43
2012 using 2013 (d2A)						
(n=50, mean = 17.03, SD = 1.73)	Seg	13	0.23	3.48	1.92	0.36
May 12 using Nov 12, Mar 13 and Aug 13)/Abs **(n=24, mean = 15.70, SD = 0.94)**	Full	15	0.46	3.61	3.20	0.96
May 12 using Nov 12, Mar 13 and Aug 13)/d^2A						
(n=24, mean = 15.70, SD = 0.94)	Full	11	0.34	3.04	2.54	0.64
Nov 12 using f May 12, Mar 13 and Aug 13/Abs						
(n=26, mean = 18.26, SD = 1.34)	Full	15	-0.27	2.25	-0.92	-0.26
Nov 12 using May 12, Mar 13 and Aug 13/d^2A						
(n=26, mean = 18.26, SD = 1.34)	Full	11	-0.28	2.68	-0.19	-0.43
Mar 13 using May 12, Nov 12 and Aug 13/Abs **(n=26, mean = 18.81, SD = 1.40)**	Full	16	-0.26	2.12	0.29	-0.25
Mar 13 using May 12, Nov 12 and Aug 13/d^2A						
(n=26, mean = 18.81, SD = 1.40)	Full	11	-0.12	1.81	0.13	-0.09
August 13 using May 12, Nov 12 and Mar 13/Abs						
(n=26, mean = 18.31, SD = 1.25)	Full	17	0.75	3.21	2.91	1.21
August 13 using May 12, Nov 12 and Mar 13/d^2A						
(n=26, mean = 18.31, SD = 1.25)	Full	12	-0.12	2.08	-0.63	-0.14

Appendix D: Conference presentations and proceedings

A4.1 Potential Use of *Agave tequilana* for Bioethanol Production

Poster presented at XII International Symposium on Environmental Geotechnology, Energy and Global Sustainable Development, Los Angeles, CA, June 27–29, 2012.



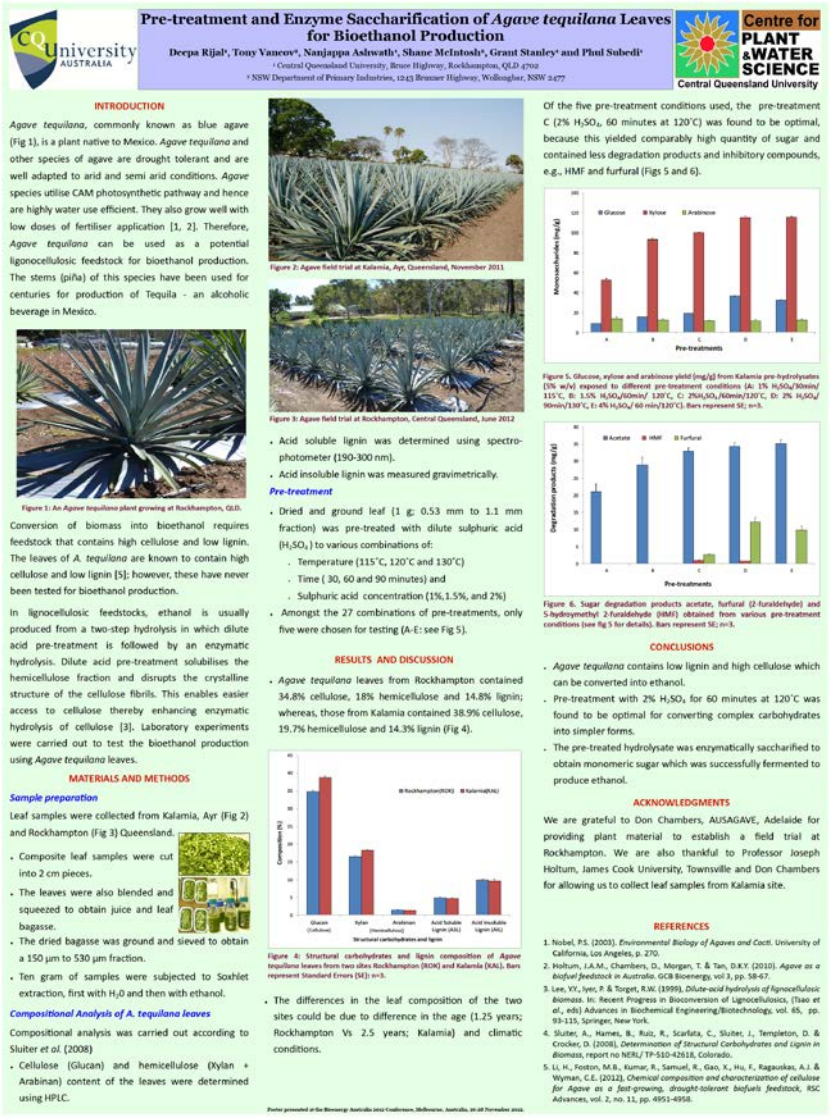
Conference Proceedings:

Rijal, D and Ashwath, N, "Potential use of *Agave tequilana* for bioethanol production in the sugarcane belt of Queensland, Australia" in XII International Symposium on Environmental Geotechnology, Energy and Global Sustainable Development (ISEG 2012), vol. 3, pp. 212–223**.**

http://www.isegnet.org/2012/proceedings_ISEG_2012_Vol_III.pdf

A4.2 Pretreatment and Enzyme Saccharification of *Agave tequilana* Leaves for Bioethanol Production

Poster presented at the Bioenergy Australia 2012 Conference, Melbourne, Australia, 26–28 November 2012.



Pre-treatment and Enzyme Saccharification of *Agave tequilana* Leaves for Bioethanol Production

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Centre for PLANT & WATER SCIENCE, Central Queensland University

INTRODUCTION

Agave tequilana, commonly known as blue agave (Fig 1), is a plant native to Mexico. *Agave tequilana* and other species of agave are drought tolerant and are well adapted to arid and semi arid conditions. *Agave* species utilise CAM photosynthetic pathway and hence are highly water use efficient. They also grow well with low doses of fertiliser application [1, 2]. Therefore, *Agave tequilana* can be used as a potential lignocellulosic feedstock for bioethanol production. The stems (piña) of this species have been used for centuries for production of Tequila - an alcoholic beverage in Mexico.

Figure 1: An *Agave tequilana* plant growing at Rockhampton, QLD.

Conversion of biomass into bioethanol requires feedstock that contains high cellulose and low lignin. The leaves of *A. tequilana* are known to contain high cellulose and low lignin [5]; however, these have never been tested for bioethanol production.

In lignocellulosic feedstocks, ethanol is usually produced from a two-step hydrolysis in which dilute acid pre-treatment is followed by an enzymatic hydrolysis. Dilute acid pre-treatment solubilises the hemicellulose fraction and disrupts the crystalline structure of the cellulose fibrils. This enables easier access to cellulose thereby enhancing enzymatic hydrolysis of cellulose [3]. Laboratory experiments were carried out to test the bioethanol production using *Agave tequilana* leaves.

MATERIALS AND METHODS

Sample preparation

Leaf samples were collected from Kalamia, Ayr (Fig 2) and Rockhampton (Fig 3) Queensland.

- Composite leaf samples were cut into 2 cm pieces.
- The leaves were also blended and squeezed to obtain juice and leaf bagasse.
- The dried bagasse was ground and sieved to obtain a 150 µm to 530 µm fraction.
- Ten gram of samples were subjected to Soxhlet extraction, first with H_2O and then with ethanol.

Compositional Analysis of A. tequilana leaves

Compositional analysis was carried out according to Sluiter *et al.* (2008)

- Cellulose (Glucan) and hemicellulose (Xylan + Arabinan) content of the leaves were determined using HPLC.

Figure 2: Agave field trial at Kalamia, Ayr, Queensland, November 2011

Figure 3: Agave field trial at Rockhampton, Central Queensland, June 2012

- Acid soluble lignin was determined using spectrophotometer (190-300 nm).
- Acid insoluble lignin was measured gravimetrically.

Pre-treatment

- Dried and ground leaf (1 g; 0.53 mm to 1.1 mm fraction) was pre-treated with dilute sulphuric acid (H_2SO_4) to various combinations of:
  - Temperature (115˚C, 120˚C and 130˚C)
  - Time (30, 60 and 90 minutes) and
  - Sulphuric acid concentration (1%, 1.5%, and 2%)
- Amongst the 27 combinations of pre-treatments, only five were chosen for testing (A-E: see Fig 5).

RESULTS AND DISCUSSION

- *Agave tequilana* leaves from Rockhampton contained 34.8% cellulose, 18% hemicellulose and 14.8% lignin; whereas, those from Kalamia contained 38.9% cellulose, 19.7% hemicellulose and 14.3% lignin (Fig 4).

Figure 4: Structural carbohydrates and lignin composition of *Agave tequilana* leaves from two sites Rockhampton (ROK) and Kalamia (KAL). Bars represent Standard Errors (SE); n=3.

- The differences in the leaf composition of the two sites could be due to difference in the age (1.25 years; Rockhampton Vs 2.5 years; Kalamia) and climatic conditions.

Of the five pre-treatment conditions used, the pre-treatment C (2% H_2SO_4, 60 minutes at 120˚C) was found to be optimal, because this yielded comparably high quantity of sugar and contained less degradation products and inhibitory compounds, e.g., HMF and furfural (Figs 5 and 6).

Figure 5. Glucose, xylose and arabinose yield (mg/g) from Kalamia pre-hydrolysates (5% w/v) exposed to different pre-treatment conditions (A: 1% H_2SO_4/30min/ 115˚C, B: 1.5% H_2SO_4/60min/ 120˚C, C: 2%H_2SO_4/60min/120˚C, D: 2% H_2SO_4/ 90min/130˚C, E: 4% H_2SO_4/ 60 min/120˚C). Bars represent SE; n=3.

Figure 6. Sugar degradation products acetate, furfural (2-furaldehyde) and 5-hydroxymethyl 2-furaldehyde (HMF) obtained from various pre-treatment conditions (see fig 5 for details). Bars represent SE; n=3.

CONCLUSIONS

- *Agave tequilana* contains low lignin and high cellulose which can be converted into ethanol.
- Pre-treatment with 2% H_2SO_4 for 60 minutes at 120˚C was found to be optimal for converting complex carbohydrates into simpler forms.
- The pre-treated hydrolysate was enzymatically saccharified to obtain monomeric sugar which was successfully fermented to produce ethanol.

ACKNOWLEDGMENTS

We are grateful to Don Chambers, AUSAGAVE, Adelaide for providing plant material to establish a field trial at Rockhampton. We are also thankful to Professor Joseph Holtum, James Cook University, Townsville and Don Chambers for allowing us to collect leaf samples from Kalamia site.

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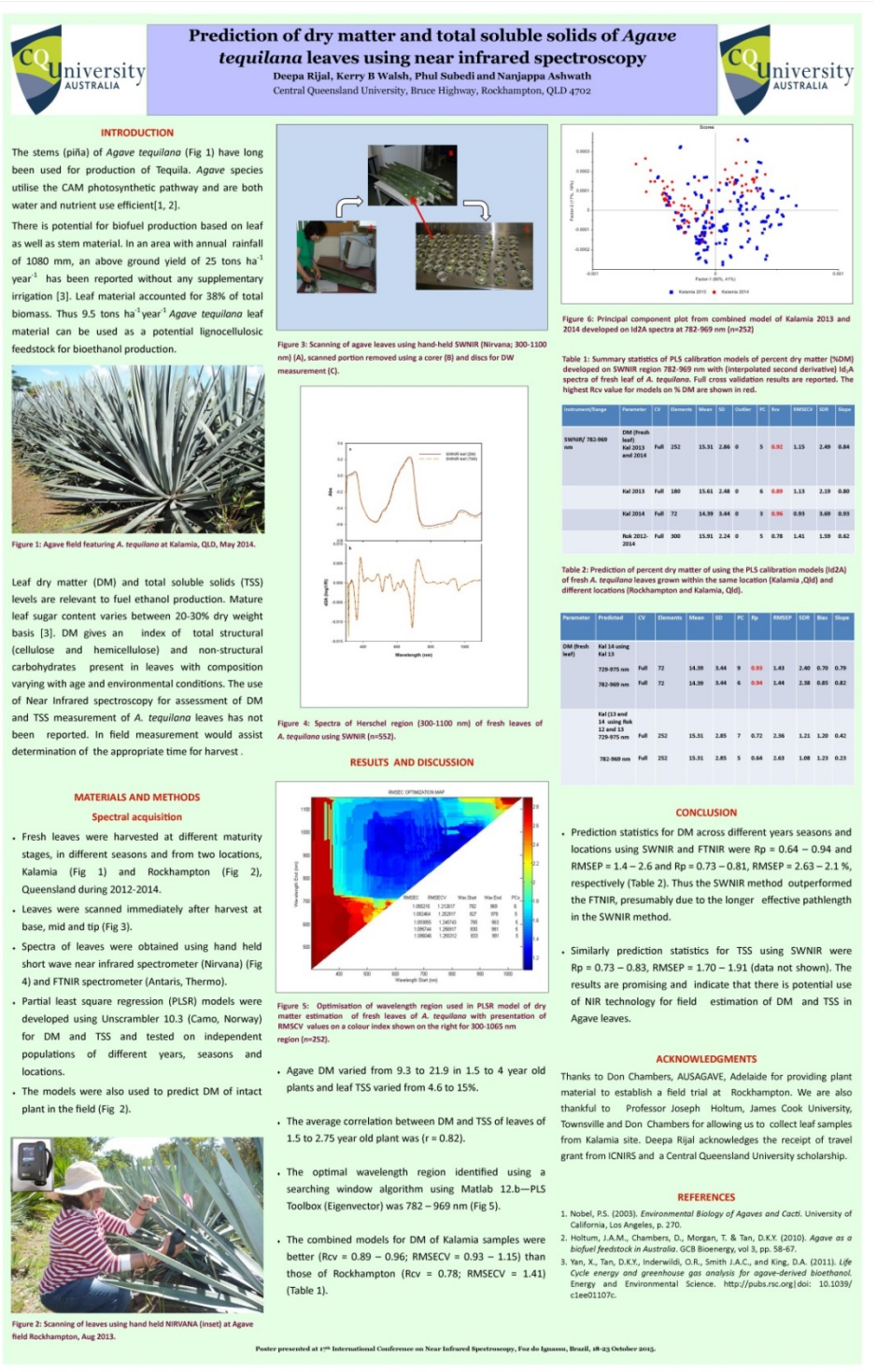
This poster won the first prize and received the “Best Poster Award. The expanded version of the contents of this poster has been published.

Rijal, D, Vancov, T, McIntosh, S, Ashwath, N and Stanley, G 2016, ‘Process options for conversion of *Agave tequilana* leaves into bioethanol’, *Industrial Crops and Products*, vol. 84**,** pp. 263–272.

A4.3 Prediction of Dry Matter and Total Soluble Solids of *Agave tequilana* Leaves Using Near Infrared Spectroscopy

Poster presented at 17th International Conference on Near Infrared Spectroscopy, Foz do Iguassu, Brazil, 18–23 October 2015.



Prediction of dry matter and total soluble solids of *Agave tequilana* leaves using near infrared spectroscopy

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INTRODUCTION

The stems (piña) of *Agave tequilana* (Fig 1) have long been used for production of Tequila. *Agave* species utilise the CAM photosynthetic pathway and are both water and nutrient use efficient[1, 2].

There is potential for biofuel production based on leaf as well as stem material. In an area with annual rainfall of 1080 mm, an above ground yield of 25 tons ha^{-1} $year^{-1}$ has been reported without any supplementary irrigation [3]. Leaf material accounted for 38% of total biomass. Thus 9.5 tons $ha^{-1}year^{-1}$ *Agave tequilana* leaf material can be used as a potential lignocellulosic feedstock for bioethanol production.

Figure 1: Agave field featuring *A. tequilana* at Kalamia, QLD, May 2014.

Leaf dry matter (DM) and total soluble solids (TSS) levels are relevant to fuel ethanol production. Mature leaf sugar content varies between 20-30% dry weight basis [3]. DM gives an index of total structural (cellulose and hemicellulose) and non-structural carbohydrates present in leaves with composition varying with age and environmental conditions. The use of Near Infrared spectroscopy for assessment of DM and TSS measurement of *A. tequilana* leaves has not been reported. In field measurement would assist determination of the appropriate time for harvest .

MATERIALS AND METHODS

Spectral acquisition

- Fresh leaves were harvested at different maturity stages, in different seasons and from two locations, Kalamia (Fig 1) and Rockhampton (Fig 2), Queensland during 2012-2014.
- Leaves were scanned immediately after harvest at base, mid and tip (Fig 3).
- Spectra of leaves were obtained using hand held short wave near infrared spectrometer (Nirvana) (Fig 4) and FTNIR spectrometer (Antaris, Thermo).
- Partial least square regression (PLSR) models were developed using Unscrambler 10.3 (Camo, Norway) for DM and TSS and tested on independent populations of different years, seasons and locations.
- The models were also used to predict DM of intact plant in the field (Fig 2).

Figure 2: Scanning of leaves using hand held NIRVANA (inset) at Agave field Rockhampton, Aug 2013.

Figure 3: Scanning of agave leaves using hand-held SWNIR (Nirvana; 300-1100 nm) (A), scanned portion removed using a corer (B) and discs for DW measurement (C).

Figure 4: Spectra of Herschel region (300-1100 nm) of fresh leaves of *A. tequilana* using SWNIR (n=552).

RESULTS AND DISCUSSION

Figure 5: Optimisation of wavelength region used in PLSR model of dry matter estimation of fresh leaves of *A. tequilana* with presentation of RMSCV values on a colour index shown on the right for 300-1065 nm region (n=252).

- Agave DM varied from 9.3 to 21.9 in 1.5 to 4 year old plants and leaf TSS varied from 4.6 to 15%.
- The average correlation between DM and TSS of leaves of 1.5 to 2.75 year old plant was (r = 0.82).
- The optimal wavelength region identified using a searching window algorithm using Matlab 12.b—PLS Toolbox (Eigenvector) was 782 – 969 nm (Fig 5).
- The combined models for DM of Kalamia samples were better (Rcv = 0.89 – 0.96; RMSECV = 0.93 – 1.15) than those of Rockhampton (Rcv = 0.78; RMSECV = 1.41) (Table 1).

Figure 6: Principal component plot from combined model of Kalamia 2013 and 2014 developed on Id2A spectra at 782-969 nm (n=252)

Table 1: Summary statistics of PLS calibration models of percent dry matter (%DM) developed on SWNIR region 782-969 nm with (interpolated second derivative) Id2A spectra of fresh leaf of *A. tequilana*. Full cross validation results are reported. The highest Rcv value for models on % DM are shown in red.

Instrument/Range	Parameter	CV	Elements	Mean	SD	Outlier	PC	Rcv	RMSECV	SDR	Slope
SWNIR/ 782-969 nm	DM (Fresh leaf) Kal 2013 and 2014	Full	252	15.31	2.86	0	5	0.92	1.15	2.49	0.84
	Kal 2013	Full	180	15.61	2.68	0	6	0.89	1.13	2.19	0.80
	Kal 2014	Full	72	14.39	3.44	0	3	0.96	0.93	3.69	0.93
	Rok 2012-2014	Full	300	15.91	2.24	0	5	0.78	1.41	1.59	0.62

Table 2: Prediction of percent dry matter of using the PLS calibration models (Id2A) of fresh *A. tequilana* leaves grown within the same location (Kalamia ,Qld) and different locations (Rockhampton and Kalamia, Qld).

Parameter	Predicted	CV	Elements	Mean	SD	PC	Rp	RMSEP	SDR	Bias	Slope
DM (fresh leaf)	Kal 14 using Kal 13										
	729-975 nm	Full	72	14.39	3.44	9	0.93	1.43	2.40	0.70	0.79
	782-969 nm	Full	72	14.39	3.44	6	0.94	1.44	2.38	0.85	0.82
	Kal (13 and 14 using Rok 12 and 13 729-975 nm	Full	252	15.31	2.85	7	0.72	2.36	1.21	1.20	0.42
	782-969 nm	Full	252	15.31	2.85	5	0.64	2.63	1.08	1.23	0.23

CONCLUSION

- Prediction statistics for DM across different years seasons and locations using SWNIR and FTNIR were Rp = 0.64 – 0.94 and RMSEP = 1.4 – 2.6 and Rp = 0.73 – 0.81, RMSEP = 2.63 – 2.1 %, respectively (Table 2). Thus the SWNIR method outperformed the FTNIR, presumably due to the longer effective pathlength in the SWNIR method.
- Similarly prediction statistics for TSS using SWNIR were Rp = 0.73 – 0.83, RMSEP = 1.70 – 1.91 (data not shown). The results are promising and indicate that there is potential use of NIR technology for field estimation of DM and TSS in Agave leaves.

ACKNOWLEDGMENTS

Thanks to Don Chambers, AUSAGAVE, Adelaide for providing plant material to establish a field trial at Rockhampton. We are also thankful to Professor Joseph Holtum, James Cook University, Townsville and Don Chambers for allowing us to collect leaf samples from Kalamia site. Deepa Rijal acknowledges the receipt of travel grant from ICNIRS and a Central Queensland University scholarship.

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Poster presented at 17th International Conference on Near Infrared Spectroscopy, Foz do Iguassu, Brazil, 18-23 October 2015.