Exploring Cornstalk and Corn Biomass Silage Retting as a New Biological Fibre Extraction Technique

by

Rachel G. Campbell Murdy

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Chemical Engineering

Waterloo, Ontario, Canada, 2013

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Presently there are two forms of biological fibre extraction, water retting or dew retting, which use bacteria or fungi, respectively. Microbial action results in release of the cellulose fibres due to modification of the pectin, hemicellulose and lignin content from parenchyma cells and the middle lamellae. Water retting results in pollution, high costs associated with labour and drying, as well as significant waste water production, while disadvantages to dew retting include the need for appropriate climates, variable and inferior fibre quality, risks of over-retting as well as health effects due to dust and fungal contaminants.

The overall objective of this research was to explore silage retting as a new pre-processing technique allowing use of available farm infrastructure and contained retting conditions to produce plant-derived fibres with improved physical and chemical characteristics suitable for application in biocomposites. The corn processing ability of the hemp retting agents *Clostridium felsineum* and *Bacillus subtilis* was also investigated. Pleiotropic and/or crop management practices were assessed by comparing the physico-mechanical properties and the microbial populations during silage fermentation of genetically equivalent conventional, Roundup Ready® (RR) and *Bt*-Roundup Ready® (*Bt*-RR) corn isolines. Potential recovery of volatile organic acids in silage retting effluent as value-added chemicals was also explored.

The results indicated that *C. felsineum* is an effective corn retting agent given the effective release of the fibre bundles from the corn pith, with *B. subtilis* contributing to the retting process by reducing the oxygen content and providing the required anaerobic conditions for clostridial growth. The native microflora present in the plant phyllosphere also showed some retting ability. Composition, thermostability and mechanical properties of the biocomposites produced using the fibres from the retted corn were all found to vary depending on the variety of corn. Specifically, retted *Bt*-RR cornstalk showed a 15^{o}C increase in onset of degradation. Divergences between corn silage microbial communities analyzed by community-level physiological and enzyme activity profiling indicated that metabolic shifts were time-, region-, and contaminant-sensitive. Acetic and butyric acid production in silage

retting effluent was found to be highest under anaerobic conditions and was also influenced by corn hybrid variety, although a specific variety was not identified as most or least favourable for organic acid production due to high variability.

Bt-RR cornstalk material was found to have higher cellulose content and better thermostability with an onset of degradation of up to 45^{o}C higher than its genetic RR and conventional counterparts. However, fibres from the RR corn isoline produced biocomposites with the highest flexural strength and modulus. RR cornstalk-reinforced polypropylene showed a 37 and 94% increase in flexural strength and modulus, respectively when compared to the mechanical properties of the pure polypropylene. The *Bt*-RR and conventional varieties produced biocomposites with an average increase of 26.5% in flexural strength and 83.5% in flexural modulus.

The thermostability of ensiled corn biomass was found to be influenced by region, use of inoculants and silage treatment, while the silage treatment accounted for most of the variability in corn biomass composition. Polypropylene matrix biocomposites produced with (30 wt%) pre- and post-silage corn did not show significant differences in mechanical properties. However, ensiled corn resulted in an increase in fibres and potential microbial biomass of smaller particle sizes with more optimal thermostability and purity, producing biocomposites with higher flexural strength and modulus especially at higher extrusion temperatures.

Cornstalk is an effective reinforcement material, producing biocomposites with higher flexural strength, flexural modulus and impact strength. Whole corn biomass presents a potential alternative to other plant fibres, especially as filler material. Silage retting resulted in fibres with a higher thermostability and smaller particle size distribution that, given their already smaller aspect ratio, could result in better mechanical properties in thermoplastics with a higher melting temperature or biocomposites requiring higher shear for mixing.

Acknowledgements

I would first and foremost like to thank my supervisor, Dr. Raymond Legge, for his encouragement and assistance throughout my research. He has been my guide and mentor, sharing with me many of my life-changing events.

I would like to acknowledge the interest and helpful recommendations of my advisory committee members, Dr. Christine Moresoli, Dr. Leonardo Simon and Dr. Kesen Ma. I also thank in advance the attention of my external examiner Dr. Lope Galindo Tabil for his participation in my defence.

I am especially grateful to my lab colleagues, past and present, who were always willing to help me with lab equipment and analyses, providing useful suggestions. I feel very fortunate to have been surrounded by such wonderful people. A special thank you to the following individuals: Peter Smith for his assistance in finding corn material, Dr. Barbara Guettler and Dr. Mohammad Arif for their help with biocomposite preparation and testing, Dr. Sarah Meunier for her time with the method development and analysis of the organic acid data, Dr. Kela Weber for his advice on the statistical analysis, as well as Dr. Muhammad Javaid for his contribution to the enzyme analysis. The various farmers across Southwestern Ontario who supplied the corn silage for the field trial are also gratefully acknowledged. I am also appreciative of the funding I have received from NSERC and BioCar.

I would finally like to thank my family and friends for their continued support. A special thank you goes out to my new family, Shawn and Laurean, for their unwavering commitment to my academic career and their patience while I chased my ambitions.

Table of Contents

List of Figures

List of Tables

List of Symbols

®	Registered trademark
™	Trademark
A_i	Absorbance value of well *i*
A_k	Standardization of well *k*
$\overline{A_k}$	Pretreated value for the variable
A_0	Absorbance reading of the blank well
A'	Value of the transformed variable
a	Sampling factor
b	Slope
$\%C_c$	Percent crystallinity
D	Maximum deflection at the centre of specimen (mm)
d	Specimen thickness (mm)
E	Modulus of elasticity (MPa)
F	Applied load (N)
ΔH^o	Standard heat of enthalpy (J/g)
ΔH	Heat of enthalpy (J/g)
ΔH_c	Heat of enthalpy of crystallization (J/g)
ΔH_m	Heat of enthalpy of melting point (J/g)
L	Specimen length (mm)
n	Number of observations
OD_{590}	Absorbance at 590 nm
T5	Temperature at 5% weight loss (^oC)
T10	Temperature at 10% weight loss (^oC)
T_g	Glass transition temperature (^oC)
T_m	Melting temperature (^oC)
S (or SD)	Standard deviation
$SE_{kurtosis}$	Standard error of kurtosis
$SE_{skewness}$	Standard error of skewness

w	Specimen width (mm)
$\bar{y}$	Mean of a sample
y_i	Value for the transformed variable
$z_{kurtosis}$	z-value of kurtosis
$z_{skewness}$	z-value of skewness
ε_f	Flexural strain (mm/mm)
σ	Flexural stress or strength (MPa)

List of Abbreviations

ABE	Acetone-butanol-ethanol
AD	Acid detergent
ADF	Acid detergent fibre
AO	Antioxidant
ASTM	American Society for Testing and Materials
AWCD	Average well colour development
BOD	Biological oxygen demand
BSTFA	bis-(trimethylsilyl)trifluoroacetamide
BuOH	Butanediol
CA	Coupling agent
CAR	Center for Automotive Research
CFU	Colony forming units
CLPP	Community-level physiological profiling
CSUC	Carbon source utilization curves
CSUP	Carbon source utilization pattern
CWDE	Cell wall degrading enzyme
DMA	Dynamic mechanical analyzer
DNS	Dinitrosalicylic acid
DSC	Dynamic scanning calorimetry
DSMZ	Deutsche Sammlung von. Mikroorganismen und Zellkulturen
FID	Flame ionization detector
FTIR	Fourier-transform infrared
GC	Gas chromatography
gds	Gram dry substrate
GPS	Global positioning system
HDPE	High-density polyethylene
hPP	homopolymer polypropylene
IS	Internal standard
LAB	Lactic acid bacteria

LLDPE	Linear low-density polyethylene
MAPP	Maleic anhydride polypropylene
MFI	Melt flow index
MS	Mass spectrometry
ND	Neutral detergent
NDF	Neutral detergent fibre
NIR	Near infrared spectroscopy
NSERC	National Science and Engineering Research Council
PC	Principle component
PCA	Principle component analysis
PG	Polygalacturonase
PL	Pectin lyase
PLFA	Phospholipid fatty acid
PMC	Polymer-matrix composites
PP	Polypropylene
PYT	Peptone, yeast extract and tryptone
OEM	Original Equipment Manufacturer
OMAFRA	Ontario Ministry of Agricultural, Food and Rural Affairs
RR	Roundup Ready®
SEM	Scanning electron microscopy
TGA	Thermogravimetric analysis
VOA	Volatile organic acid

Chapter 1
Introduction

Increasing awareness of environmental sustainability and waste management is leading to research and industrial interest in biocomposite materials. Biocomposites consist of a synthetic polymer as the matrix material reinforced with plant fibres or natural fibres from other natural sources. These biofibres add strength and stiffness to the material due to their cellulose microfibrils, which are the structural components of plants. For use in biocomposites, plant-derived cellulose fibres must first be extracted from the supporting lignin and hemicellulose fractions, as these non-cellulose components will decrease the strength of the fibre and the resulting biocomposite making it more susceptible to biological, ultraviolet and thermal degradation (Mohanty *et al.*, 2000). Current fibre extraction techniques include biological, chemical (including enzymatic) and physical separations (Faruk *et al.*, 2012), although combinations of these are often required for processing.

Biological fibre extraction is known as water retting and dew retting, which use bacteria and fungi, respectively. The fibre bundles are separated from the core, epidermis and cuticle, and into smaller bundles and individual fibres. Bacteria, such as *Bacillus* sp. and *Clostridium* sp., and fungi, such as *Rhizomucor pusillus* and *Fusarium lateritium*, separate the lignin, pectin and hemicelluloses from parenchyma cells and the middle lamellae, leaving the cellulose fibre (Henriksson *et al.*, 1997; Reddy and Yang, 2005). Water retting involves fermentation of the matrix polysaccharides, which results in pollution including odour nuisance and high costs of labour and drying as well as water waste and high oxygen demand (Henriksson *et al.*, 1997; Meijer *et al.*, 1995; Morrison III *et al.*, 2000; Tahir *et al.*, 2011). Dew retting has slowly replaced water retting, where harvested crops are left out in the field and exposed to fungal organisms that partly degrade the same polysaccharides releasing the fibres (Meijer *et al.*, 1995; Henriksson *et al.*, 1997; Akin *et al.*, 1998). Disadvantages of dew retting include the need for appropriate climates, variable and inferior fibre quality in comparison to water

retting, risks of overretting as well as health problems due to dust and fungal contaminants (Sharma and Faughey, 1999; Tahir *et al.*, 2011; Van Sumere, 1992).

This thesis investigates a new approach to biological fibre extraction using solid state fermentation in silage facilities as a means of converting the plant polysaccharides into organic acids thereby releasing the fibre. This new microbial preprocessing could theoretically combine the advantages of water and dew retting while addressing some of the shortcomings of both methods. Silage is an example of solid state fermentation where microbes ferment soluble carbohydrates, such as glucose, into organic acids which preserve the biomass for feed. There is no information on the effect of silage retting on the properties of plant fibres, therefore microbially processed corn biomass should be incorporated into a polymer matrix to determine whether it provides value as a reinforcement material. By virtue of the "enclosed" nature of the silage process, extraction and use of other value-added materials for industrial purposes such as organic acids may be realized.

1.1 Objectives

The four main objectives, and their respective secondary objectives, of this research are as follows:

1. Investigate silage retting as a new microbial pre-processing technique by exploring the potential use of *Clostridium felsineum* and *Bacillus subtilis* as corn retting agents.
    - To ascertain the retting ability of *Clostridium felsineum*, *Bacillus subtilis* and combinations of the two in aerobic and anaerobic conditions.
    - To explore the retting ability of the indigenous microbial population in the aerobic and anaerobic conditions, in addition to potentially identifying organisms of interest.
2. Collect and analyze fermentation by-products produced from silage effluent as potential value-added products.

- To develop the methodology required for identifying and quantifying organic acid production by gas chromatography-mass spectrometry.
- To monitor organic acid production as an indicator of bacterial growth and metabolic activity.

3. Assess the thermal and compositional characteristics of cornstalk fibres from 3 varieties representing genetically equivalent isolines, as well as determine the mechanical properties of cornstalk-reinforced polypropylene biocomposites.
    - To determine thermal stability of the cornstalk fibres by thermogravimetric analysis.
    - To quantitatively assess cellulose, hemicelluloses and lignin content in addition to other plant constituents by spectroscopic techniques or wet chemistry.
    - To produce and test cornstalk-reinforced polypropylene biocomposites to determine potential impact of corn variety on the resulting mechanical properties.
4. Evaluate the potential for large scale applications in silage batch-reactors using farm infrastructure in a set of field trials.
    - To determine corn biomass composition and thermal characteristics from inoculated and non-inoculated silos.
    - To produce and determine the mechanical properties of corn biomass-reinforced polypropylene biocomposites from the microbially processed plant material.

1.2 Thesis Organization

This thesis consists of 5 research-based chapters, preceded by a general introduction and literature review. Conclusions and recommendations are presented as the final chapter of the thesis. Briefly, chapters 3 to 5 investigate the use of the hemp retting bacteria *Bacillus subtilis* and *Clostridium felsineum* as well as the indigenous microbial population as potential corn processing agents. Chapters 6 and 7 give an overview of the thermal and compositional analyses of cornstalk and corn biomass varieties, respectively. Chapter 6 outlines the mechanical properties resulting from cornstalk-reinforced polypropylene biocomposites,

while Chapter 7 gives an overview of the mechanical properties of whole corn biomass-reinforced polypropylene biocomposites. A schematic overview of the research is presented in Figure 1.1.

Chapter 3 describes the bench scale trials exploring the cornstalk processing ability of the bacteria *Bacillus subtilits* and *Clostridium felsineum*, two hemp retting agents. This preliminary research was used as a proof of concept for the subsequent investigation of the potential pleiotropic effects of corn variety by studying 3 Pioneer corn isolines, which are genetically equivalent conventional, Roundup Ready® and *Bt*-Roundup Ready® varieties, and the set-up for the silage retting field trial.

Chapter 4 describes the bacterial community dynamics of corn during ensiling by community-level physiological profiling (CLPP). This chapter fulfills the objective of characterizing the ability of *C. felsineum* and *B. subtilis* to compete with the indigenous population present in the plant phyllosphere. Crop management practices were found to affect the robustness of the plant-associated microorganisms, thereby influencing the impact of the microbial inoculant. This chapter is in preparation for submission (May, 2013).

Chapter 5 summarizes a study describing the methodology used to identify and quantify volatile organic acids as potential value-added materials. A techno-economic analysis is also presented to determine the commercial feasibility of extracting these compounds.

Chapter 6 presents a comprehensive examination of the thermal and mechanical properties of 3 corn isolines (varieties from a hybrid family thought to be genetic equivalents).

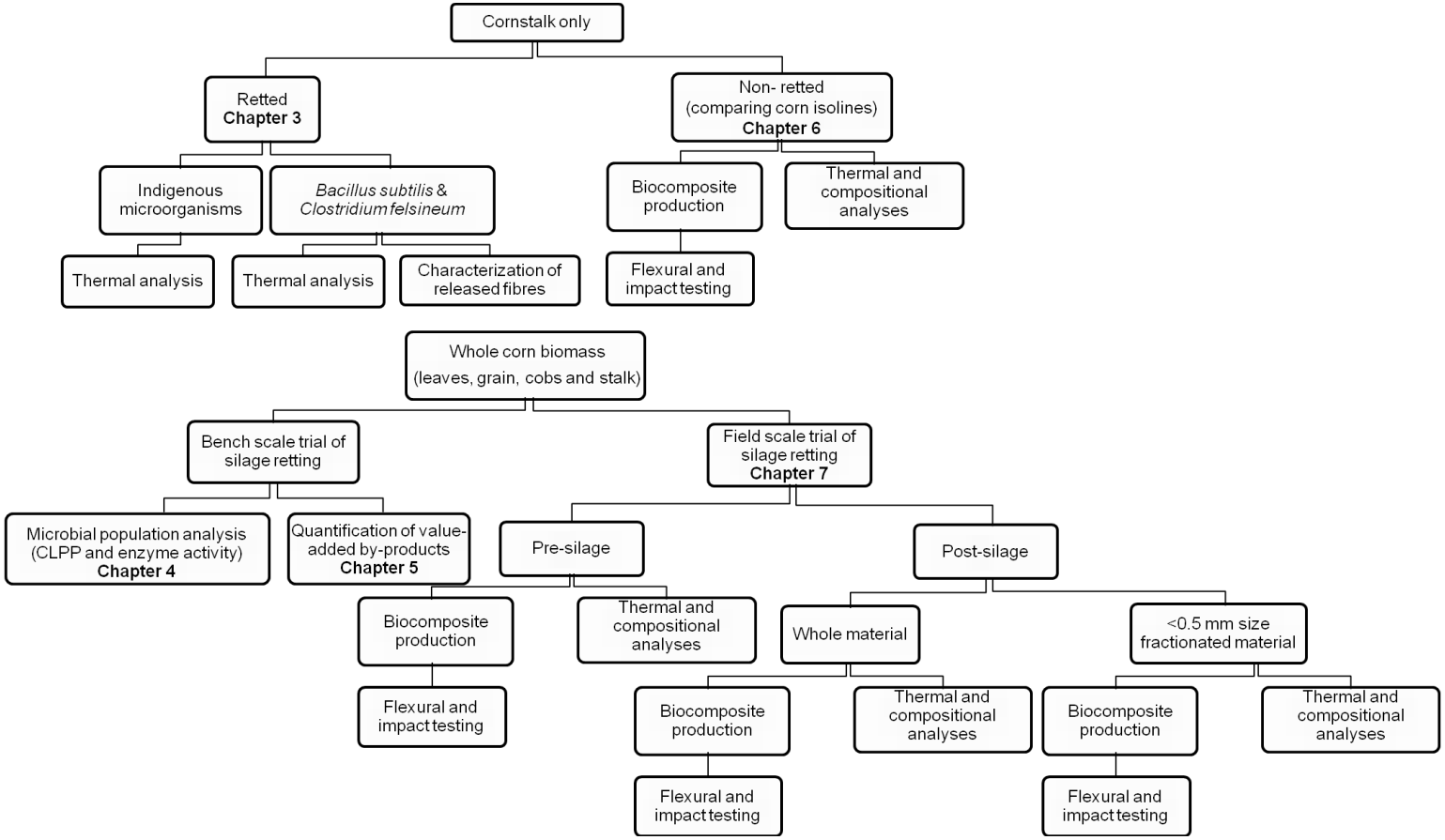


Figure 1.1 Schematic representation of the investigation of the microbial processing of cornstalk and corn biomass material.

Significant differences in the mechanical properties of the corn isolines tested could have implications for the potential use of corn fibres as filler in biocomposites. This chapter is in preparation for submission (May, 2013).

Chapter 7 presents the potential use of ensiling as a pre-processing technique for corn biomass as a feedstock source for the biocomposite industry. Farms across Southwestern Ontario participated by supplying corn silage material which was characterized by compositional analysis and thermal behavior. Comparisons between polypropylene reinforced with pre- and post-ensiled biomass indicated that both resulted in an increase in mechanical properties.

Chapter 2

Literature Review

Research into the manufacture and use of biocomposites is building momentum due to increasing environmental concerns associated with synthetic materials and emerging cost benefit aspects associated with biocomposites (Faruk *et al*., 2012). "Green" biocomposites consist of a biodegradable plant-based polymer as the matrix material reinforced with plant fibres. Research in the area of plant fibres is focusing on optimizing the efficiency of fibre production, extraction and binding of the fibre to the matrix for the production of biocomposites.

Biofibres are classified into two categories: wood fibres used in the construction industry and non-wood fibres used in biocomposites. The non-wood fibres are also divided into straw fibres, bast, leaf, seed/fruit and grass/reed fibres. Primary plants (jute, hemp, kenaf, and sisal) are grown for their fibre content while secondary plants (pineapple, oil palm and coir) produce fibres as a by-product (Faruk *et al.*, 2012). Bast fibres are most commonly used in biocomposites due to their high strength and stiffness qualities. The major structural and chemical constituents of biofibres are cellulose, hemicellulose and lignin, with secondary components of pectin and waxes. Cellulose is a hydrophilic, linear polymer of D-anhydroglucose units, which contain alcoholic hydroxyl groups, joined by β-1,4-glycosidic linkages (Mohanty *et al.*, 2000). The hemicellulose component is a highly branched polysaccharide attached to the cellulose. Lignin is a phenolic compound that forms the matrix sheath, giving structural support to the plant by cross-linking the spaces between the cellulose and hemicellulose during lignification. Pectins are heteropolysaccharides consisting of α-1,4-linked galacturonic acid units, while waxes consist of various alcohols (Eichhorn *et al.*, 2001).

The structural components come together to form the primary wall of the plant. Cellulose, hemicellulose and lignin can each vary in percent content depending on the species and age of the plant (Table 2.1). A plant with high cellulose content is desirable given its more

Table 2.1 Cellulose, hemicellulose and lignin composition of biofibres (Adapted from Faruk *et al.*, 2012, and Reddy and Yang, 2005)

Fibre Source	Cellulose %	Hemicellulose %	Lignin %
Corn stover	38-40	28	7-21
Pineapple leaf fibre	70-82	18	5-12
Coir	36-43	0.15-0.25	41-45
Bagasse	32-55	17-24	23-32
Banana	60-65	6-8	5-10
Wheat straw	33-38	26-32	17-19
Rice straw	28-36	23-28	12-14
Sorghum stalks	27	25	11
Barley straw	31-45	27-38	14-19
Bamboo	26-43	30	21-31
Flax	71	18.6-20.6	2.2
Kenaf	72	20.3	9
Jute	61-71	14-20	12-13
Hemp	68	15	10
Ramie	68.6-76.2	13-16	0.6-0.7

favourable mechanical properties. However, the degree of polymerization of the cellulose also affects the properties of the fibre, with bast fibres (flax, ramie, kenaf, hemp and jute) showing the highest degree of polymerization (Mohanty *et al.*, 2000). The hemicellulose and lignin can be detrimental to the integrity of the cellulose fibre and are largely removed from the microfibrils during extraction. It is therefore conceivable that a plant with less cross-linking would have cellulose fibres that are more easily extractable. The following are known to influence the quality of the fibre and its crystallinity: (i) diameter and length, where an increase in diameter corresponds with a decrease in modulus, (ii) the microfibrillar angle (angle between axis and fibril of the fibre) affects the strength and stiffness, with a smaller angle having higher mechanical properties, (iii) lignin content will affect the fibres' structure, properties and morphology, (iv) waxes will affect the wetability and compatibility of the fibre with the matrix (Mohanty *et al.*, 2000). It is therefore important to consider the content

of each component with respect to the end use of the fibre. For example, coir and flax show low and high tensile strength, respectively, due to their respective cellulose content and microfibrillar angle (Bledzki *et al.*, 1996). Based on the mechanistic properties of adequate tensile strength, Young's modulus and elongation at break, hemp and flax remain popular choices as reinforcing biofibres for biocomposites. In fact, hemp is currently subsidized as a non-food agriculture crop in the European Union (Faruk *et al.*, 2012).

An important factor in fibre variability may be attributed to the altered lignin content of genetically modified crops. For example, corn genetically engineered to express the *cry1Ab* gene from *Bacillus thuringiensis* has been shown to have higher lignin content than its isogenic non-*Bt* counterpart (Saxena and Stotzky, 2001). Using the acetyl bromide method (Hatfield *et al.*, 1999), Saxena and Stotzky determined that the content of lignin of the same portion of the corn stems was 33-97% higher in all hybrids of *Bt*-corn (2001). It should be noted that the acetyl bromide assay for lignin quantification was originally developed for woody plant species (Johnson *et al.*, 1961). The assay's application in herbaceous plants has been fraught with inconsistent results due to interference of xylan (a hemicellulose component) degradation resulting in overestimation of lignin (Hatfield *et al.*, 1999). The lignin content of plants genetically engineered to express the *cp4 epsps* gene conferring tolerance to the herbicide glyphosate has not been studied. However, there is potential for higher deposition of lignin in these Roundup Ready® varieties as expression of the gene affects the shikimate pathway, which leads to the over production of phenylalanine (Bentley, 1990, Padgette *et al.*, 1995). Phenylalanine is a precursor in the phenylpropanoid pathway, leading to the formation of the phenolic compounds present in lignin.

2.1 Overview of use of plant fibres in the automotive industry

The use of natural reinforcing fibres derived from renewable and sustainable plant resources as alternatives to traditional glass, carbon and aramid composites is gaining popularity in industrial applications from construction material to the automotive industry. According to GreenVehicleDisposal.com 5 to 6% of the Canadian automotive fleet is taken off the road

every year, translating to between 400,000 and 500,000 vehicles in Ontario. Between 10 and 11 million vehicles are discarded as end-of-life vehicles each year in the United States, with 25% of the vehicles by weight non-recyclable (Mohanty *et al.*, 2002). The European Union end-of-life directive will allow a quota of only 5% incineration for discarded passenger cars and light commercial motor vehicles by 2015 (Directive 2000/53/EC, 2000). Based on the increasing cost of disposal of end-of-life vehicles, which must be stripped or wrecked due to mechanical failure, the automotive industry is looking for environmentally friendly alternatives to petroleum-based plastics and synthetic fibres. For example, numerous original equipment manufacturers including Toyota, Honda, Ford and General Motors, are researching and/or implementing the use of various natural fibres in their vehicles, both as seat covers and reinforcing material. Advantages of the biofibres include: low cost, low density, high toughness, adequate strength properties, reduced tool wear, reduced dermal and respiratory irritation, good thermal properties, ease of separation, enhanced energy recovery and biodegradability (Mohanty *et al.*, 2000). However, natural fibres have many variable features that affect their mechanical properties, including chemical properties such as cellulose, lignin and hemicellulose content and physical properties such as density, diameter, tensile strength, Young's modulus and elongation at break.

The properties of the extracted cellulose fibres in comparison to their E-glass (or electrical grade glass, fiberglass), Kevlar and carbon counterparts are compared in Table 2.2. The density of plant fibres is generally lower or equivalent to that of synthetic fibres, resulting in a lower weight material. The range of the diameter of plant fibres is highly variable, with most having a much higher diameter than the synthetic fibres. However, the method used to extract the fibre from the plant will affect its diameter, such that an extraction that will yield a smaller or larger diameter can be selected based on the anticipated use of the fibre. The tensile strength of the fibres is the measure of tensile stress required to pull the fibre until it reaches its breaking point. The tensile strength of synthetic fibres can be 10 times higher than that of natural fibres. However, when taking into account the lower density of the plant fibres, the tensile strengths are comparable on a mass basis. The Young's modulus, which

measures the stiffness of a given material, ranges from 4-6 GPa for coir up to 128 GPa for ramie, which is comparable to the Young's modulus of E-glass and Kevlar (Bismarck *et al*., 2005). However, carbon fibres are 4 to 6 times stiffer than hemp. The choice of the stiffness of the fibre will depend on its eventual application. The percent elongation at break, which measures the ability of the fibre to stretch without breaking, shows natural fibres to be within a comparable range to synthetic fibres.

Table 2.2 A comparison of the mechanical properties of common plant fibres with their synthetic counterparts (Adapted from Bismarck *et al.* (2005), Hill *et al.* (2012) and Faruk *et al.* (2012))

Fibre	**Density (g/cm^3)**	**Diameter (µm)**	**Tensile strength (MPa)**	**Young's Modulus (GPa)**	**Elongation at Break (%)**	**Price (US/kg)**
Flax	1.5	40-600	345-1500	27.6	2.7-3.2	1.5
Hemp	1.47	25-500	690	70	1.6	0.6-1.8
Jute	1.3-1.49	25-200	393-800	13-26.5	1.16-1.5	0.35
Ramie	1.55		400-938	61.4-128	1.2-3.8	1.5-2.5
Sisal	1.45	50-200	468-700	9.4-22	3-7	0.6-0.7
Abaca	1.5		430-760	12	3-10	1.5-2.5
Cotton	1.5-1.6	12-38	287-800	5.5-12.6	7-8	1.5-2.2
Coir	1.15-1.46	100-460	131-220	4-6	15-40	0.25-0.5
E-glass	2.55	< 17	3400	73	2.5	1.3
Kevlar	1.44		3000	60	2.5-3.7	
Carbon	1.78	5-7	3400[a]-4800[b]	240[a]-425[b]	1.4-1.8	
Polyethylene (HDPE)	0.95		28		30	
Polypropylene	0.9		200		35	
Polystyrene (high impact)	1.05		15		35	

[a]Ultra High Modulus carbon fibres
[b]Ultra High Tenacity carbon fibres

2.1.1 Biocomposites in the automotive industry

Bio-based composites are currently being used by the automotive industry in a variety of products in interior applications such as door panels and trunk liners (Alves *et al.*, 2010). Natural fibres are appealing to the automotive industry because they are considered biodegradable, non-abrasive to processing equipment, CO_2 neutral, and offer acoustic and thermal dampening (Peijs, 2002). With the rising cost of crude oil and consumer awareness, Original Equipment Manufacturers (OEMs) and their suppliers are investigating these new natural and renewable products. Germany has been using natural fibres in biocomposites in the automotive production sector for close to 2 decades. From 1996 to 2003, use of biofibres from hemp, flax and exotic plants increased from 4,000 to 18,000 tons per year in Germany (Karus *et al.*, 2004), predicted to increase to 100,000 tons by 2010 (Bledski *et al.*, 2006). Toyota, Daimler Chrysler and Ford are now following Germany's lead using natural fibres or plastics in some of their vehicles. For example, Toyota announced in 2008 its goal of replacing 20% of the plastics used in its vehicles with bio-based plastics by 2015 (Hill *et al*., 2012; Otani, 2008). Ford is currently using a polypropylene biocomposite reinforced with 20% (w/w) wheat straw for the storage bins in the 2010 Ford Flex (Hill *et al*., 2012). A description of other OEMs' use of bio-based materials can be found in the 2006 Automotive Plastic Report Card by Juska *et al.* (2006). For example, Daimler Chrysler is using biofibres from flax, coconut and abaca in their Mercedes vehicles (Juska *et al.*, 2006). According to the OEM, the Mercedes A-Class includes 26 components weighing 23 kg made from natural materials. The Mercedes S-Class has 27 bio-based components weighing 43 kg (Juska *et al.*, 2006). Other automotive manufacturers using biofibres include Audi, BMW, Fiat, Opel, Saab, Volkswagon, Volvo and Mitsubishi (Bledzki *et al.*, 2006). Use of natural fibres in automotive components is expected to increase over 50% per year (Alves *et al.*, 2010). These biocomposites are primarily used in front door liners (1.2 – 1.8 kg), rear door liners (0.8 – 1.5 kg), boot liners (1.5 – 2.5 kg), and seat backs (1.6 – 2.0 kg) (Bledzki *et al.*, 2006).

In Ontario, the Ontario BioCar Initiative (BioCar, 2011) and the Ontario BioAuto Council (BioAuto Council, 2011) focus on the development of bio-based materials by linking the

industry sector with universities and the provincial government. Federally, AUTO21 has also funded research in bio-based materials (AUTO21, 2011). More recently, the Centre for Automotive Research (CAR) published a study on the adoption of bio-based materials in the automotive industry in the Great Lakes region by examining three case studies (Hill *et al*., 2012). Beyond the traditional uses bio-based materials in wood trim, cotton textiles and leather seats, the two primary non-traditional uses emphasized were polymers or reinforcement/filler (Table 2.3). For example, certain Ford vehicles contain interior door panels made of a 50 percent kenaf fiber-reinforced polypropylene biocomposite (Fernyhough and Markotsis, 2011; Hill *et al.*, 2012). The case studies reported by Hill *et al*. (2012) include: (1) wheat straw reinforced biocomposite in a storage bin of the Ford Flex, (2) bio-based material commercialization fund managed by the Ontario BioAuto Council, and (3) castor oil based nylon in the radiator end tank of the Toyota Camry.

Table 2.3 Current bio-based components in domestic vehicles (Adapted from Hill et al., 2012).

Model(s)	**Feedstock**	**Material**	**Application**
Cadillac DeVille	Wood	Polypropylene	Seatbacks
Chevrolet Impala	Flax	Polypropylene	Trim, rear shelf
Ford Flex	Wheat straw	Polypropylene	Interior storage bins
Ford vehicles	Soy	Polyurethane	Foam seating, headrests, headliner
GMC Terrain	Cotton, kenaf	Polyester	Acoustic insulator, ceiling liner

2.2 Fibre extraction and modification techniques

Widespread industrial use of plant fibres is hindered by 2 main factors: (1) non-cellulose components that decrease the strength and thermostability of the cellulose fibres, and (2) poor compatibility between the hydrophilic filler and hydrophobic matrix. The structural components of plants reside in their cellulose microfibrils, which are further supported by lignin and hemicellulose. In order to use the natural fibres in industrial applications, the

lignin and hemicellulose must be extracted from the crystalline cellulose as these will decrease the strength of the fibre as well as increase susceptibility to biological, ultraviolet and thermal degradation (Mohanty *et al.*, 2000). The nature of the plant source will dictate the techniques necessary for fibre extraction or surface modification due to differences in the cellulose, hemicellulose, lignin and wax content. Research into various fibre extraction and surface modification techniques has led to many fibre processing methods, which can be categorized as chemical and physical, enzymatic or biological.

2.2.1 Surface modification of natural fibres

Modification of plant fibres may involve mercerization (alkaline) and silane treatments, in addition to acetylation and use of maleated coupling agents. Given the polarity of cellulose fibres due to their free hydroxyl groups, compatibility between filler and matrix can be achieved by introducing a third material which acts by modifying the morphology of the interface, the acid-base reactions in the interface, the surface energy and wetting (Faruk *et al.*, 2012; George *et al.*, 2001).

Mercerization, also known as alkaline treatment, is an old method of treating textiles by soaking the fibres in a dilute solution of sodium hydroxide, which improves the surface adhesion by removing impurities resulting in a rough surface topography (Bisanda and Ansell, 1991). The alkaline treatment disrupts the hydrogen bonding in the cellulose structure. For example, alkali treatment in 0.5 N solution of sodium hydroxide of sisal-epoxy biocomposites resulted in a material with higher rigidity and density in addition to lower porosity (Bisanda, 2000, Faruk *et al.*, 2012). In other research, Bisanda and Ansell (1991) noted that although the compressive strength of sisal-epoxy biocomposites improved, the flexural strength and stiffness were not affected. Improvements in treated pineapple leaf fibre (Huda *et al.*, 2008), ramie (Goda *et al.*, 2006), and jute (Ray *et al.*, 2001) also showed greater interfacial bonding and adhesion between matrix and filler (Faruk *et al.*, 2012). Alkalization followed by an enzymatic treatment of corn husks showed good pliability, moderate strength, high elongation at break (Reddy and Yang, 2005). Yilmaz (2012) used alkalization to extract

fibres from corn husk and reported lower moisture content due to a decrease in hydroxyl groups.

Silane treatment forms stable covalent bonds to the fibre surface and the resin, which would improve the contact angle against water by increasing the hydrophobicity and decreasing the hygroscopicity (Bisanda and Ansell, 1991). As described by Bisanda and Ansell (1991), a silane chemical reacts with water to form a silanol and an alcohol. In the presence of moisture, the silanol reacts with the hydroxyl groups of the cellulose molecules, bonding itself to the cell wall. Faruk *et al.* (2012) describe the latest research involving silane treatment as plant fibre surface modifiers for kenaf, abaca, flax, oil palm, and coir. In each case, silane was effective at reducing the polarity of the plant fibres and improving the interfacial adhesion of the biocomposites, resulting in better mechanical properties.

The acetylation treatment increases the hydrophobicity of the plant fibres by replacing the hydroxyl group with an acetyl functional group. As with other chemical treatments, a decrease in hydrophilicity leads to lower moisture absorption and better interfacial adhesion. Recent research into acetylated plant fibres (Faruk *et al.*, 2012) is summarized in Table 2.4.

Table 2.4 Summary of recent research into plant fibres treated by acetylation.

Fibre source	Results	Reference
Flax	Decrease of degree of polymerization of cellulose Lower moisture absorption	Bledzki *et al.*, 2008
Abaca	Improvement in tensile strength, tensile modulus and impact strength	Seena *et al.*, 2005
Flax, hemp and wood	Removal on non-crystalline plant constituents Change in surface topography Change in fibre surface free energy Improvement of stress transfer	Tserki *et al.*, 2005
Coir and oil palm	Higher bio-resistance Better tensile strength (in comparison to silane treatment)	Hill *et al.*, 2000 Khalil *et al.*, 2001

The use of maleated coupling agents to improve filler and matrix adhesion is increasingly popular. For example, maleic anhydride-grafted polypropylene (MAPP) has the same molecular structure as polypropylene with the maleic anhydride group attached to the backbone (Soleimani *et al.*, 2012). This treatment targets both the plant fibre as well as the matrix for better interfacial bonding resulting in higher mechanical properties by using a graft polymer (example polyethylene or polypropylene) with a maleated anhydride. A reference search of the use of coupling agents in biocomposites yielded over 3,700 publications ranging from vegetable-based to vinyl isocyanate coupling agents spanning four decades from 1969 to 2013.

Modification of plant fibres by physical methods includes either the corona or plasma treatments, which are similar techniques for surface oxidation activation. The coronas treatment arose in 1951 from the need to bond normally incompatible polymers by using surface activation techniques by plasma discharge treatment, either at below 100 torr (glow discharge), or near atmospheric pressure with low (corona discharge) or high (arc plasmas) current densities (Blais *et al.*, 1971). Improvement in interfacial adhesion has been proposed to originate as a combination of 4 mechanisms: (1) mechanical interlocking, (2) adsorption due to dispersion forces, (3) electrostatic attraction, and (4) interdiffusion (Blais *et al.*, 1971). The plasma treatment produces a variety of reactive free radicals and groups, thereby changing surface cross-linking. More recently, corona discharge and plasma treatments have shown improvements in compatibilization between hydrophilic fibres and hydrophobic matrices (Faruk *et al.*, 2012, George *et al.*, 2001). A summary of the latest research on physical modification of plant fibres is presented by Faruk *et al.* (2012) in their in-depth review of biocomposites reinforced with natural fibres.

2.2.2 Enzymatic extraction techniques

Enzymatic processing of plant fibres for their use in biocomposites is becoming increasingly popular for a multitude of reasons, including (1) enzymes being regarded as environmentally friendly, (2) specific and focussed reactions, (3) cost reduction, (4) energy and water savings,

(5) improved product quality and performance (Aehle, 2004; Bledzki *et al.*, 2010). As with other plant fibre modification techniques, enzyme treatment of hemp, flax, wool, cotton and abaca improves the surface properties by removing non-cellulosic components such as lignin, fats, waxes and proteins. The processing enzymes include cellulases, hemicellulases, pectinases, proteases, lipases and laccases. Recently, Bledzki *et al.* (2010) explored the use of fungamix, a commercially available mixture of enzymes (Novozyme, Denmark) as a processing agent for abaca fibres. The tensile strength of modified abaca biocomposites was found to increase 5 to 45% due to the enzymatic treatment. Fungamix in combination with a maleic anhydride polypropylene coupling agent resulted in abaca fibre biocomposites showing about 35% better flexural properties when compared to unmodified fibre biocomposites (Bledzki *et al.*, 2010). Other commercial cellulose and xylanase enzymes include Celluclast® and Pentopan® Mono BG, respectively (Novozyme, Denmark) (Yilmaz, 2012). As commercial enzyme mixes become more widely available, their industrial use in processing plant fibres is expected to increase.

2.2.3 Biological extraction techniques

There are two forms of biological fibre extraction, water retting or dew retting, which use bacteria or fungi, respectively. Fibre bundles, mostly from hemp and flax, are separated from the core, epidermis and cuticle, and into smaller bundles and individual fibres. Bacteria, such as *Bacillus* sp. and *Clostridium* sp., and fungi, such as *Rhizomucor pusillus* and *Fusarium lateritium*, remove the lignin, pectin and hemicelluloses from parenchyma cells and the middle lamellae, leaving the cellulose fibre (Henriksson *et al.*, 1997; Reddy and Yang, 2005).

2.2.3.1 Water retting

Water retting, or tank retting, involves bacterial fermentation of the plant matrix polysaccharides while submerged in water. Clostridia, and to a lesser extent bacilli, are the main water retting agents, due to the activity of the extracellular enzyme polygalacturonase (Chesson, 1978; Akin *et al.*, 2001; Zhang *et al.*, 2000; Tamburini *et al.*, 2003).

Early research showed a retting process that could be divided into three phases: the establishment of an anaerobic flora in the initial phase (0-20 hours), followed by a period of rapid pectinolysis (20-50 hours), with a terminating period of slow pectinolysis (Chesson, 1978). Donaghy *et al.* (1990) further characterized the process by following the succession of bacteria during anaerobic flax retting. The anaerobic retting process was divided into four phases: (1) a lag phase from 0 to 5 hours, characterized by low bacterial count consisting mostly of staphylococci, (2) an early phase from 5 to 40 hours, during which *Lactobacillus*, *Cellulomonas* and *Bacillus* spp. reached their highest population, (3) a mid-phase from 40 to 80 hours, where a dramatic increase in clostridia was seen, and a final (4) late phase, during which clostridial populations fell, while *Lactobacillus* and *Bacillus* spp. remained relatively constant (Donaghy *et al.*, 1990). The microbial population fluxes were most likely responding to changes in dissolved oxygen, whereby residual oxygen was quickly eliminated during the first 20 hours. Clostridial growth was accompanied by a corresponding production of acetic and butyric acid (Donaghy *et al.*, 1990). The strains with the highest retting ability were found to be *Bacillus licheniformis* and *Bacillus subtilis*, succeeded by *Clostridium acetobutylicum* (renamed *Clostridium saccharobutylicum*) and *Clostridium felsineum*.

Further water retting research has been performed on hemp, studying duration and temperature effects of the process. Di Candilo *et al.* (2000) and Tamburini *et al.* (2003) found that the addition of *C. felsineum* significantly reduced the duration of retting from 12 to 6 days. Mechanical decortications, termed scutching, was also found to enhance the process for hemp stems retted with the microorganism, leading to a lighter, softer and finer fibre (Di Candilo *et al.*, 2000). Physico-mechanical benefits were observed with the clostridial species leading to a higher modulus value, indicating higher cellulose content. This observation was confirmed by Fourier-transform infrared (FTIR) spectroscopy, where the signal intensity at 1734 cm^{-1} (Rau, 1963; Garcia-Jaldon, 1997), representing non-cellulosic components, was significantly less when retting with *C. felsineum* (Di Candilo *et al.*, 2000). More recently, Di Candilo *et al.* (2010) reported that inoculating hemp with the water-retting agents

Clostridium sp. L1/6 and *Bacillus* sp. ROO40B significantly sped up the process to 3-4 days while improving the fibre quality.

A recent study compared the effects of water retting, alkalization and enzymatic treatment of corn husk fibres (Yilmaz, 2012). The effects of the different extraction processes on the physical, thermal and chemical properties on the corn husk fibres were reported. The highest moduli results were obtained from the water-retted corn husks, although infrared measurements indicated higher amounts of lignin and hemicelluloses. The enzymatic treatment and alkalization improved the thermal behavior of the fibres (Yilmaz, 2012).

Although water retting has been found to produce higher quality fibres than dew retting, the high costs of labour and drying as well as the pollution associated with this biological fibre extraction technique has encouraged research in alternative retting processes (Henriksson *et al.*, 1997; Meijer *et al.*, 1995; Morrison III *et al.*, 2000; Tahir *et al.*, 2011).

2.2.3.2 Dew retting

Between 1965 and 1980, flax retting was almost completely replaced by dew retting because of its advantages which include that it can be fully mechanized and the reduction in waste water production (Meijer *et al.*, 1995). In dew retting, the stems are pulled and spread out in fields where aerobic filamentous fungi colonize the plants and degrade the polysaccharides in cell walls and middle lamellae with pectinolytic enzymes (Meijer *et al.*, 1995; Henriksson *et al.*, 1997; Akin *et al.*, 1998). The quality of the dew-retted straw is dependent on flax cultivar, soil nutrients, crop management and weather conditions (Sharma and Faughey, 1999; Trove, 1996; Vlaswinkel, 1996)

Due to regional variability in native soil fungi, many studies have been dedicated to identifying retting microorganisms and evaluating their retting efficiencies. Table 2.5 lists some of fungal species isolated in Europe and the United States, along with their retting activity. Bacteria that have also been identified on flax stems include *Bacillus mycoides*, *B.*

subtilis, *Erwinia carotovora*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Micrococcus* sp. (Sharma, 1986b). Sharma (1986b) found that *B. subtilis* produced cell wall degrading enzymes (CWDEs) such as pectin-lyase and xylanase, which were suspected to be important during the later retting stages, with maximum activity detected 4 weeks after spraying. Polygalacturonase, the predominant CWDE in dew retting produced by numerous fungal species (Akin *et al.*, 2002), was not detected in cultures of *B. subtilis*, indicating the importance of the fungal population (Sharma, 1986b). The bacterial population was also found to decrease in response to an increase in fungal growth.

Table 2.5 Examples of fungi isolated from dew retted flax in Europe and the United States.

Organism	Location	Retting efficiency	Reference
Fusarium equiseti	South Carolina	Less efficient	Henricksson *et al.*, 1997
Rhizomucor pusillus	South Carolina	Very efficient	Henricksson *et al.*, 1997; Akin *et al.*, 1998
Trichoderma virens	South Carolina	Very efficient	Henricksson *et al.*, 1997
Alternaria alternate	South Carolina	Less efficient	Henricksson *et al.*, 1997
	Lambeg, Ireland	Not reported	Sharma, 1986a
Fusarium lateritium	South Carolina	Very efficient, with potential over-retting	Henricksson *et al.*, 1997; Akin *et al.*, 1998
Fusarium lateritium	Connecticut	Very efficient	Henricksson *et al.*, 1997
Cladosporium herbarum	Connecticut	Not reported	Henricksson *et al.*, 1997
	Lambeg, Ireland	Not reported	Sharma, 1986a
Fusarium oxysporum	France	Less efficient	Henricksson *et al.*, 1997
Epicoccum nigrum	Netherlands	Very efficient, with potential over-retting	Henricksson *et al.*, 1997; Akin *et al.*, 1998
	Lambeg, Ireland	Not reported	Sharma, 1986a
Aspergillus sojae	Culture collection, University of Georgia	Very efficient	Henricksson *et al.*, 1997
Fusarium culmorum	Lambeg, Ireland	Not reported	Sharma, 1986a
Phoma spp.	Lambeg, Ireland	Not reported	Sharma, 1986a
Mucor spp.	Lambeg, Ireland	Not reported	Sharma, 1986a
Rhizopus spp.	Lambeg, Ireland	Not reported	Sharma, 1986a

Research focusing on fungal retting agents has shown that, as in water retting, a succession of species occurs with variations due to retting treatment. In general *Cladosporium herbarum*, *Epicocum nigrum* and yeast appeared first, followed by *Alternaria alternata*, *Fusarium culmorum* and *Phoma*, *Mucor* and *Rhizopus* sp. (Sharma, 1986a). Growth of *A. alternata*, *F.culmorum* and *Phoma* spp. has resulted in overetting due to the production of cellulases, resulting in the recommendation of recovering the stems before their appearance (Brown and Sharma, 1984; Sharma, 1986a). Ground retted flax was also compared to flax stems left standing. Ground retted flax was found to be colonized more rapidly than stems left standing (Sharma, 1986a). It was suggested that ground retting provided higher moisture and temperature, accelerating enzyme activity. However, stand-retting produced fibres of greater strength, which was attributed to slower growth of the aggressive cellulose-producing fungal species (Sharma, 1986a). The over-retting activity of fungi was further confirmed by Akin *et al.* (1998), who found that *F. lateritium* and *E. nigrum* degraded the fibre cell walls in addition to the parenchyma tissues. Henricksson *et al.* (1997) found large differences among the growth characteristics of the different fungi, including mycelia, spores and fruiting bodies as well as coloured-staining of the stems and agar plates.

Variability in dew-retted flax straw from different cultivars was studied qualitatively and quantitatively by Sharma and Faughey (1999). Four flax cultivars, Ariane, Evelin, Laura and Viola, were grown, harvested and laid out in the field for dew-retting. The resulting retted straw was analyzed chemically for neutral detergent fibre (NDF) and acid detergent fibre (ADF), while lignin content was determined by sulphuric acid treatment followed by refluxing in acid detergent solution. Thermogravimetric analysis was also performed to measure thermal stability variations. It was found that the cultivar Laura produced the best fibre, although further genotypic comparisons were not performed (Sharma and Faughey, 1999).

Research on fungal retting has been extended to wheat straw. In a small scale study, wheat straw was cut and placed in bottles with nutrient broth (Sain and Panthapulakkal, 2006). After autoclaving and inoculation with a culture of *Apospheria* sp., the treatments were incubated aerobically for 3 weeks and the wet straw was defibrillated. Retted fibres were found to have better mechanical properties with a smaller diameter and greater strength, due to partial removal of the hemicelluloses and lignin (Sain and Panthapulakkal, 2006). Degradation characteristics of the mechanically processed and microbial retted fibres are presented in Table 2.6. Microbial retted wheat straw showed an increased thermal stability, an important attribute for processing fibres into biocomposites. An increased peak temperature, the temperature measured at the maximum rate of degradation, was attributed to a reduction in the diffusion of degradation products, which also elevated the rate of degradation. Sain and Panthalulakkal (2006) also hypothesized that the decrease in residue remaining at 550^{o}C could be due to partial removal of silica from the wheat straw during the retting treatment.

Table 2.6 Thermal degradation characteristics of wheat straw processed mechanically and microbially (Adapted from Sain and Panthapulakkal, 2006)

Wheat straw fibre	**Moisture content (%)**	**Onset of degradation (^{o}C)**	**Peak temperature (^{o}C)**	**Rate of degradation (wt.%/min)**	**Residue after 550^{o}C (%)**
Mechanical processing	5	217	324.7	8.2	25.8
Microbial retting	4	231	351.5	11.6	9.3

In their comprehensive review of retting processes, Tahir *et al.* (2011) compared the various retting types used for the production of long bast fibres. Their results are summarized in Table 2.7. Enzymatic retting is included as a basis for comparison given that many formulations are prepared by isolating and extracting enzymes from retting microorganisms.

Table 2.7 A comparison of biogical fibre extraction processes used for the production of long bast fibres (adapted from Tahir *et al.*, 2011).

Retting Type	Description	Advantages	Disadvantages	Duration of retting	References
Dew	Plant stems are cut and left on the field	Pectin material can be removed by bacteria/fungi	Reduced strength, low and inconsistent quality Climatic restrictions Product contamination with soil	2-3 weeks	Van Sumere, 1992 Sharma and Faughey, 1999
Water	Plant stems are immersed in water	Produces fibres of greater uniformity and quality	Extensive pollution from anaerobic fermentation High cost Low-grade fibres	7-14 days	Van Sumere, 1992 Sharma, 1987 Yu and Yu, 2007 Cochran *et al.*, 2000 Banik *et al.*, 2003 Rome, 1998
Enzymatic	Enzymes (pectinases, xylanases, etc.) are used to degum the bast	Easier refining, with faster and cleaner processing	Lower fibre strength	12-24 hours	Van Sumere, 1992 Akin *et al.*, 2007

2.3 Microbial community profiling

Given the importance of the activity of the microbial community in biological fibre extraction, a thorough analysis of the metabolic profiling of the organisms during dew and water retting would be of benefit. Research on retting microorganisms has so far been limited to culturing and molecular characterization, mostly based on pectinolytic activity. For example, Di Candilo *et al.* (2000) isolated 258 spore-forming bacterial strains in attempts to culture *Clostridium felsineum* from hemp, flax and soil samples. Community level physiological profiling (CLPP) (Lehman *et al.*, 1995) is a technique that is increasingly being used to track mixed microbial communities based on their metabolic profile. BIOLOG™ microplates, which are plates with 96 wells containing a different carbon source and a redox dye indicator, are used to compare communities based on sole carbon source utilization patterns (CSUPs). BIOLOG EcoPlates™ consist of 31 different carbon sources and a blank triplicate with a tetrazolium dye to track the catabolic profile of a community, thereby giving an idea of both the catabolic activity and range of metabolic function of a community (Weber and Legge, 2010). The carbon source utilization profiles (CSUPs) can be analyzed by principal component analysis (PCA) and guild analysis (Weber and Legge, 2009; Weber *et al.*, 2007; Zak *et al.*, 1994).

When performing CLPP, plates are inoculated and absorbance readings at 590 nm are collected during the incubation. Given the potential for large amounts of data, analysis can quickly become overwhelming. It is most useful to select a metric based on absorbance values for statistical analysis (Weber and Legge, 2010). Such values may include a specific time point, an average well colour development (AWCD) for that plate or a logistic curve fitting value for growth curve analysis. An appropriate time point will preserve the greatest variance between well responses while retaining the maximum number of wells within the linear absorbance range (below 2.0) (Weber and Legge, 2010). Once a metric is selected, the data will require standardization and its underlying structure (heterogeneity, normality) will need to be assessed. Upon this assessment, a data transformation may be required.

A thorough description of the CLPP analysis is presented by Weber and Legge (2010). The following is a brief summary of said publication. If a time point absorbance is used, standardization of the data will reduce bias due to varying inoculum densities in BIOLOG™ microplates (Garland, 1997; Weber and Legge, 2010). Represented by Equation 2:1, standardization of the data involves adjusting each collected absorbance value by its corresponding blank, and then dividing by the average well colour development for that time point. The standardization of well *k* can be calculated as:

$$\overline{A_k} = \frac{A_k - A_0}{\sum_{i=1}^{31}(A_i - A_0)}, \qquad (2:1)$$

where A_i represents the absorbance value of well *i* and A_0 represents the absorbance reading of the blank well (Weber and Legge, 2010). Negative values, which are considered meaningless, are coded as zero.

PCA assumes two fundamental properties: normality and homoscedasticity (equal variance), which must be met for proper data evaluation. Normality of BIOLOG™ microplate data can be evaluated by assessing the standard errors of kurtosis and skewness, represented by equations 2:2 and 2:3, respectively (Weber *et al.*, 2007):

$$SE_{kurtosis} = \sqrt{24/n}\,, \qquad (2:2)$$

$$SE_{skewness} = \sqrt{6/n}\,, \qquad (2:3)$$

where *n*, is the number of observations. Kurtosis describes the peakedness or flatness relative to a normal distribution curve, while skewness characterizes the asymmetry of distribution around the mean of the data. The corresponding *z* values can be calculated as:

$$z_{kurtosis} = \frac{\text{kurtosis}}{SE_{kurtosis}}, \qquad (2:4)$$

$$z_{skewness} = \frac{\text{skewness}}{SE_{skewness}}. \qquad (2:5)$$

These *z* values can then be used to test the null hypothesis that the data is normally distributed. A 2-tailed test is used and the null hypothesis is rejected if $|z| > 1.96$ (95% confidence interval).

Homoscedasticity describes the homogeneity of variance, based on the assumption that all variables within a data set have variance. Given the impracticality of assessing the homogeneity of the variance by scatter plot of paired variables (Weber and Legge, 2010), a variance ratio can be calculated:

$$\text{variance ratio} = \frac{\text{highest variance}}{\text{lowest variance}} \quad (2:6)$$

A lower separation between the maximum and minimum variance of any one variance will indicate more homogeneity between variance of the whole data set.

Linear correlation between variables must also be evaluated for each carbon source by counting the number of correlation coefficients greater than Pearson's critical *r* value (Weber and Legge, 2010). If 2 variables are considered to be linearly correlated within a 95% confidence level, data transformation can be used to significantly reduce the number of linear correlations.

According to Weber *et al.* (2007), two transformations are commonly used for the analysis of ecological data and can also be employed on data collected from BIOLOG ™ microplates, namely the Taylor Power transformation and the natural logarithmic transformation.

The Taylor transformation (Taylor, 1961) is commonly used to stabilize variances and meet the assumption of normality (Legendre and Legendre, 1998). It is based on the assumption that:

$$S^2 = a\bar{y}^2 \quad (2:7)$$

where S is the standard deviation of a sample, $\bar{y}$ is the mean of a sample and a is the sampling factor. This leads to the following equation:

$$\log S^2 = \log a + b \log \bar{y}^2 \quad (2:8)$$

where the slope b can be obtained by plotting the linear regression of all variables. Finally, this leads to the transformation:

$$y_i^{'} = y_i^{(1-\frac{b}{2})} \; for\; b \neq 2, \qquad (2:9)$$

$$y_i^{'} = \log y_i \; for\; b = 2, \qquad (2:10)$$

where y_i is the value for the transformed variable (Weber and Legge, 2010).

A logarithmic transformation (Legendre and Legendre, 1998) can also be used to normalize skewed data. A common logarithmic transformation used is (Weber *et al.*, 2007):

$$A^{'} = \ln(\overline{A_k} + 1) \quad (2:11)$$

where $A^{'}$ is the value of the transformed variable and $\overline{A_k}$ is the pretreated value for the variable.

Many other data transformations can be used to analyze data sets in order to meet the assumptions required for PCA.

Based on the preceding analytical methods for statistical analysis, use of CLPP in combination with PCA allows characterization of the stability and metabolic activity of a microbial community (Amador and Görres, 2007; Bucher and Lanyon, 2005; Garland and Mills, 1991; Garland *et al.*, 2010; He *et al.*, 2012; Heuer and Smalla, 1997; Weber *et al.*, 2007, 2008; Weber and Legge, 2009, 2010, 2011; Widmer *et al.*, 2001).

2.4 Silage fermentation

The silage process is a preservation method used to minimize losses in nutrients of harvested forage crops for later usage. The plant material is enclosed in a pit, tower, bunker, trench or plastic silo, ensuring an anaerobic environment to prevent spoilage by aerobic microorganisms (Woolford, 1984). Although used for millennia, the fermentation in silos is a relatively uncontrolled process. The general ensilage begins with a rapid removal of air by plant respiration. The lactic acid bacteria present on the plant material or added as inoculants then ferment the sugars into a variety of organic acids, resulting in a rapid decrease in the pH. Silage fermentation is essentially a solid-state fermentation, due to its low moisture content. As opposed to a submerged fermentation in which the water content reaches over 95%, for solid-state fermentation the water content ranges between 40 and 80% (Shuler and Kargi, 1997). Although silage fermentations vary in water content depending on the crop and wilting period, water content can range from approximately 50 to 80% (Woolford, 1984; McDonald *et al.,* 1991). Disadvantages of high moisture content silage fermentation include: higher clostridial growth due to higher pH, low nutritional value due to lower dry matter intake, production of high volumes of effluent, and greater difficulty in handling and processing (McDonald *et al.*, 1991).

There are parallels between silage and water or dew retting. The first is that fermentation is involved in both processes. The microorganisms found on the plant material rapidly convert the plant polysaccharides into sugars and organic acids, which depend on the organisms present. Bacteria can be grouped into lactic acid bacteria, clostridia, fungi, molds, bacilli, listeria, acetic acid bacteria and propionic acid bacteria. Lactic acid bacteria (LAB) dominate in successful silage, producing large amounts of lactic acid by the Embden-Meyerhof-Parnas (glycolytic) pathway. The group is composed of a variety of genera including *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* (McDonald *et al.*, 1991). Clostridia and bacilli involved in retting are also often implicated in silage. These bacteria are often indicative of poor silage by maintaining a higher pH, thereby preventing growth of LAB.

Clostridia are present as spores on plant material and in soil, requiring an oxygen-free atmosphere for vegetative growth. Certain clostridia grow exponentially during the first few days of ensiling, although extensive growth usually only occurs in late silage (McDonald *et al.*, 1991). If the pH is not reduced rapidly by the LAB, a secondary clostridial fermentation may lead to an increase in production of butyric acid, resulting in reduced cattle intake due to unpalatable silage (Schroeder, 2004). Bacilli are also found in low populations on plant material and soil. However, their growth does not appear to be significant during the fermentation process (Gibson *et al.*, 1958). Many bacilli produce lactic and acetic acids, contributing to the decrease in pH, although less efficient in the production of lactic acid than LABs (McDonald *et al.*, 1991). Growth of clostridia and bacilli, and the corresponding production of organic acids from clostridia and bacilli, is not encouraged during ensiling, therefore studies targeting biological fibre extraction in silage has yet to be pursued.

2.4.1 The silage bioreactor

Silage material is stored with the main objective of rapidly reaching anaerobic conditions. Various methods of storage have been explored and hermetically sealing the material has been found to be the most efficient (McDonald *et al.*, 1991). In a sealed container, termed a silo, the respiratory enzymes in the degrading plant material trap the oxygen, thereby decreasing aerobic microbial activity and subsequent plant decay while promoting lactic acid production.

Silos vary in sizes and volume capacity from commercial tower silos to single large bale silos. For example, the Ontario Ministry of Agricultural, Food and Rural Affairs (OMAFRA) lists the most common tower silo sizes for alfalfa, whole-plant corn silage and forages ranging from 3.7 to 9.1 metres in diameter and 9.1 to 33.5 metres in settled depths. These dimensions translate from close to 30 to over 2000 tonnes capacities (Jofriet and Daynard, 1988). Other commercial silos include clamp silos with and without retaining walls, which can reach from 2 to 3 metres in height, and the popular plastic sausage silo with a diameter of

2.4 metres but lengths ranging up to 30 metres (McDonald *et al.*, 1991). The widely used individual bale silos can contain material weighing from 0.5 to 0.75 tonnes (McDonald *et al.*, 1991).

Regardless of size and capacity, the silage bioreactor is essentially a batch reactor without mixing. The chopped plant material is heaped and sealed with little manipulation during the reaction. The plant cells quickly respire carbohydrates while oxygen remains available, with most of the energy being dissipated as heat, causing an increase in reactor temperature from an ideal initial temperature of 22^{o}C up to 30^{o}C (McDonald *et al.*, 1991; Woolford, 1984). Although temperatures between 38 and 40^{o}C were found to hasten the onset of fermentation, these high temperatures were also attributed with an increase in clostridial growth (Woolford, 1984). As oxygen becomes limiting, the facultative anaerobes produce lactic acid, ethanol and alanine from glycolysis as well as an accumulation of organic acids, leading to a decrease in pH to approximately 4 (McDonald *et al.*, 1991). Due to the acidic environment, bacterial growth is arrested and steady state is achieved, preserving the plant material until later exposure to oxygen.

Silage seepage also presents concerns of pollution of land and water, corrosion and deterioration of the silo (Clarke and Stone, 2004). Silage effluent is of particular concern due to its high biochemical oxygen demand (BOD) of 12,000 to 90,000 mg/L, which is 60 to 450 times greater than domestic sewage (Clarke and Stone, 2004; McDonald *et al.*, 1991). Silage seepage is produced within 5 days of loading and is composed of nutrients, acids, minerals and bacteria (Table 2.8). Silage effluent must be stored on site, and can be used as fertilizer or as feed for farm animals (Clarke and Stone, 2004; McDonald *et al.*, 1991).

Table 2.8 Silage seepage composition and components (Clarke and Stone, 2004).

Seepage Characteristics/Components	**Details**
Lactic Acid	4%-6%
Acetic Acid	1%-2%
Butyric Acid	Normally less than 1%
Acidity	4
pH	3.5-5.5

Volatile organic acids (VOAs) in silage effluents are under review as potential value-added by-products for diversification of the current agriculture economy. For example, the organic compounds in grass silage liquor were found to complex heavy metals including cadmium, zinc, copper and nickel leading to sanitation of polluted soils (Leidmann *et al.*, 1993). Raw silage effluent produced from whole corn silage at 35% moisture at harvest was also found to contain a variety of alcohols in the range of 3 g/L, of which over half was ethanol (Lesperance *et al.*, 2008).

2.5 Mechanical properties and chemical characterization of biocomposites

Thermoplastic biocomposites are formed when using a thermoplastic matrix and natural fibre filler. These products are commonly used in industrial applications given their tensile, flexural and impact strengths as well as their thermal stability and chemical resistance. Polyethylene, polyurethane and polypropylene composites are especially common in the automotive sector. This section will focus on polypropylene biocomposite processing and testing with respect to its use in automotive applications.

2.5.1 Biocomposite processing

Thermoplastic composites must be processed for use in industrial applications. Specifically, processing most often includes extrusion of the material followed by injection moulding (Maier and Calafut, 1998). During extrusion, the thermoplastic is melted and compounded with fillers and other additives such as coupling agents, antioxidants (to prevent oxidative damage) and dyes. Features of polypropylene include low processing temperatures, low density and good chemical resistance (Maier and Calafut, 1998). Extruders consist of a motorized rotating screw (either single or double) which will mix the material as it melts. Typically, single screw extruders are used when rigorous mixing is not required. As the

material passes from the feeding region to the melting region, the screws will progressively mix and shear the melted thermoplastic with the filler. The pelletized biocomposite material is then available for further processing.

Injection moulding is commonly used to mould the extruded pellets into either testing material or its final shape. In comparisons to compression moulding, injection moulding is easier for up and down scaling, minimal warping and shrinkage, high function integration and use of recycled material (Faruk *et al.*, 2012; Scherübl, 2005). Other moulding techniques used for natural fibre reinforced thermoplastics in industry include compression moulding and flow compression moulding, as well as other long fibre thermoplastic-direct (LFT-D) methods, which involve direct feeding of long fibres into either an extruder or an injection moulding machine (Faruk *et al.*, 2012).

2.4.2 Biocomposite testing

Biocomposites with any end-use industrial application must meet certain predetermined requirements. For automotive applications, the most important parameters are the flexural characteristics and the Izod impact. The flexural modulus and strength of a material is a measure of its stiffness. These parameters are evaluated by a three-point-bend test according to ASTM standard method D790-07 (ASTM International, 2013). Force is applied onto a test sample, resulting in a stress/strain curve that can be analyzed to give the flexural performance of the biocomposite (Figure 2.1). Flexural stress is based on specimen thickness, bending and the moment of inertia of the cross-section (Callister 2001). The areas of elastic and plastic deformation are separated by a point which identifies the maximum force that can be applied before a material deforms permanently. The slope of the elastic region represents the modulus of elasticity (E-modulus). For a test bar, the flexural properties are obtained using the following formulas:

Flexural stress or strength, $\sigma = \frac{3FL}{2wd^2}$ (2: 12)

Flexural strain, $\varepsilon_f = \frac{6Dd}{L^2}$ (2:13)

Modulus of elasticity, $E = \frac{\text{flexural stress}}{\text{flexural strain}}$ or $E = \frac{L^3 m}{4wd^2}$, within elastic limit (2:14)

where, *F* is the applied load, and *L*, *w* and *d* are the specimen length, width and thickness, respectively. *D* is the maximum deflection at the centre. In order for a biocomposite to feasibly replace its glass fibre reinforced composite counterpart, it should meet or surpass the equivalent mechanical properties (Table 2.2).

The Izod impact test characterizes a material's brittleness by measuring the energy to break a specimen and is described by ASTM D256-06. The resistance of a material is determined by using a pendulum swing, which hits a notched sample creating a stress concentration. The Izod impact strength is presented in J/m (energy absorbed per unit of specimen width or per unit of cross-sectional area).

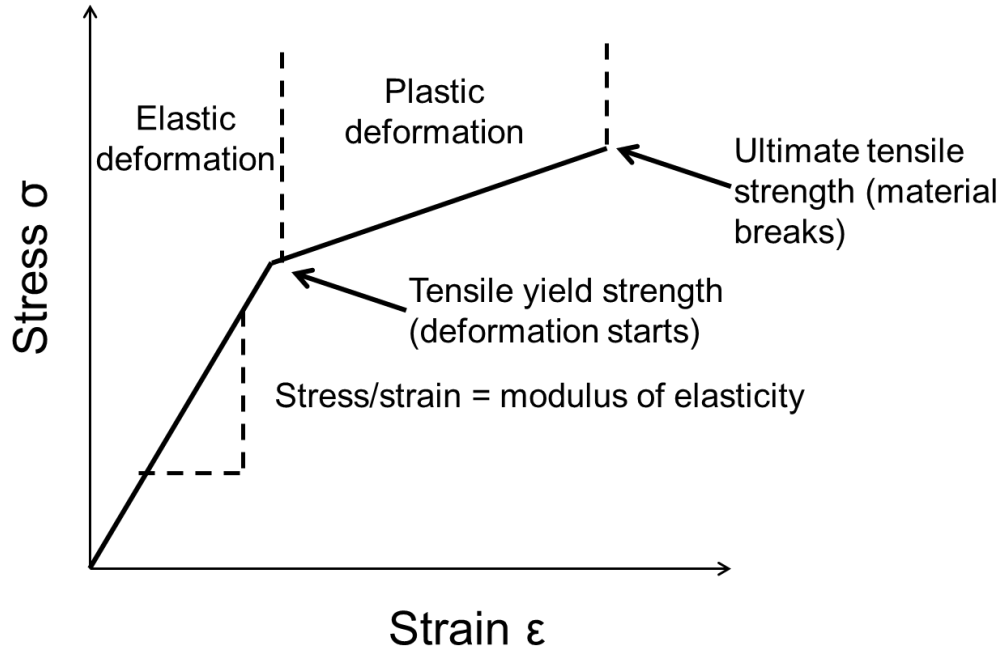


Figure 2.1 Typical stress/strain diagram resulting from a flexural test.

2.4.3 Characterization of a filler or biocomposite

Numerous tests are used to determine fibre quality. Fibre compositional analysis by wet chemistry is used to quantify acid detergent fibre (ADF) and neutral detergent fibre (NDF) for measurement of cellulose, hemicelluloses and lignin fraction, which can also be determined by near infrared spectroscopy (NIR). Other characterization techniques for filler and composite properties include thermogravimetric analysis (TGA) to determine thermal stability and Fourier transform infrared spectroscopy (FTIR) analysis to study the physico-chemical and conformational properties of the fibres (Sain and Panthapulakkal, 2006; Sharma *et al.*, 1999). Along with these characterization techniques, scanning electron microscopy offers a qualitative analysis of the interfacial adhesion between the polymer matrix and the plant fibre.

Acid detergent (AD) and neutral detergent (ND) methods are used to determine the cellulose, hemicellulose and lignin in samples, with the AD fibre fraction containing cellulose and lignin and the ND fibre fraction containing cellulose, hemicelluloses and lignin. The procedure is presented in detail by Sharma *et al.* (1999). The lignin fraction can be determined by the sulfuric acid method followed by refluxing in the AD fibre solution. The cellulose and hemicellulose fractions are then determined by finding the difference between AD and ND. For example when comparing water, dew and enzyme retted flax fibres, the hemicellulose content was not found to be significantly different (Sharma *et al.*, 1999). However, the cellulose content was highest in enzyme retted fibres, while lignin was highest in water retted fibres. It is expected that higher cellulose content will result in better mechanical properties.

Thermogravimetric analysis (TGA) is used to determine numerous thermal stability traits, including onset of degradation, degradation rates and ash content. The system can be either flushed with air or a gas such as helium, argon or nitrogen. In flax, the major weight loss occurring from 240-400^{o}C is attributed to the pectin, hemicellulose and cellulose. Decomposition from 400-520^{o}C may be attributed to lignin (Sharma *et al.*, 1999). However,

it has been reported that the activation energy for thermal degradation for different cellulosic, hemicellulose and lignin samples is in the range 36-60, 15-26, and 13-19 kcal/mole, respectively (Ramiah, 1970), and that lignin would begin to decompose first. As described above, Sain and Panthapulakkal (2006) found that retted fibres had a higher onset of degradation, peak temperature and rate of degradation than mechanically separated wheat straw fibres.

FTIR spectroscopy was reportedly first used in retting studies to determine the changes in retted flax (Himmelsbach *et al.*, 1998, 2002). Sain and Panthapulakkal (2006) used FTIR for comparing the physico-chemical changes attributed to retting of wheat straw. Their results are summarized in Table 2.9. The findings indicate the partial removal of hemicellulose and

Table 2.9 Summary of selected Fourier transform infrared spectroscopy wavenumbers and their corresponding chemical assignments.

Wavenumber (cm^{-1})	**Assignment**	**Findings**	**Reference**
3422	Stretching vibrations of O-H		Sun *et al.*, 2000
1056	Stretching vibrations of C-O		Sun *et al.*, 2000
1648	Absorbed water molecules associated		Himmelsbach *et al.*, 2002
1737	Carboxyl groups in the acids and esters of acetic, *p*-coumeric, ferulic and uronic acid (hemicellulose)	Intensity decreased in microbial retted fibres	Himmelsbach *et al.*, 2002
1456, 1366, 1312, 1242	C-H, O-H and CH_2 bending		Himmelsbach *et al.*, 2002
900, 1730	Indicate associated hemicelluloses	Intensity decreased in microbial retted fibres	Sun *et al.*, 2000
1505	Aromatics (lignin)	Low intensity indicating partial removal during retting	Himmelsbach *et al.*, 2002
1000-1500	Hemicellulose	Lower intensity indicate partial removal	Mwaikamboo and Ansell, 2002

lignin in microbial retted fibres (Sain and Panthapulakkal, 2006). Sgriccia *et al.* (2008) also showed that alkali treatment of kenaf, flax and hemp removed hemicelluloses and lignin for the fibre surfaces by FTIR. Adapa *et al.* (2011) recently used FTIR to predict differences in cellulose, hemicelluloses and lignin in non-treated and steam exploded agricultural biomass feedstocks including barley, canola, oat and wheat straw.

Along with mechanical testing, material characterization is typically a good predictor of biocomposite performance. For example, plant fibres that contain a high percentage of cellulose will often produce better mechanical properties in biocomposites. Hemp, which contains approximately 68% cellulose, has a tensile strength of 690 MPa (Faruk *et al.*, 2012). However, bamboo, at 26-43% cellulose, shows only a tensile strength of 140-230 MPa. Similarly, the Young's modulus ranges from 70 GPa for hemp to 11-17 GPa for bamboo (Faruk *et al.*, 2012).

Dynamic scanning calorimetry (DSC) is used to study the thermal properties, including the glass transition temperature (T_g), melting temperature (T_m), the percent crystallinity and phase changes of a polymer (Rudin, 1999). Essentially, a material is subjected to heating and cooling and an analysis of the phase transitions provides the amount of heat absorbed or released. Upon heating, a polymer will melt due to a breakdown in its crystallinity transitioning from solid to liquid. When cooled, the polymer will re-crystallize while undergoing an exothermic transition. Using DSC, the impact of a filler on a polymer's crystallinity can be quantified and compared to the pure resin.

Chapter 3

Bench Scale Trials Exploring the Effectiveness of *Bacillus subtilis* and *Clostridium felsineum* as Corn Retting Agents

Summary

Silage retting was evaluated as a new pre-processing technique for the extraction of plant fibres from cornstalks. The hemp retting agents *Bacillus subtilis* and *Clostridium felsineum* were used for retting of cornstalk material from conventional and genetically engineered corn isolines. The physical properties of the extracted fibres were compared. *C. felsineum* and *B. subtilis* demonstrated retting ability for cornstalk material and their suitability for fibre bioprocessing. Bacterial treatment and absence of grain resulted in fibres with a difference in their cellulose to lignin ratio of up to 3%, indicative of higher cellulose purity. An increase in onsets of degradation ranged from 3^{o}C to 15^{o}C in *Bt*-Roundup Ready® cornstalk-only and bacterial treatments, respectively, indicating higher thermostability of this treated isoline. Indigenous bacteria also showed retting ability in the non heat-treated controls.

Keywords: Cellulose; *Clostridium felsineum*; cornstalk; plant fibre; silage retting; thermostability

3.1 Introduction

Increasing pressure for environmental sustainability is leading to an interest in biocomposite materials, which consist of a matrix material reinforced with biofibres. Plant fibres add strength and stiffness to the material due to their cellulose microfibrils. In order for plant-derived fibres to be used in biocomposites, the fibres must first be extracted from the surrounding lignin and hemicellulose, as these non-cellulose components decrease the strength of the fibre and make the resulting biocomposite more susceptible to biological, ultraviolet and thermal degradation (Mohanty *et al.* 2000).

Biological fibre extraction is known as water retting and dew retting. Water retting involves bacterial degradation of the plant matrix polysaccharides while submerged in water. Clostridia and bacilli are the main water retting agents due to the production of the extracellular enzyme polygalacturonase (Zhang *et al.*, 2000; Akin *et al.*, 2001; Tamburini *et al.*, 2003). Water retting results in pollution, high costs associated with labour and drying, as well as significant waste water production. Dew retting has slowly replaced water retting, where the crops are left out in the field following harvest and exposed to fungal organisms that partly degrade the same polysaccharides releasing the fibres (Meijer *et al.*, 1995; Henriksson *et al.*, 1997; Akin *et al.*, 1998). Disadvantages of dew retting include the need for appropriate climates, variable and inferior fibre quality in comparison to water retting, risks of over-retting as well as health effects due to dust and fungal contaminants (Van Sumere 1992).

A new approach to biological fibre extraction is presented which uses solid state fermentation from a traditional silage system. The end purpose of the research is to develop a process which could make use of existing farm infrastructure at the farm gate under contained retting conditions. This new microbial preprocessing could combine the advantages of water and dew retting while addressing some of the shortcomings. Two agents, *Clostridium felsineum* and *Bacillus subtilis*, which are traditionally used in hemp retting, were evaluated as suitable retting agents for corn by comparing three Pioneer corn

isolines in a bench-scale silage retting setup. Therefore, the objective of this research was to investigate the corn retting ability of *C. felsineum* and *B. subtilis* as potential agents for silage retting, in addition to exploring the indigenous microbial population for new retting agents. The physical properties of the resulting retted fibres were analyzed and compared.

3.2 Materials and Methods

3.2.1 Preliminary Trial

Bacillus subtilis ROO2A, first isolated by Tamburini *et al.* (2003) as a hemp water retting agent, was sent by Dr. Giorgio Mastromei (University of Florence, Florence, Italy) and *Clostridium felsineum* DSM 794 was obtained from DSMZ (Braunschweig, Germany). *B. subtilis* ROO2A was cultured in a peptone, yeast extract and tryptone (PYT) medium at 30^{o}C under aerobic conditions and *Clostridium felsineum* DSM 794 was cultured in PYT supplemented with glucose and cysteine at 37^{o}C in an Oxoid anaerobic jar (Oxoid Ltd., Thermo Fisher Scientific, UK). Air dried cornstalks (Pioneer 39K37 RR) were cut into 0.5 inch pieces and autoclaved at 121^{o}C for 20 minutes. Five grams of sterilized and non-sterilized material was distributed into 250 ml Erlenmeyer flasks with 40 ml of M9 minimal salts medium (Miller 1972). Each flask was inoculated in triplicates with overnight cultures of either *B. subtilis* ROO2A or *C. felsineum* DSM 794; negative controls were not inoculated. The flasks were incubated at 30^{o}C with shaking at 200 rpm for six days, with observations every 24 hours. Hand-sections of the post-retted corn samples were stained with phloroglucinol for lignin content (Liljegren, 2010) and examined microscopically.

Mechanical properties of individual retted corn fibres were characterized with a DMA 2980 Dynamic Mechanical Analyzer (TA Instruments, New Castle, DE) with a preload force of 0.010 N, an isothermal temperature of 35^{o}C, a soak time of 0.10 min, a force ramp rate of 0.100 N/min and an upper force limit of 18 N.

3.2.2 Bench Scale Trial

C. felsineum DSM 794 and *B. subtilis* DSM 15029 (obtained from DSMZ, Braunschweig, Germany) were selected for further research. For vegetative growth, the organisms were grown according to Tamburini *et al.* (2003). Briefly, *B. subtilis* was grown on agar plates of medium composed of 0.5% yeast extract, 0.5% peptone, 1% tryptone at 30^{o}C. *C. felsineum* was grown on the same medium supplemented with 2% glucose and 0.05% cysteine at 37^{o}C in a CO_2 atmosphere in an in an Oxoid anaerobic jar (Oxoid Ltd., Thermo Fisher Scientific, UK) (Tamburini *et al.*, 2003).

Three Pioneer corn isolines were studied to determine any varietal impact: 38N85, 38N86 and 38N87, representing a Roundup Ready® (RR), a conventional and a *Bt*-Roundup Ready® (*Bt*-RR) stacked variety (Pioneer Hi-Bred Limited, 2008), respectively. The whole plants were harvested at or in the vicinity of the Elora Research Centre (Elora, Ontario) in mid-September. Cornstalks were collected in early-November from the same fields for the post-harvest trial. The plant material was chopped and heat-treated by autoclaving at 121^{o}C for 20 min. Experiments were conducted using a 2x3x8 factorial design, representing harvest time, corn variety and treatment (Table 3.1). Aerobic and anaerobic treatments were inoculated with 12 hr cultures of *B. subtilis, C. felsineum* or combinations of the two at concentrations of approximately 1 x 10^4 and 1 x 10^8 cfu ml^{-1}, respectively, along with the addition of 10 ml of M9 medium (Miller 1972) in 250 ml Erlenmeyer flasks. Heat-treated and non heat-treated controls were included. Anaerobic treatments were maintained using a gas-trap. All flasks were incubated at 30^{o}C for seven days. Fibre bundles were separated from the corn pith by hand and allowed to air dry prior to analysis.

Thermogravimetric analysis **(**TGA) experiments were performed using an SDT 2960 (TA Instruments, New Castle, DE) with a heating rate of 10^{o}C/min from 30^{o}C to 400^{o}C in a nitrogen environment. TGA plots were analyzed for: (i) moisture content determined from percent weight loss occurring at 100^{o}C; (ii) three onset points of degradation at 0.5 wt%/min,

1.0 wt%/min and 2.0 wt%/min; (iii) the maximum rate of degradation and it's corresponding peak temperature and (iv) the residue at run termination. Three onsets of degradation were selected to reduce the subjectivity of selecting an onset from the initial slope.

Table 3.1 2x3x8 experimental design for cornstalk retting trials. Treatments were performed in triplicate.

Harvest Time	Plant Variety	Condition	Treatment
Pre (whole plant) and Post (cornstalk only)	Conventional (Conv) Roundup Ready (RR) *Bt*-Roundup Ready (*Bt*-RR)	Aerobic	1. *B. subtilis* 2. *B. subtilis* + *C. felsineum* 3. Sterile control 4. Non-sterile control
		Anaerobic	5. *C. felsineum* 6. *C. felsineum* + *B. subtilis* 7. Sterile control 8. Non-sterile control

A qualitative analysis of fibre composition was determined using wavenumber assignment based on Sigmacell cellulose and lignin (alkali or kraft, low sulfonate content) standards (Sigma-Aldrich, St. Louis, MO). Spectra were acquired using a Tensor 27 FTIR spectrometer (Bruker Optik GmbH, Germany), along with OPUS 4.2 software (Bruker Optik GmbH, Germany). Wavenumber assignments for cellulose and lignin were 1035 cm^{-1} and 615 cm^{-1} for cellulose and lignin, respectively. The ratio of these two peaks was used as an indicator of the cellulose/lignin composition (Pandey and Pitman, 2003).

3.3 Results and Discussion

3.3.1 Preliminary Trial

Corn sections exposed to *C. felsineum* DSM 794 showed significant fibre separation compared to those inoculated with *B. subtilis* ROO2A and the negative controls (Figure 3.1). Although *B. subtilis* ROO2A did not show retting ability, the organism is believed to

contribute to the process by reducing the oxygen content to levels required for growth of anaerobes (Tamburini *et al.*, 2003).

Figure 3.1 Corn biomass after a 6 day incubation period: (A) non-inoculated control, (B) *B. subtilis* ROO2A and (C) *C. felsineum* DSM 794.

Staining with phloroglucinol further indicated an apparent reduction in lignin in cross-sections of corn exposed to *C. felsineum* DSM 794 in comparison to the other treatments (Figure 3.2).

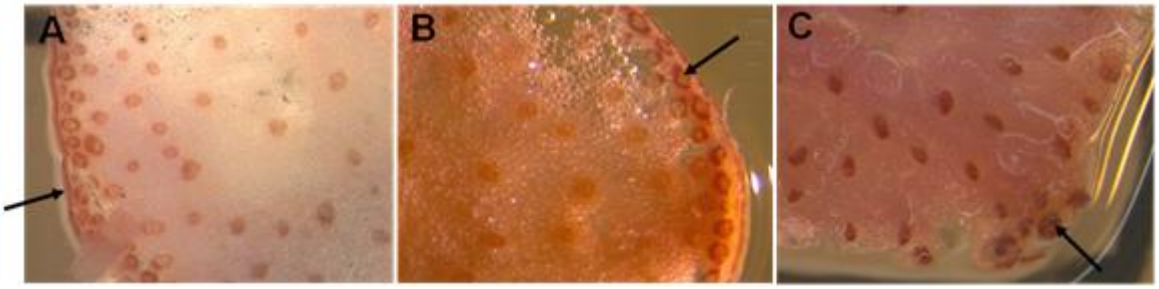


Figure 3.2 Air-dried cross-sections of corn after incubation with (A) non-inoculated control, (B) *B. subtilis* ROO2A and (C) *C. felsineum* DSM 794, stained with phloroglucinol to compare lignin content.

Physico-mechanical tests performed on the fibres released by clostridial retting showed a diameter of 200 µm, a tensile modulus of about 3.5 GPa with a break at 38.7 MPa. These

values are comparable with the mechanical properties of coir, which has a diameter range of 100 to 460 µm, a tensile modulus of 4 to 6 GPa and a tensile strength of 131 to 220 MPa (Bismarck *et al.*, 2005). Given that individual fibres were not released in the non-inoculated sample and the corn treated with *B. subtilis* ROO2A, mechanical testing was not possible.

3.3.2 Bench Scale Trial

There were no overall significant differences between treatment means, potentially due to high standard deviations (SD). The overall means and SD of cornstalk fibres determined by TGA are presented in Table 3.2. Treatments were therefore considered separately and analyzed as complete 2^2 factorial designs with triplicates with Design Expert 6.0.6 software (Stat-Ease, Inc., Minneapolis, MN).

Table 3.2 Overall thermal cornstalk fibre characteristics determined by thermogravimetric analysis.

	Moisture Content (%)	Onset of degradation (wt%/min)			Peak temperature (^oC)	Max. rate of degradation (wt%/min)	Residue after 400^oC (%)
		0.5	1.0	2.0			
Mean	4.7	196.4	234.6	262.6	324.7	9.4	33.1
SD[a]	0.8	22.7	13.4	8.8	8.7	1.2	4.6

[a]SD: Standard deviation

3.3.3 Influence of Crop Variety on Retted Fibres

The "*Clostridium+Bacillus* anaerobic" treatment appeared to have a consistent effect on cornstalk fibre characteristics (Table 3.3). In this treatment the *Bt*-RR variety resulted in fibres with higher onsets of degradation and peak temperatures, which was surprising given its reportedly higher lignin content (Saxena and Stotzky 2001). This treatment also produced post-harvest fibres of higher thermostability (Figure 3.3). This observation may be attributed

to the absence of grain as a source of readily assimilated carbon in the post-harvest trials, resulting in the bacteria utilizing more soluble sugars than glucose, which may have lead to the higher cellulose to lignin content of these extracted fibres for stalks retted with *C. felsineum* (Figure 3.4). The indigenous bacterial population with the non heat-treated treatments also appeared to result in fibres with increased thermostability for the post-harvest conventional variety. Certain treatments produced pre-harvest fibres with a higher peak temperature, although this observation was not consistent. Treatments did not appear to have a predictable effect on the moisture content or the amount of residue at 400^{o}C. Although interactions between harvest time and variety were found to be significant, these were also inconsistent.

3.3.4 Influence of Growth Conditions on Retted Fibres for Heat-Treated Controls

Heat-treated controls were compared to determine influence of the presence of oxygen on the pre- and post-harvest extracted fibres. Fibres extracted from the conventional variety had a significantly higher 0.5 wt%/min onset of degradation (P = 0.0265) in the presence of oxygen, as indicated by regression analysis ($y = 199.26 + 12.66 * \text{oxygen}$). A higher cellulose to lignin ratio was also seen in post-harvest conventional corn (P = 0.0268, $y = 0.91 - 0.027 * \text{harvest time}$), which was not observed in RR and *Bt*-RR corn. The presence of oxygen, however, led to a higher residue at 400^{o}C in RR corn (P = 0.0378, $y = 31.07 + 1.93 * \text{oxygen}$). Due to oxygen's significance in the 3 factors in non-*Bt* corn, aerobic and anaerobic treatments were considered separately for all further analyses.

3.3.5 Influence of Bacterial Treatment on Retted Fibres

Bt-RR corn had significantly higher 0.5 wt%/min and 1.0 wt%/min onsets of degradation in the "*Clostridium+Bacillus* anaerobic" treatment, $y = 197.79 + 15.33 * \text{treatment}$ and $y = 234.06 + 7.44 * \text{treatment}$, respectively (Table 3.4). Similarly, the fibres extracted from

Table 3.3 Significant terms between corn varieties and harvest time. Significance of plant variety and harvest time were analyzed as complete 2^2 factorial designs with triplicates. Treatments are defined in Table 3.1.

Treatment	Onset of Degradation*	Peak Temperature	Rate of Degradation	Residue	Cellulose to Lignin Ratio
Roundup Ready vs Conventional					
1					E
2					E
3					
4	A				
5					
6					
7					
8				A	
Roundup Ready vs *Bt*-Roundup Ready					
1					E
2					E, I(-ve)
3					
4		D	E		
5				B, I(-ve)	C
6	C	B, D		E	
7				B, E, I(-ve)	
8					
Conventional vs *Bt*-Roundup Ready					
1					A, E
2					
3					
4					
5					C
6	C	C, E, I(+ve)			E
7				A, E, I(-ve)	
8					

*0.5 wt%/min onset of degradation

Conventional	A	Interaction between	I
Roundup Ready	B	variety and harvest time	
Bt-Roundup Ready	C		
Pre-harvest	D		
Post-harvest	E		

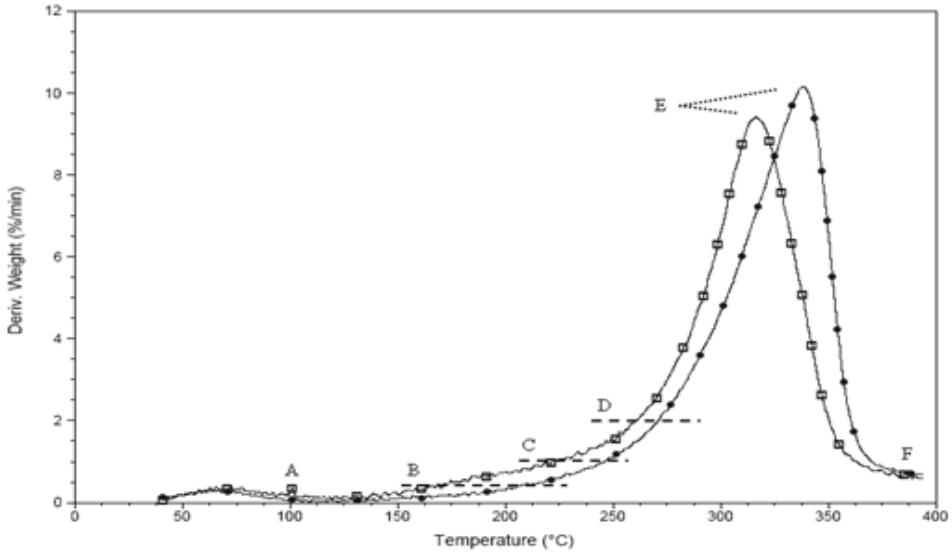


Figure 3.3 Comparison of the first derivative plot of thermogravimetric analysis curves resulting from *Bt*-Roundup Ready cornstalk material. Fibres retted anaerobically with *C. felsineum* and *B. subtilis* (●); heat-treated anaerobic control (□). Data extracted from the plot includes (A) moisture content (%) represented by the weight loss at 100°C; (B) 0.5 wt%/min onset of degradation; (C) 1.0 wt%/min onset of degradation; (D) 2.0 wt%/min onset of degradation; (E) maximum rate of degradation (wt%/min) and peak temperature (°C) and (F) residue at 400°C (%). Refer to Appendix F for a weight loss versus temperature plot.

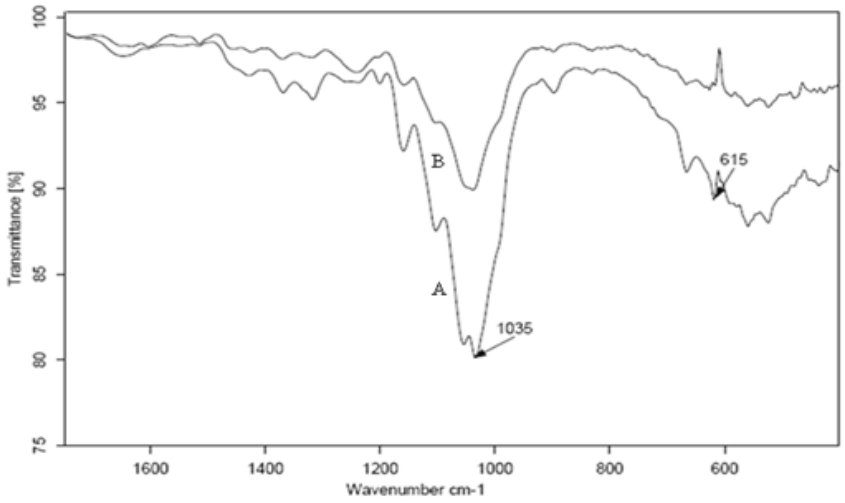


Figure 3.4 Comparison of the Fourier transform infrared spectroscopy spectra from retted and non-retted *Bt*-Roundup Ready cornstalk fibres. Spectra represent fibres (A) retted with *C. felsineum* and (B) a heat-treated anaerobic control. Peaks occurring at wavenumbers 1035 cm^{-1} and 615 cm^{-1} are representative of cellulose and lignin, respectively.

Table 3.4 Significant terms between corn treatment and harvest time as compared to the heat-treated controls. Significance of bacterial treatment and harvest time were analyzed as complete 2^2 factorial designs with triplicates. Treatments are defined in Table 3.1.

Treatment	Onset of Degradation*	Peak Temperature	Rate of Degradation	Residue	Cellulose to Lignin Ratio
Roundup Ready					
1			B		
2		A			
4	C	A	B		
5					
6					
8				C	
Conventional					
1					B
2					
4					
5					
6		B			B
8			B	D	
Bt-Roundup Ready					
1					
2					
4					D
5				B	
6	C	I(-ve)		B	
8	C			B	

*0.5 wt%/^{o}C onset of degradation

Pre-harvest	A
Post-harvest	B
Treatment	C
Control	D
Interaction	I

the anaerobic non heat-treated control of *Bt*-RR showed an increase in 0.5 wt%/min onset of degradation ($y = 194.91 + 12.45$*treatment). Where harvest time was significant, post-harvest corn had a higher 2.0 wt%/min onset of degradation, maximum degradation rate, residue at 400^{o}C and cellulose to lignin ratio. With regards to thermostability, cornstalk fibres were found to behave in a comparable fashion to those obtained for mechanically processed wheat straw (Panthapulakkal *et al.*, 2006).

3.4 Conclusions

C. felsineum was shown to be an effective corn retting agent. *B. subtilis* contributed to the retting activity of *C. felsineum* by reducing the oxygen content and providing the required anaerobic conditions for clostridial growth. In addition to bacterial treatment, absence of grain also resulted in fibres with a higher cellulose purity, higher onsets of degradation. Indigenous bacteria also showed retting ability in the non heat-treated controls. These outcomes are significant and show sufficient promise to warrant further evaluation, although quality of the resulting fibres may be dependent on year-to-year variation, crop variety and silage retting culture conditions.

Acknowledgements

Funding from the Ontario BioCar Initiative is gratefully acknowledged. Rachel Campbell Murdy was a recipient of an NSERC postgraduate scholarship during the course of this work. We thank Mr. Peter Smith for his assistance in finding and supplying the corn samples. The authors are also grateful to Professor Giorgio Mastromei (Universita di Firenze, Firenze, Italy) for providing the microbial reference strains.

Preamble to Chapter 4

Chapter 4: Characterization of the Metabolic and Enzymatic Profiles of Microbial Communities during Corn Ensiling is being submitted to the Journal of Agriculture, Ecosystems & Environment as an evaluation of the impact of crop management practices and silage contaminants on the stability and metabolic activity of the resident bacterial communities by community-level physiological profiling (CLPP). The original objective of this research was to investigate whether the retting agents *Clostridium felsineum* and *Bacillus subtilis* could compete with the indigenous microbial population in corn silage. Impact of these bacteria was found to be region- and variety-dependent, indicating that certain varieties of corn may benefit more from an introduced silage inoculants than others depending on the robustness of the microbes present in the phyllosphere. The changes in metabolic activity of the epiphytic bacterial communities could be detected by CLPP.

The findings of this research indicate that CLPP is a useful technique in optimizing the silage process by determining when bacterial inoculants may be necessary to produce higher quality silage. Clostridia are silage contaminants and would not be considered appropriate as silage inoculants. Therefore for publication purposes of this chapter as an independent research paper, the retting agents herein were described as bacterial contaminants.

Chapter 4

Characterization of the Metabolic and Enzymatic Profiles of Microbial Communities during Corn Ensiling

Summary

The phyllosphere of six varieties of ensiled corn was studied to evaluate the impact of crop management practices and silage contaminants on the stability and metabolic activity of the resident bacterial communities. Community-level physiological profiling and a suite of enzymatic activities were used to observe and quantify temporal divergence and regional microbial community differences in corn stover silage trials conducted in Southwestern Ontario, Canada. Corn varieties studied included two conventional (38A56 and 38N86), two Roundup Ready (38A55 and 38N85), and two *Bt*-Roundup Ready (38A57 and 38N87) isolines. Plant material was inoculated with *Bacillus subtilis* and *Clostridium felsineum*, two spore formers, to explore the impact of silage microbial contaminants. In comparing the community level physiological profiling results and enzymatic profiles, there were clear indications of differences between the microbial communities present on the corn isolines indicating that plant genotype and crop management affect plant-associated microbial communities. Temporal shifts were also evident during the fermentation, characteristic of the silage process. The impact of spore-forming spoilage organisms was dependent on region with the phyllosphere from isolines of the corn hybrid family 38N86 showing a more pronounced metabolic shift. Plant variety, either as a result of crop management practices or pleiotropic effects of the genetic manipulation, and presence of spore-forming spoilage organisms resulted in detectable changes in the metabolic activity of the epiphytic bacterial communities which could be used to optimize the silage process.

Keywords: Community-level physiological profiling, corn silage, enzyme activity, isoline, phyllosphere, principal component analysis

Note: The following chapter entitled "Characterization of the Metabolic and Enzymatic Profiles of Microbial Communities during Corn Ensiling" co-authored by Rachel G. Campbell Murdy, Nathaniel Hamelin, Muhammad Javaid Asad, Kela P. Weber and Raymond L. Legge will be submitted for publication as a refeered journal article.

4.1 Introduction

Silage is the product of solid state fermentation formed when plant material is enclosed anaerobically in storage structures such as towers, bunkers and bag silos to prevent fungal and bacterial spoilage. Quality silage is characterized by the bacterial production of organic acids from the breakdown of simple sugars. Microbial production of lactic acid and acetic acid lead to a drop in pH in the ensiled plant material thereby preventing growth of spoilage microorganisms and nutritional losses. Acetic and lactic acid-producing bacteria require anaerobic conditions for growth, which occurs in the initial three to five days of ensiling and may require two to three weeks for completion (Schroeder, 2004). During the initial stage of ensiling, the pH drops to between 3.8 and 4.2 as a result of lactic acid production by lactic acid bacteria (LAB) in the storage structure, at which point further bacterial action substantially decreases although residual activity by *Lactobacillus buchneri* has been detected for up to a year (Kleinschmit and Kung, 2006) and proteolytic activity remains beyond 3 months of storage (Der Bedrosian *et al*., 2012). This active fermentation period was found to be the most significant parameter for high quality silage (Mohd-Setapar *et al*., 2012).

During typical corn silage fermentation, bacterial growth follows a predictable succession differentiated by separate phases. Each of the successive phases in silage fermentation has distinct microbiological features that may be examined to improve and control the overall quality of the process. Culturing of microorganisms using selective media for standard plate counts remains useful for identification and isolation of specific organisms (Bolsen *et al.*, 1992; Lin *et al.*, 1992; Pasebani *et al.*, 2010). In addition, current molecular fingerprinting and other culture-independent profiling techniques have been explored for monitoring microbial population succession in environmental conditions (Malik *et al.*, 2008; Saito *et al.*, 2007). In fact, culture-independent methodologies have shown that the majority of plant-associated bacteria have yet to be cultured, limiting the usefulness of plate counts (Araújo *et al.*, 2002; Kent and Triplett, 2002; Malik *et al.*, 2008; Saito *et al.*, 2007; Yang *et al.*, 2001). The four primary fingerprinting techniques used to monitor microbial communities include

denaturing gradient gel electrophoresis, single strand conformation polymorphism, terminal restriction fragment length polymorphism, and automated ribosomal intergenic spacer analysis (Ikeda *et al.*, 2006; Kirk *et al.*, 2004; Pancher *et al.*, 2012; Saito *et al.*, 2007). In addition to the equipment and expertise requirements for these analyses, limitations of many of these techniques include sampling, DNA/RNA extraction as well as PCR amplification and primer design (Saito *et al.*, 2007). Phospholipid fatty acid (PLFA) analysis has also been used to show differences in microbial communities in soil and rhizosphere environments (Ramsey *et al.*, 2006, Söderberg *et al.*, 2004, Widmer *et al.*, 2001). However, comparative data analyses combining different techniques were shown to be of importance when attempting to interpret results, given the high degree of variability of microbiological analyses (Widmer *et al.*, 2001).

Soil quality is often correlated with microbial community so that methods to detect and monitor soil health require microbial-based indicators (Anderson, 2003). The use of community-level physiological profiling (CLPP) in combination with principal component analysis (PCA) allows characterization of the stability and metabolic activity of a microbial community (Amador and Görres, 2007; Bucher and Lanyon, 2005; Garland and Mills, 1991; Garland *et al.*, 2010; He *et al.*, 2012; Heuer and Smalla, 1997; Weber *et al.*, 2007, 2008; Weber and Legge, 2009, 2010, 2011; Widmer *et al.*, 2001). The technique uses BIOLOG EcoPlates™, which consists of 96 well plates containing 31 different carbon sources and a blank triplicate with a tetrazolium dye to track the catabolic profile of a community, thereby giving an idea of both the catabolic activity and range of metabolic function of a community (Weber and Legge, 2010). The purpose of this study was to use CLPP in combination with an analysis of enzymatic activity to investigate temporal and regional microbial community dynamics in corn silage trials. Impact of crop management and presence of silage contaminants characterized by the stability and metabolic activity of the bacterial communities could offer insight in process optimization and development of inoculants.

4.2 Materials and Methods

4.2.1 Inoculant Development

Bacilli and clostridia belong to spore-forming genera considered silage contaminants (Te Giffel *et al.*, 2002). Spores of *Bacillus subtilis* subsp. *spizizenii* (DSM 15029) and *Clostridium felsineum* (DSM 794) (DSMZ, Braunschweig, Germany) were cultured according to Di Candilo *et al.* (2010) with small modifications. Briefly, 10 plates per strain were prepared on sporulation medium. *C. felsineum* was cultured on Reinforced Clostridial Agar (RCA) and incubated for 7 days in an Oxoid anaerobic jar (Oxoid Ltd., Thermo Fisher Scientific, UK) in CO_2 atmosphere (Oxoid AnaeroGen, Thermo Fisher Scientific, UK) at 37°C, while *B. subtilis* was cultured on peptone, yeast extract and tryptone (PYT) medium (0.5% yeast extract, 0.5% peptone, 1% tryptone) and grown aerobically at 37°C. Colonies on the plates were resuspended by adding 1 mL sterilized tap water to the plate and the inoculum was injected into potatoes with a syringe. Potatoes were added to 1 L of sterile water, autoclaved for 10 minutes at 100^{o}C and incubated at 30^{o}C for 7 days. Colony forming units were enumerated using 3M Petrifilm Aerobic Count Plates 6400 (3M Microbiology Products, St. Paul MN, USA) aerobically for *B. subtilis* and anaerobically for *C. felsineum*. Inoculant solutions contained *B. subtilis* and *C. felsineum* at concentrations of 1.64 x 10^8 cfu/mL and 3.65 x 10^4 cfu/mL, respectively. Non-inoculated potatoes were also autoclaved and incubated for use as a control for the silage trial to replicate the sugar content from the potato extract. The inoculant solutions were stored at 4^{o}C until further use.

4.2.2 Preparation of Plant Material

Six Pioneer varieties comprised of the 2 corn (*Zea mays*) hybrid families 38N86 (heat unit 2700) and 38A57 (heat unit 2850), each comprised of 3 isolines were investigated in duplicated lab-scale silage trials: 38N85 and 38A55 (Roundup Ready abbreviated RR), 38N86 and 38A56 (Conventional), 38N87 and 38A57 (*Bt*-Roundup Ready abbreviated *Bt*-RR) (Pioneer Hi-Bred International, Inc., Johnston IA, USA). The varieties 38A55-57 were

grown in Woodstock, Ontario (Plant Hardiness Zone 5b), while varieties 38N85-87 were grown in Elora, Ontario (Plant Hardiness Zone 5a). Freshly harvested corn stover was shredded using a gas-powered Troy-Bilt Chipper Shredder, Model No. 47044 (Garden Way Incorporated, Troy NY, USA) and inoculated with 500 mL spore solutions described above using a spray bottle containing 250 mL/strain diluted in 2.5 L water. Non-inoculated controls were sprayed with the bacteria-free 500 mL solution in 2.5 L water. The plant material was then enclosed in anaerobic bags (12 x 16, 3 Mil Clear Poly Biolable, Bag Poly Disposables Ltd., Kitchener ON, Canada) with a modified Handy-Lok Closure Set (Glas-Col LLC, Terre Haute IN, USA). Bags were stored at 4^{o}C until needed.

4.2.3 Laboratory-Scale Silage Trials

Ten grams of cornstalk material from each isoline was incubated statically at 28^{o}C with 100 mL of modified M9 minimal salt solution (containing per litre: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl with 10 mL each of filtered 0.01 M and 0.1 M solutions of $CaCl_2$ and $MgSO_4 \cdot 7H_2O$, respectively) in 500 mL Erlenmeyer flasks. The flasks were all flushed with CO_2 and enclosed with a rubber stopper and gas trap to replicate anaerobic conditions. Ten mL samples were collected on days 0, 3, 6, 10 and 14 and immediately stored at 4^{o}C to minimize changes in microbial populations. After each sampling, the flasks were flushed with CO_2 to maintain anaerobic conditions.

4.2.4 Community-Level Physiological Profiling

Each well of the BIOLOG™ EcoPlates (BIOLOG Inc., Hayward CA, USA) was inoculated with 100 µl of sample diluted to an OD_{420} of approximately 0.2 for a target inoculation density of 10^5 cells/mL to reduce lag times (Weber and Legge, 2010). Anaerobic conditions were maintained during EcoPlate inoculation and analysis by working within Glove Bag™, Model X-37-27 (Glas-Col, Terre Haute IN, USA) under a nitrogen environment at room temperature and sealing the plates with tape. The absorbance at 590 nm (OD_{590}) of the plates was read every 4-8 hours for 36 hours with a microplate reader (Synergy 4 Hybrid Multi-

Mode Microplate Reader, Biotek®, Winooski VT, USA). The data was recorded using GEN 5™ software from Biotek® (Biotek®, Winooski VT, USA) and analyzed with Microsoft Office Excel Version 2007 (Microsoft Corp., Redmond WA, USA) and Statistica 8.0 software (StatSoft Inc., Tulsa OK, USA).

4.2.5 Statistical Analysis

The carbon source utilization profiles (CSUPs) were analyzed by principal component analysis (PCA) and guild analysis (Weber and Legge, 2009; Weber *et al.*, 2007; Zak *et al.*, 1994) with Microsoft Excel 2007 (Microsoft Corp., Redmond WA, USA) and Statistica 8.0 software (StatSoft Inc., Tulsa OK, USA). After subtracting the blank well reading from each of the carbon source well readings, a time point of 24h was selected for analysis (Garland, 1996; Weber *et al.*, 2007). This selection was based on maximizing the informational output from the 31 well OD readings while minimizing the number of well OD readings greater than 2.0 (which is outside the linear absorbance range). Two transforms were considered to help improve the suitability of data for principal component analysis: Taylor power and a natural logarithmic transformation (Legendre and Legendre, 1998; Taylor, 1961; Weber *et al.*, 2007). In each case, normality, homoscedasticity, and linear correlations within the data sets were evaluated to choose the best transformation for further analysis. Principal components (PCs) were extracted from the data covariance matrix (Weber *et al.*, 2007) and PCA ordinations used to identify groupings.

4.2.6 Enzyme Analysis

The activity of 5 enzymes, namely α-amylase, glucoamylase, cellulase, pectin lyase (PL) and polygalacturonase, was assayed as a secondary analysis of metabolic profiling. The activities are presented as U/gds (unit/gram dry substrate).

The activity of α-amylase was assayed by incubating 0.5 mL enzyme with 0.5 mL soluble starch (Sigma) (1%, w/v) prepared in 0.1 M sodium phosphate buffer (pH 7). After

incubation at 37^{o}C for 60 minutes, the reaction was stopped by the addition of 2 mL of Dinitrosalicylic acid (DNS) reagent (Bernfeld, 1955) and absorbance was measured in a UV/Vis spectrophotometer. One unit (U) is defined as the amount of enzyme which releases 1 µmol of reducing end groups per minute in 0.1 M sodium phosphate buffer (pH 7) with 0.5% w/v soluble starch as substrate at 37^{o}C.

Glucoamylase activity was determined in a reaction mixture containing 0.1 mL of enzyme, 0.5 mL of 0.016 M sodium acetate buffer (pH 4.8), and 0.5 mL of starch solution (Sigma) (Bernfeld, 1955). DNS reagent was used as coloring reagent and absorbance was read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme which releases 1 µmole of glucose in 1 minute.

Cellulase activity was measured according to Gadgil *et al.* (1995). One mL of diluted enzyme solution was incubated for 30 minutes with 1 mL of 1.0 percent CMC (Sigma) and 1 mL of 0.1 M citrate buffer of pH at 4.8 at 50^{o}C. The reaction was terminated by adding 3 mL of DNS reagent and the mixture was boiled for 15 minutes and cooled on ice. After cooling, the absorbance was read at 540 nm.

Assay of pectin lyase was performed by the method described by Preiss and Ashwell (1963). 0.5 mL of enzyme was incubated for 1 hour with 0.5 mL of 0.5% pectin (Sigma) and 1 mL of 50 mM tris HCl buffer (pH 8) and 1 mL of 0.2 mM $CaCl_2$. After 1 hour incubation the absorbance was measured at 548 nm. One unit of pectin lyase activity was defined as the amount of enzyme present in 1 mL of original enzyme solution which released 1 µM of galacturonic acid in 1 minute (Preiss and Ashwell, 1963).

The activity of polygalacturonase was measured by the method according to Friedrich *et al.* (1989). The activity of polygalacturonases (PGs) was assayed by measuring the reducing groups released from polygalacturonic acid (Sigma) using the 3, 5-Dinitrosalicylic acid (DNS) method with D-galacturonic acid monohydrate (Sigma) as the standard. One unit of

PGs activity was defined as the amount of enzyme which yielded 1 μmol reducing ends per minute at 40 ^{o}C and pH 5.0.

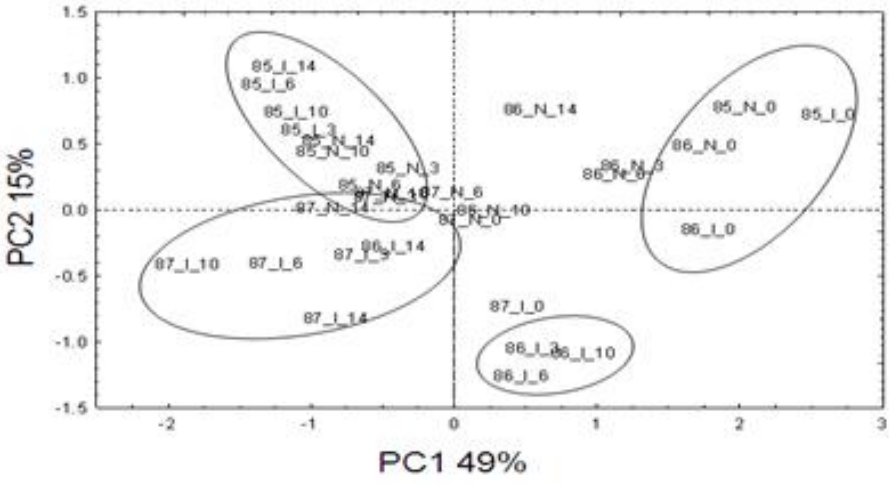
4.3 Results and Discussion

4.3.1 Community-Level Physiological Profiling

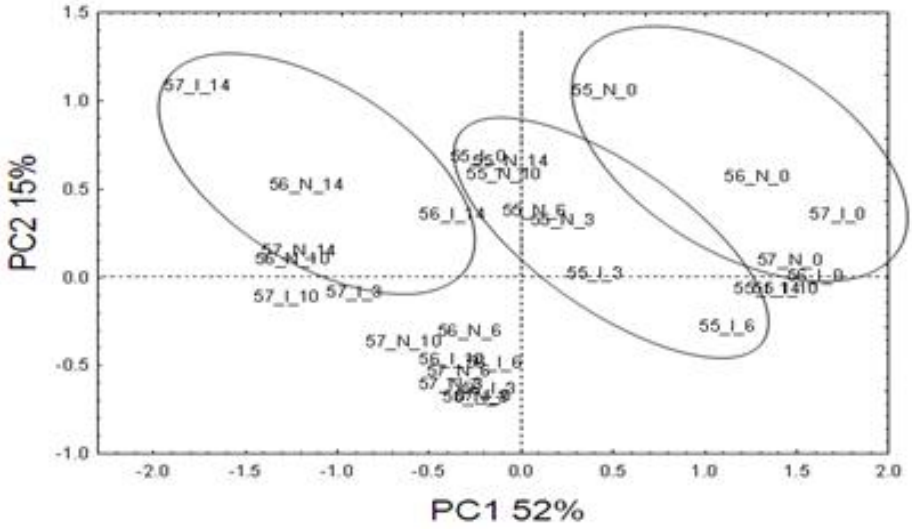
From the 31 different carbon source utilization curves (CSUCs) it was seen that certain inoculated wells showed very little or no response in all trials. Cell 18, which contained 2-hydroxy benzoic acid, was the most prominent non-responsive well. There was also minimal responses in Cells 10 and 22, D-Malic acid and i-Erythritol, respectively, indicating that the mixed microbial community was unable to utilize these particular carbon sources for growth or as an energy source.

Distribution of the microbial community showed a very clear grouping and separation between the 38N85-87 and the 38A55-57 isolines along the second PC (Figure 4.1). These groupings (indicated by encircling based on manual groupings) would indicate that bacterial communities on corn grown in zones 5a and 5b potentially co-varied based on region and variety. There was also a noticeable grouping of samples collected from day 0, indicating an overall change in the metabolic function of the community from initial ensiling. This observation coincides with the initial phase during which plant cells continue to respire contributing to anaerobiosis conditions needed for growth of acetic and lactic acid-producing bacteria (Schroeder, 2004). There were also distinctions between inoculated and non-inoculated plates which became more apparent when analyzed separately, indicating that the spore-forming organisms impacted the physiological profiles of the communities. Average well color development as a measure of activity and richness confirmed these observations.

Samples collected from the isolines were also analyzed via separate PCAs to determine if differences occurred within regions. Data from 38N85 to 87 indicated a clear grouping and segregation of each isoline, with a separate group at Day 0 (Figure 4.1a). Data from isolines



(a)



(b)

Figure 4.1 Principal components analysis ordination of the microbial communities in inoculated and non-inoculated corn isolines of (a) 38N85-87 and (b) 38A55-57. Breakdown of code: Variety_Use of inoculant (I: inoculated, N: non-inoculated)_Day of incubation. Circles shown are based on manual groupings. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)

38A55 to 57, showed a similar distinction for Day 0 samples, while 38A55 separated from 38A56 and 57 on PC1 (Figure 4.1b). Samples from Day 14 could also be grouped indicating temporal variations in communities. These observations suggest that different plant management strategies affect the bacterial communities present in the plant phyllosphere and endosphere, which may be due to application of Roundup® given that RR and *Bt*-RR crops would allow application of the herbicide. Crop management procedures such as the application of fertilizers and pesticides have been shown to cause structural changes to plant-associated microorganisms (Green *et al.*, 2006; Marschner and Baumann, 2003; Marschner *et al.*, 2001, 2004; Pancher *et al.*, 2012; Saito *et al.*, 2007; Seghers *et al.*, 2004; Tan *et al.*, 2003; Yang and Crowley, 2000). Plant genotypic variations have also been shown to impact microbial communities in rice (Knauth *et al.*, 2005; Saito *et al.*, 2007). In fact when studying the phyllosphere communities of different potato varieties and sugar beet by CLPP, Heuer and Smalla (1997) found quantitative differences in catabolic profiles of the plant-associated microorganisms. However, these differences were not evident when comparing transgenic versus nontransgenic potatoes (Heuer and Smalla, 1997).

Figure 4.2 shows a comparison between genetically equivalent isolines. The RR and conventional corn varieties (38A55-38N85 and 38A56-38N86, respectively) are presented in Figure 4.2a and b. A separation between 38A55 and 38N85 as well as 38A56 and 38N86 are noticeable along PC1. However, 38A57 and 38N87 grouped along the PC2 axis, indicating less variability (Figure 4.2c). In all comparisons the initial samples at Day 0 were distinct. These observations further confirm regional and varietal differences in plant-associated bacteria.

Individual varieties were also analyzed by PCA to establish temporal and treatment impacts. The pattern from variety 38N85 is presented in Figure 4.3a and was found to be representative for the three varieties from the same region. However, treatment influences were not as evident in the corn varieties from a different region. Although there was a clear separation along PC1 between inoculated and non-inoculated isoline 38A55 (Figure 4.3b),

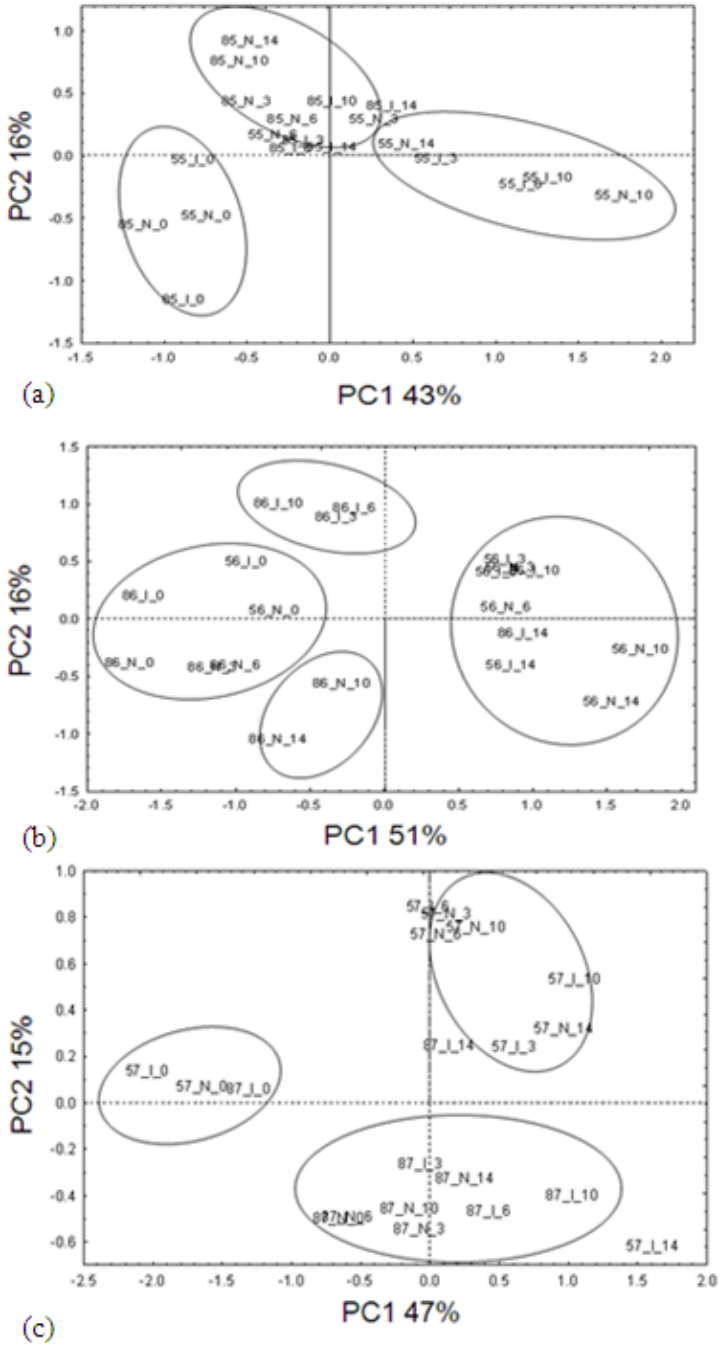


Figure 4.2 PCA ordination of the microbial communities in inoculated and non-inoculated corn isolines of (a) 38A55-38N85, (b) 38A56-38N86 and (c) 38A57-38N87. Breakdown of code: Variety_Use of inoculant (I: inoculated, N: non-inoculated)_Day of incubation. Circles shown are based on manual groupings. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)

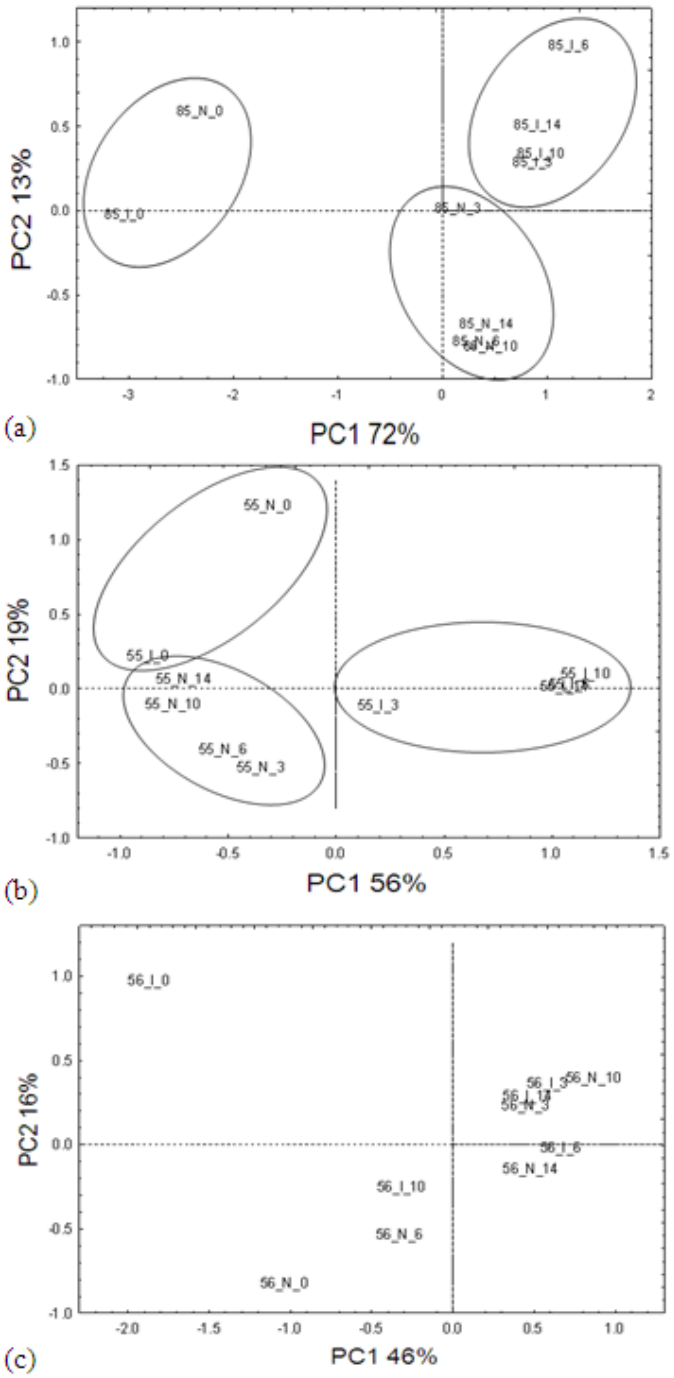


Figure 4.3 PCA ordination of the microbial communities in inoculated and non-inoculated corn of (a) 38N85, (b) 38A55 and (c) 38A56. Breakdown of code: Variety_Use of inoculant (I: inoculated, N: non-inoculated)_Day of incubation. Circles shown are based on manual groupings. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)

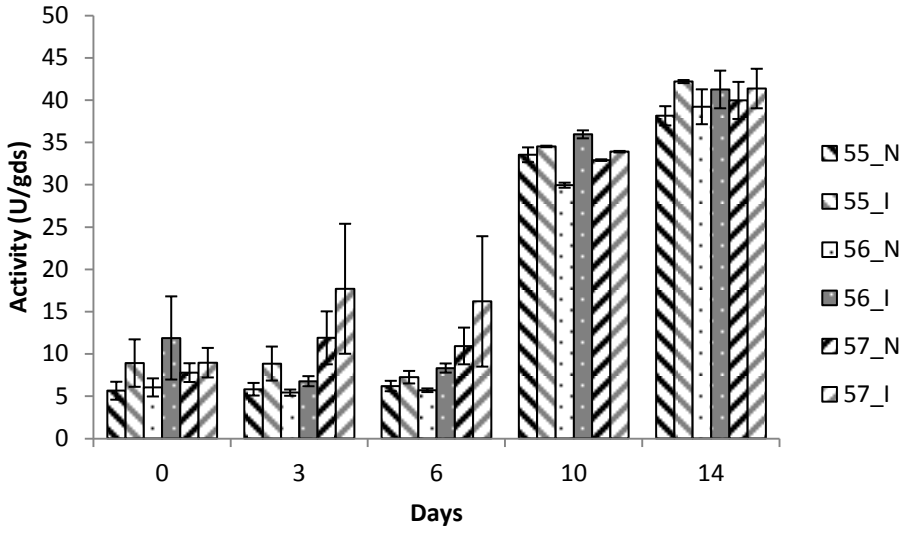
there were no clear groupings found for 38A56 (Figure 4.3c). However, again Day 0 samples could be grouped separately from the majority of the sample days according to the PC1 or PC2. These results show CLPP can be used to distinguish between bacterial community function and dynamics in silage and that a robustness analysis of the plant phyllosphere prior to ensiling may indicate the need for customized inoculants to improve silage quality and optimization.

4.3.2 Enzyme Analysis

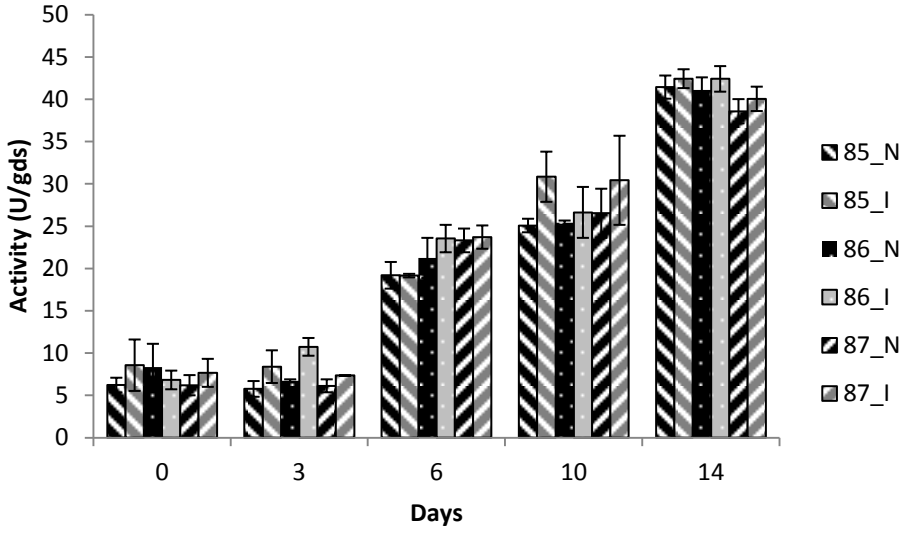
α-Amylase is an enzyme that hydrolyses alpha-bonds of large alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose. It is present in seeds containing starch as a food reserve, and is secreted by many fungi. α-Amylase had the highest overall activity at Day 14, reaching levels of 40 U/gds (Figure 4.4). When significant differences were noted in α-amylase activity, samples inoculated with the spore-formers had higher activity than non-inoculated samples. There was a pronounced increase in activity between Day 3 and Day 6 in the hybrid family 38N86, which was delayed but found to occur between Day 6 and Day 10 in the hybrid family 38A57 with an average increase in activity of 20 U/gds.

Glucoamylase is a type of amylase that cleave α(1-6) glycosidic linkages, as well as the last α(1-4) glycosidic linkages at the non-reducing end of amylose and amylopectin, yielding glucose. Overall, inoculated samples had higher activity than non-inoculated samples, although this observation was more pronounced in the hybrid family 38A57 (Figure 4.5). Day 0 had the lowest activity (ranging between 5.2 in 86_N and 21.9 in 85_I), which gradually increased and peaked at Day 10 with an average activity of approximately 50 U/gds.

Cellulases are enzymes that catalyze cellulolysis (i.e. cellulose hydrolysis). Cellulases mainly catalyze the hydrolysis of β-1,4-D-glycosidic linkages in cellulose, lichenin and cereal β-D-glucans. Cellulase action is considered to be synergistic as all three classes of cellulase

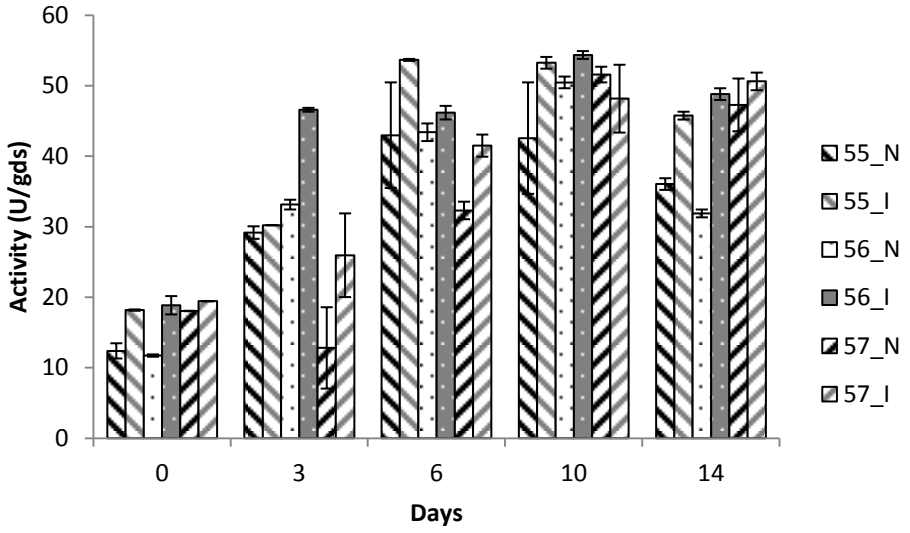


(a)

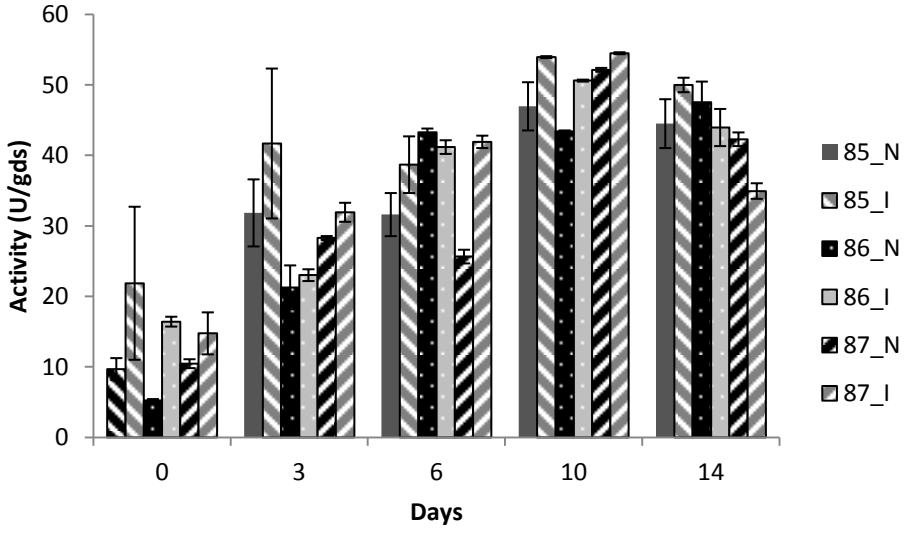


(b)

Figure 4.4 α-Amylase activity in inoculated (I) and non-inoculated (N) corn silage samples with varying incubation periods. (a) Hybrid family 38A57 and (b) hybrid family 38N86. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)

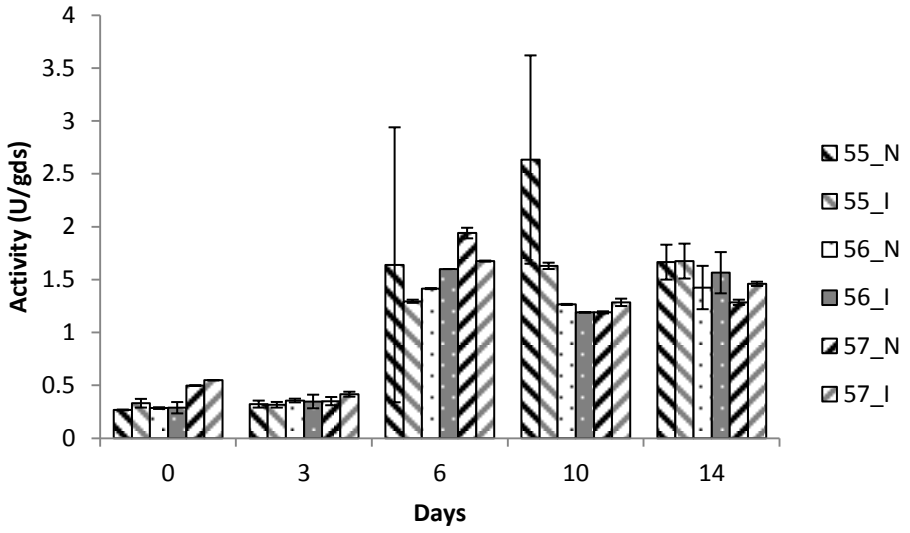


(a)

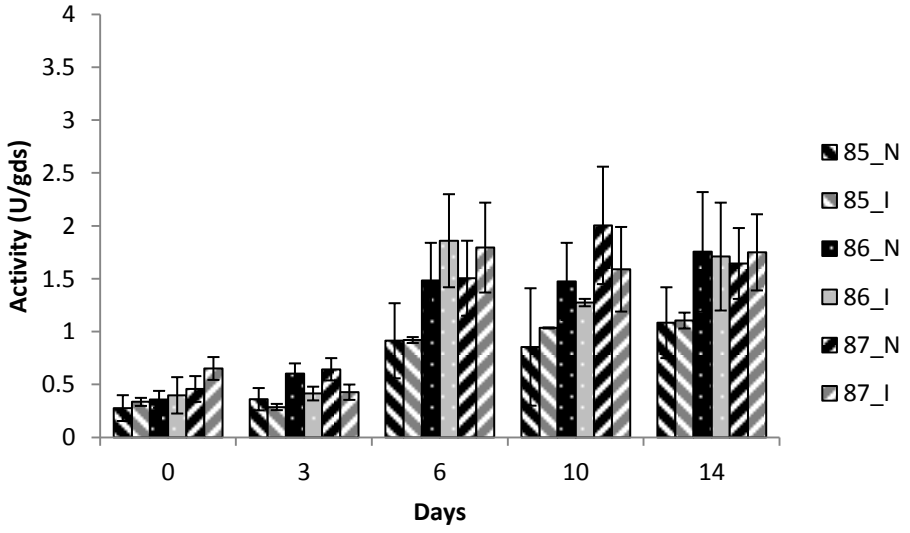


(b)

Figure 4.5 Glucoamylase activity in inoculated (I) and non-inoculated (N) corn silage samples with varying incubation periods. (a) Hybrid family 38A57 and (b) hybrid family 38N86. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)



(a)



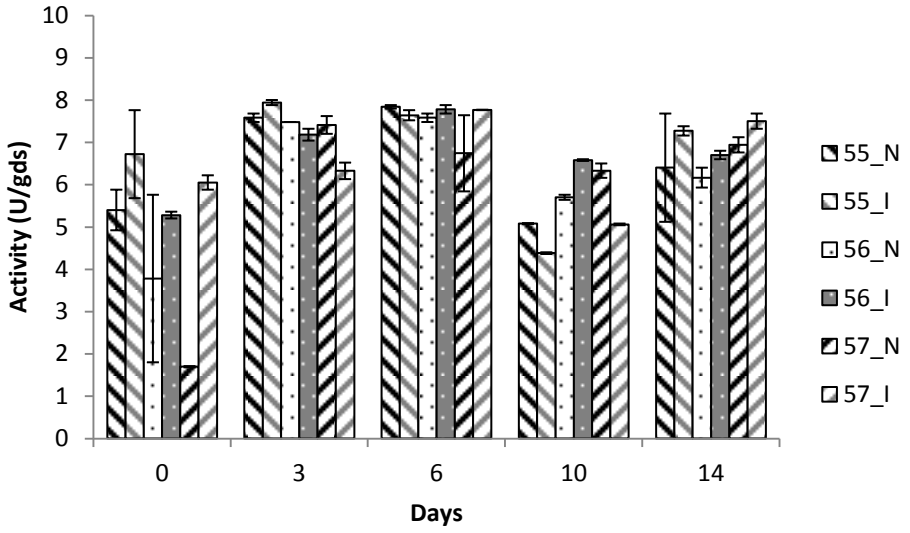
(b)

Figure 4.6 Cellulase activity in inoculated (I) and non-inoculated (N) corn silage samples with varying incubation periods. (a) Hybrid family 38A57 and (b) hybrid family 38N86. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)

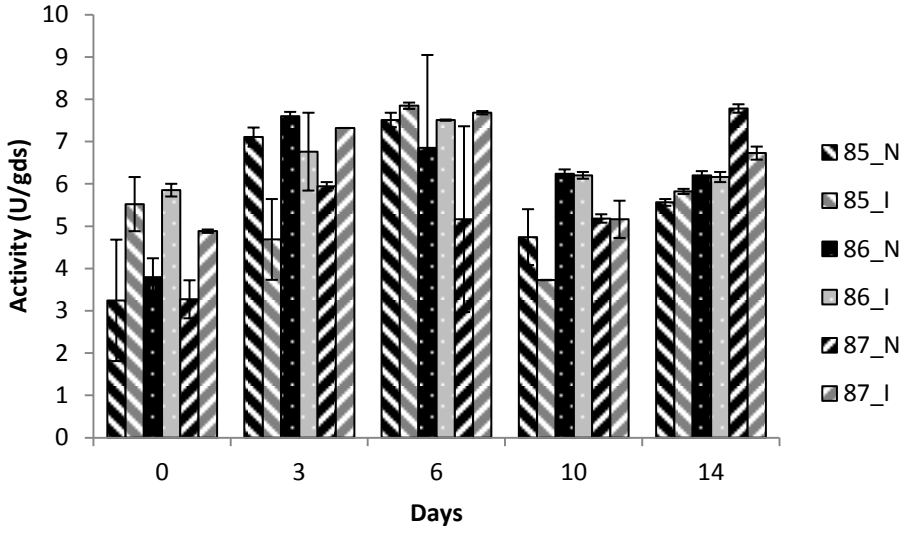
(endoglucanase, exoglucanase and β-glucosidase) can yield much more sugar than the addition of all three separately. Overall activity of cellulase was lower than the other enzyme assayed (Figure 4.6). In general there were no differences between inoculated and non-inoculated samples. Day 0 and 3 had lowest activity, with an average of 0.25 U/gds, which peaked to 1.5 U/gds by Day 6 before leveling off. No significant differences were found between the two hybrid families.

Pectin lyases (PL) are a group of enzymes that act collectively to degrade the pectin polymer into galacturonide units. Samples from Days 0 and 10 were found to have the lowest activities at wide range of 1.7 to 6.7 U/gds for 57_N and 55_I, respectively, with a resurgence at Day 14, potentially indicative of successive communities (Figure 4.7). There were no consistent differences between inoculated and non-inoculated samples, and minimal day to day fluctuations. As found with cellulase activity, there were no significant differences between the two hybrid families based on a 2-tailed t-test.

Polygalacturonase (PG) is a group of enzymes produced by selective microorganisms to degrade polygalacturonan to release galacturonic acid present in the cell wall of plants by the hydrolysis of glycosidic bond. The spore-formers used as inoculants in this study were initially isolated as hemp retting agents with high PG activity (Di Candilo *et al.*, 2010). As anticipated, inoculated samples had PG activities of more than double their non-inoculated counterparts, from an average 6 to 3 U/gds, respectively. However, 38A57_I and N had lower starting activities of 2 and 1 U/gds (Figure 4.8). By Day 3, the presumed competitive advantage of the spore-formers was lost as PG activity in non-inoculated samples reached or surpassed the levels found in those of the inoculated samples. The robustness of the microbial communities did not appear to be as high in *Bt*-RR corn given that PG activity in the inoculated samples remained higher throughout the 14 day trial for 38N87 and up to Day 6 for 38A57. This may be attributed to either crop management practices or potential secondary effects of the expression of the Cry endotoxin by *Bt* corn, which has been engineered to express the toxin isolated from *Bacillus thuringiensis*. Blackwood and Buyer

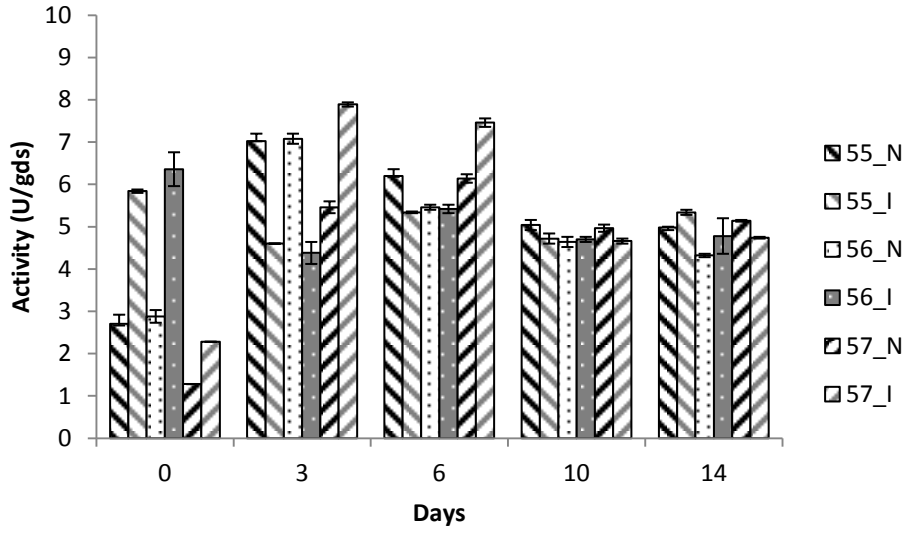


(a)

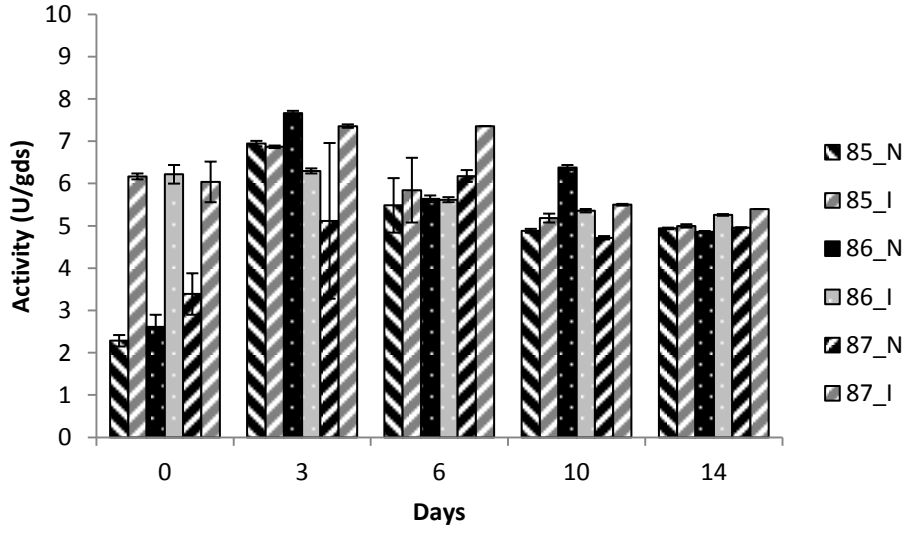


(b)

Figure 4.7 Pectin lyase activity in inoculated (I) and non-inoculated (N) corn silage samples with varying incubation periods. (a) Hybrid family 38A57 and (b) hybrid family 38N86. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)



(a)



(b)

Figure 4.8 Polygalacturonase activity in inoculated (I) and non-inoculated (N) corn silage samples with varying incubation periods. (a) Hybrid family 38A57, and (b) hybrid family 38N86. (Note: 55&85 – Roundup Ready; 56&86 – Conventional; 57&87 – *Bt*-Roundup Ready)

(2004) used CLPP and PLFA to study the soil microbial communities associated with *Bt* and non-*Bt* corn in various soils and found that expression of the Cry endotoxin and corn line had an effect on microbial profiles in high-clay soil. Castaldini *et al.* (2005) also noted differences in the rhizospheres of *Bt* corn in greenhouse experiments. In a field trial of the same study, non-harvested and plowed plant residues from *Bt* corn were also found to affect the bacterial communities in soil and the rhizosphere (Castaldini *et al.*, 2005). The results ofthis study suggest that inoculants containing lactic acid bacteria may be of more benefit to *Bt* and *Bt*-RR corn silage.

An overall analysis of enzyme activity confirms the temporal metabolic community divergence as well as the impact of the presence of spore-formers shown by the carbon substrate utilizing patterns of CLPP. However, the limitations of using enzyme analysis as a monophasic approach are substantiated by the hidden metabolic shifts within the phyllosphere of individual corn isolines, which only became evident when looking at the PCA results from CLPP analysis.

4.4 Conclusions

In conclusion, CLPP in combination with PCA was shown to be a useful analytical tool to detect differences in silage microbial community dynamics as a result of crop management, plant genotype and presence of spore-forming spoilage organisms. Although enzyme activity profiling was also effective in monitoring activity of the silage communities, CLPP provided a broader understanding of community shifts from a metabolic perspective. There were clear indications of a metabolic divergence between the microbial communities present in the 6 varieties of corn studied which was illustrated by CLPP in addition to a pronounced temporal shift in dynamics during ensiling. The bacterial spore-formers present in the inoculum were found to have a more pronounced impact on the microbial communities for the 38N86 corn hybrid family. This regional microbial population variability shows that the indigenous community in certain regions may out-compete bacterial inoculants and spoilage organisms

on cornstalk material. Monitoring the activity and stability of microbial communities of the phyllosphere pre-harvest by CLPP offers the potential to manipulate the silage process to increase the predictability and quality of the ensiled plant material.

Acknowledgements

This work was supported by the Ontario BioCar Initiative and NSERC through a postgraduate scholarship. The authors thank Peter Smith for his assistance in finding and processing the corn samples. We are grateful to Dr. Kela Weber and Dr. Muhammad Javaid Asad for their help with principal component analysis and enzyme analysis, respectively.

Chapter 5

Quantification of C_2-C_6 Volatile Organic Acids in Silage Retting Effluent

Summary

Ensiling of corn biomass results in the production of silage liquor or effluent composed of various short-chain volatile organic acids (VOAs). Production of VOAs in silage effluent was examined as a potential feedstock source for value-added organic acid production. Three Pioneer® corn isolines were included to determine whether variety had an impact on production of organic acids: 38N85, 38N86 and 38N87, representing a Roundup Ready® (RR), a conventional and a *Bt*-Roundup Ready® (*Bt*-RR) stacked variety, respectively. Treatments included inoculations with the two retting agents *Bacillus subtilis* and *Clostridium felsineum* individually or combinations under aerobic and anaerobic environments. Acetic acid was consistently present in all treatments, at approximately 400 and 800 mg/L under the aerobic and anaerobic conditions, respectively. Butyric acid was only produced under anaerobic conditions, with the anaerobic non-sterile control showing the highest levels of butyric acid of up to 8000 mg/L. In contrast, isobutyric acid was only present for certain treatments under aerobic conditions. A specific corn hybrid variety was not identified as most or least favourable for VOA production. The presence of acetic and butyric acid in corn silage effluent indicates the potential for use of this agricultural waste as a new feedstock for precursors in the chemical and biofuel industries.

Keywords: Corn silage effluent, retting agents, volatile organic acids

5.1 Introduction

Ensiling of cornstalk biomass results in the production of silage liquor or effluent composed of various short-chain volatile organic acids (VOAs). Major fermentation products include lactic, acetic, propionic, isobutyric, *n*-butyric and isovaleric acids (Canale *et al.*, 1984). Depending on the moisture content of the ensiled material, production of silage effluent can be from about 300 L to 700 L per ton (Lesperance *et al.*, 2008). Given that the volume and chemical composition of silage effluents vary based on many parameters such as dry matter, type of silo and nature and pre-treatment of the crop (McDonald *et al.*, 1991), a measure of these VOAs is useful as an assessment of the extent of the microbial fermentation (Brotz and Schaefer, 1987). For example, grass silage effluent was found to be composed of up to 1700 mg/L acetic acid, 230 mg/L propionic acid, 440 mg/L butyric acid, 110 mg/L isovaleric acid and 5730 mg/L lactic acid (Galanos *et al.*, 1995).

Given that silage liquor is often rich in nutrients, effluents are occasionally used in feedstuff for pig and beef cattle (Fischer *et al.*, 1994). However, the seepage is most often lost or diluted and discharged to soil (Galanos *et al.*, 1995), sewers and surface waters (Fischer *et al.*, 1994). Environmental detriments of this silage liquor include high biological oxygen demand (BOD), pollution, fish toxicity (Clarke and Humphreys, 1971), changes in soil redox conditions due to oxygen depletion, reduced mobility of micronutrients and trace elements as a result of complexation with organic compounds, overfertilization of soils, and lower ground water quality (Fischer *et al.*, 1994; Merriman, 1988).

VOAs in silage effluents are under review as potential value-added by-products for diversification of the current agriculture economy. For example, the organic compounds in grass silage liquor were found to complex heavy metals leading to sanitation of polluted soils (Leidmann *et al.*, 1993). Grass silage effluent was particularly effective at removing cadmium, zinc, copper, nickel, and to a lesser extent, chromium and lead from contaminated soils (Leidmann *et al.*, 1993). Raw silage effluent produced from whole corn silage at 35% moisture at harvest was also found to contain a variety of alcohols in the range of 3 g/L, of

which over half was ethanol (Lesperance *et al.*, 2008). If collected, the high nutrient content of the silage seepage made this waste product suitable for further bioconversion and/or downstream processing into a value product such as biofuels (Lesperance *et al.*, 2008). Acetic acid, in particular, shows promise as a feedstock for the production of ethanol, as it can be vaporized and hydrogenated to ethanol (Celanese Acetyl Technology, Celanese Corp., 2011). Butanol as a substitute for gasoline is more optimal than ethanol as it can replace the petroleum-base without the need for engine modification (Ramey and Yang, 2004). In a typical acetone-butanol-ethanol (ABE) fermentation, butyric and acetic acids are produced then converted into solvents (butanol, acetone and ethanol) by *Clostridium acetobutylicum* (Fond *et al.*, 1985). A feedstock containing these VOAs may eliminate the need for *de novo* production and facilitate production of butanol and ethanol.

VOAs can be analyzed by various chromatographic methods. Ion exclusion chromatography and high performance liquid chromatography have been used to quantify organic acids in silage extracts (Canale *et al.*, 1984; Fisher *et al.*, 1994; Stephens *et al.* 1996). However, gas chromatography-flame ionization detector (GC-FID) has shown high resolution and sensitivity when used to quantify organic acid production in foods (Yang and Choong, 2001). Yang and Choong (2001) were able to detect and quantify C_2-C_{12} organic acids based on their different retention time with 1,3-butanediol (BuOH) as the internal standard (IS).

In this study, VOAs produced during cornstalk fermentation of three Pioneer corn isolines were quantified to determine their potential use as value-added by-products. Cornstalk biomass was also inoculated with the hemp retting agents *Clostridium felsineum* and *Bacillus subtilis* to compare VOA production in material processed with the indigenous microbial populations and the introduced bacteria.

5.2 Materials and Methods

5.2.1 Bench Scale Fermentation Trial

Bacillus subtilis ROO2A, first isolated by Tamburini *et al.* (2003) as a hemp water retting agent, was sent by Dr. Gorgio Mastromei (University of Florence, Florence, Italy) and *Clostridium felsineum* DSM 794 was obtained from DSMZ, Braunschweig, Germany. *B. subtilis* ROO2A was cultured in a peptone, yeast extract and tryptone (PYT) medium at 30^{o}C under aerobic conditions and *Clostridium felsineum* DSM 794 was cultured in PYT supplemented with glucose and cysteine at 37^{o}C in an Oxoid anaerobic jar (Oxoid Ltd., Thermo Fisher Scientific, UK).

The whole plants were harvested at or in the vicinity of the Elora Research Centre (Elora, Ontario) in mid-September. Cornstalks were collected in early-November from the same fields for the post-harvest trial. The plant material was chopped and heat-treated by autoclaving at 121^{o}C for 20 min. Experiments were conducted using a 2x3x8 factorial design, representing harvest time, corn variety and treatment (Table 5.1). Aerobic and anaerobic treatments of 10 grams of plant material were inoculated with 12 hr cultures of *B. subtilis, C. felsineum* or combinations of the two at concentrations of approximately 1 x 10^{4} and 1 x 10^{8} cfu ml^{-1}, respectively, along with the addition of 10 ml of M9 medium (Miller 1972) in 250 ml Erlenmeyer flasks. Heat-treated and non heat-treated controls were included. Anaerobic treatments were maintained using a gas-trap. All flasks were incubated statically at 30^{o}C for seven days. Fibres were extracted by decortication and allowed to air dry prior to analysis.

Table 5.1 2x3x8 experimental design for cornstalk retting trials. Treatments were performed in triplicate.

Harvest Time	Plant Variety	Condition	Treatment
Pre (whole plant) and Post (cornstalk only)	Conventional Roundup Ready (RR) *Bt*- Roundup Ready *Bt*-RR)	Aerobic	1. *Bacillus* 2. *Clostridium* + *Bacillus* 3. Sterile control 4. Non-sterile control
		Anaerobic	5. *Clostridium* 6. *Clostridium* + *Bacillus* 7. Sterile control 8. Non-sterile control

5.2.2 Organic Acid Analysis

A modified version of the GC-FID method developed by Yang and Choong (2001) was selected as the method to quantify C_2-C_6 volatile organic acids, namely acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, hexanoic acid (or caproic acid), with the following modifications: (1) mass spectrometry (MS) was used as the detector instead of FID, and (2) the samples were derivatized with bis-(trimethylsilyl)trifluoroacetamide (BSTFA) at 100^{o}C for 1 hour to improve the detection of the organic acids. Derivatized samples taken at study initiation and termination were analyzed using a Varian CP-3800 gas chromatograph equipped with a CP-Wax 52 CB fused silica column (Varian, Inc., Palo Alto CA, USA) according to the conditions specified by Yang and Choong (2001).

As VOA production can be related to microbial metabolism of plant sugars, an increase in acid concentration should be representative of bacterial growth and metabolism. Therefore, organic acid detection was used to monitor the metabolic activity of the bacterial population as an indicator of growth as opposed to colony forming unit (CFU) count due to the potential for underestimation as a result of cell death during dilution, exposure to oxygen and dessication (Elsas *et al.*, 1997).

5.3 Results and Discussion

Acetic acid was produced in most samples tested, both pre-harvest and post-harvest, with a lower detection limit of 316 mg/L. In the aerobic treatments, acetic acid levels were found to be in the range of 400 mg/L, with an average pre-harvest (or whole plant) production of 389 mg/L (Figure 5.1). These levels were also present in the sterile control, indicating that conditions used for sterilization of the material were not effective, most likely as a result of the presence of resistant spores. The *Bacillus* post-harvest levels in *Bt*-RR corn had the highest production at over 3000 mg/L.

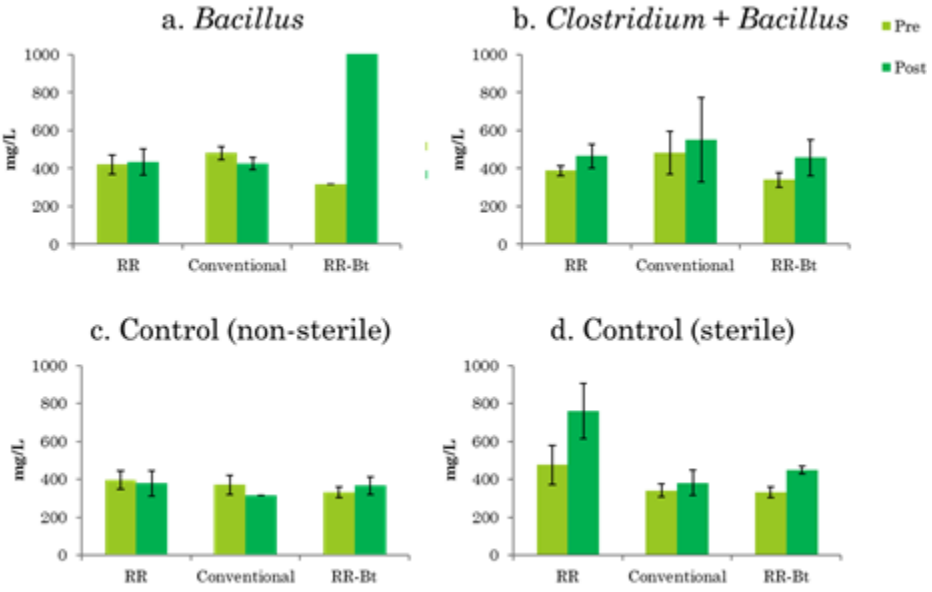
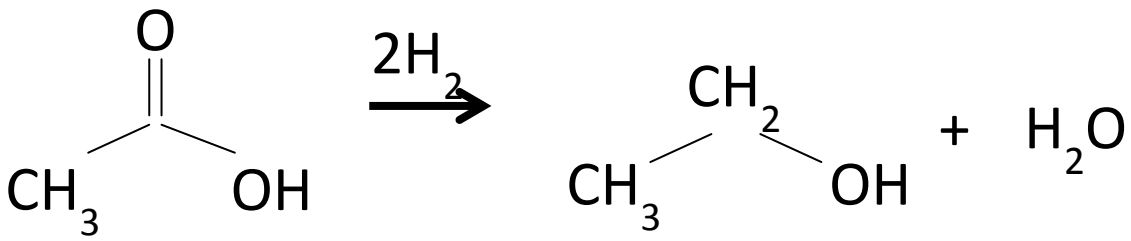


Figure 5.1 Acetic acid production (in mg/L) in pre- and post-treated samples incubated under aerobic conditions. (Note: RR – Roundup Ready; RR-Bt – *Bt*-Roundup Ready)

Acetic acid production was higher in general in the anaerobic treatments, with an average pre-harvest level of close to 800 mg/L, twice the average level of the aerobic treatments. The conventional corn variety had a consistently higher production of acetic acid after

fermentation in all anaerobic treatments (Figure 5.2). As with the aerobic treatments, the anaerobic sterile control showed high levels of acetic acid both pre- and post-harvest. Acetic acid is a highly versatile carboxylic acid with many industrial uses in its various forms, including as a vinyl acetate monomer, pure terephthalic acid as a precursor for polyethylene terephthalate, acetates such as cellulose acetate used in film, and acetic anhydride (Sano *et al.*, 1999). Global demand for acetic acid is forecasted to reach 11.3 million tons by 2015 according to the Global Industry Analysts, Inc. as reported by their Public Relations (available at http://www.prweb.com/releases/2011/1/prweb8049448.htm). Acetic acid also presents a new feedstock for the production of ethanol. In a patent submitted in 2008, Johnston *et al.* claim that hydrogenating acetic acid over a platinum and tin catalyst supported on silica-based or graphite support produces ethanol in a vapour phase at 250^{o}C based on the following reaction:



At a lower anaerobic production level of 800 mg/L, acetic acid in silage effluent could reach levels of 26 to 560 g based on 300 to 700 L effluent per ton silage (Lesperance *et al.*, 2008). At 3000 mg/L, acetic acid production could reach 900 to 2100 g per ton silage.

Propionic acid was not detected in most samples with a lower detection limit of 318 mg/L, with exceptions in two anaerobic non-sterile controls. The conventional corn variety had an average content of 429 mg/L in the post-harvest samples, while the *Bt*-RR corn variety had 413 mg/L in the pre-harvest samples.

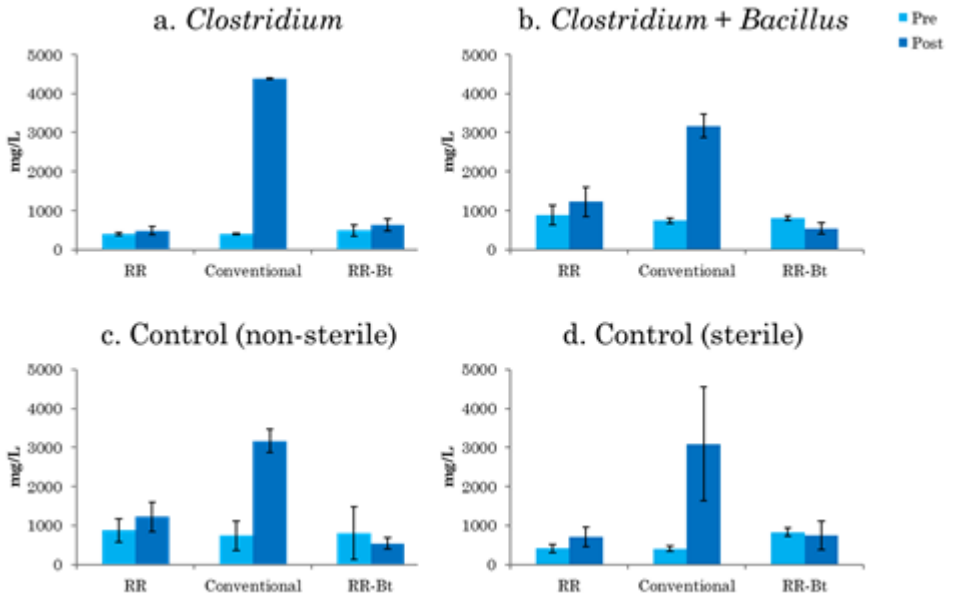


Figure 5.2 Acetic acid production (in mg/L) in pre- and post-treated samples incubated under anaerobic conditions. (Note: RR – Roundup Ready; RR-Bt – *Bt*-Roundup Ready)

Butyric acid is an indicator of clostridial fermentation, and is undesirable in typical silage (McDonald *et al.*, 1991). This VOA had a lower detection limit of 87 mg/L and was mostly found in the *Clostridium* + *Bacillus* and anaerobic non-sterile control treatments. The addition of *B. subtilis* may have contributed to the growth of *C. felsineum* and other clostridial spores by eliminating residual oxygen levels, thus allowing growth of the anaerobe. The anaerobic non-sterile control had the highest levels of butyric acid, both pre- and post-harvest of up to 8000 mg/L (Figure 5.3). At this level, theoretical production of butyric acid could reach between 2.4 to 5.6 kg per ton silage based on 300 to 700 L effluent (Lesperance *et al.*, 2008). In contrast, isobutyric acid was mostly found in the aerobic treatments and at lower levels of 150 mg/L (Figure 5.4).

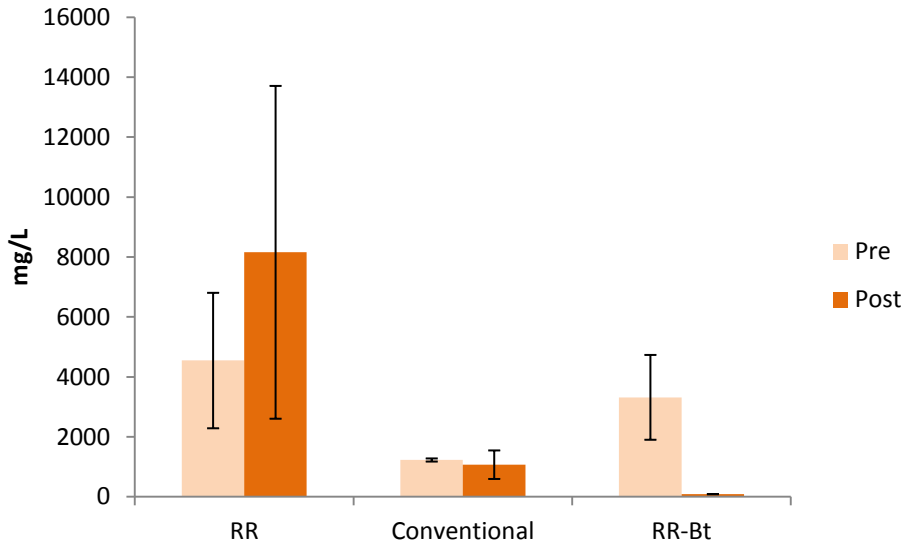


Figure 5.3 Butyric acid production for the non-sterile anaerobic control. As butyric acid was not detected post-treatment in the *Bt*-RR corn variety, it is presented at the lower detection limit of 87 mg/L. (Note: RR – Roundup Ready; RR-Bt – *Bt*-Roundup Ready)

Butyric acid can be converted to butanol by *C. acetobutylicum*, but is most often produced via petrochemical routes (Ramey and Yang, 2004). Recently, *Clostridium saccharoperbutylacetonicum* N1-4 was reported to produce 13 g/L of butanol using 10 g/L of butyric acid and 20 g/L of glucose (Al-Shorgani *et al.*, 2012). Based on these figures, production of butanol could range between 240 and 560 L per ton silage. According to Biobased Butanol Info (available at http://www.biobutanol.com/Research-Study.html), butanol's current global demand exceeds 1.2 billion gallons per year and is valued at over $6 billion. Production of bio-based butanol is hindered by 2 factors: yield from glucose is low at about 15% w/w, and production is limited by product inhibition and must be lower than 13 g/L, which makes acetone-butanol-ethanol (ABE) fermentation uneconomical (Ramey and Yang, 2004). Butyric and isobutyric from silage effluent could be used and eliminate the need for acidogenesis used by *C. acetobutylicum*, thereby requiring only the solventogenesis pathway and one bioreactor to increase the overall efficiency of the process.

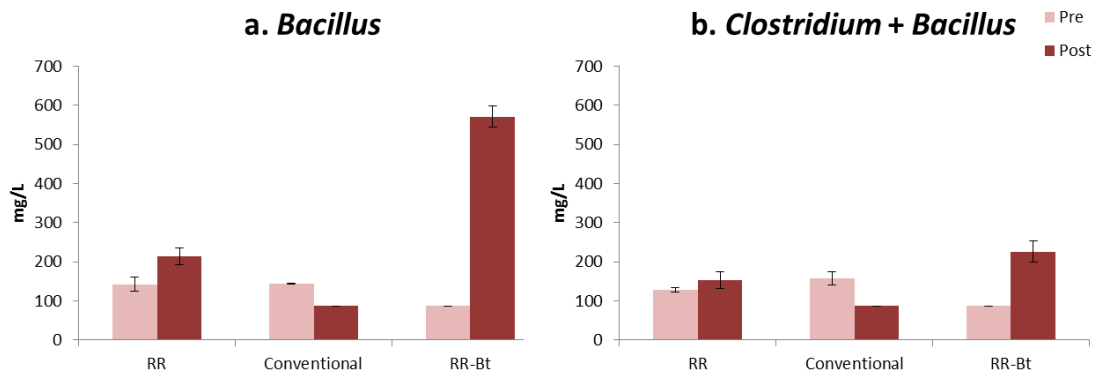


Figure 5.4 Isobutyric acid production (in mg/L) in pre- and post-treated samples incubated under aerobic conditions. . In cases where isobutyric acid was not detected, its concentration is presented at the lower detection limit of 87 mg/L. (Note: RR – Roundup Ready; RR-Bt – *Bt*-Roundup Ready)

Valeric and hexanoic acid were not detected in any sample. However, isovaleric acid was detected in the aerobic post-treatment samples at low levels, although these were inconsistent. For example, in the *Bacillus* and *Clostridium* + *Bacillus* aerobic treatments, isovaleric acid was only detected in the RR and *Bt*-RR samples, respectively. Similarly, isovaleric acid was detected in the RR corn variety in the sterile control post-treatment samples, although it was not found to be present in the conventional and *Bt*-RR corn treatments.

Total organic production was found to be highest in the conventional variety under anaerobic conditions post treatment (Table 5.2). This constitent observation was not noted under aerobic conditions or for other varieties. However, the anaerobic non-sterile control showed the highest overall production of volatile organic acids in the 3 varieties, incidating that the indigenous microorganisms were the most effective at producing potential value added fermentation by-products during silage fermentation.

Table 5.2 Total volatile organic acid production (mg/L) in pre- and post-treated corn samples.

Aerobic	PRE	POST	**Anaerobic**	PRE	POST
Bacillus			***Clostridium***		
RR[1]	590.20	813.16	RR	398.07	478.30
Conventional	623.42	511.64	Conventional	401.55	4371.73
Bt-RR[2]	403.04	3694.86	*Bt*-RR	487.49	770.70
Bacillus+Clostridium					
RR	515.71	617.06	RR	873.70	1452.92
Conventional	639.46	636.34	Conventional	995.30	3491.99
Bt-RR	424.86	845.05	*Bt*-RR	804.03	540.79
Control-Sterile					
RR	563.28	1068.23	RR	408.70	700.92
Conventional	568.99	381.86	Conventional	407.26	3090.74
Bt-RR	419.66	677.57	*Bt*-RR	827.79	750.81
Control-Non Sterile					
RR	547.563	379.22	RR	5247.22	8578.56
Conventional	548.282	316.34	Conventional	3044.89	3839.73
Bt-RR	332.757	367.57	*Bt*-RR	5208.34	1072.12

[1]RR – Roundup Ready
[2]*Bt*-RR – *Bt*-Roundup Ready

5.4 Conclusions

Currently, silage effluent is typically diluted and either added to feedstuff or collected and discharged to soil or water. Given the potential for the presence of organic compounds as value-added by-products in silage liquor, production of VOAs with emphasis on acetic and butyric acids could result in a new industrial feedstock for biofuels and other important chemical compounds. Highest acetic and butyric acid production was found to require anaerobic environments, conditions also needed for optimum silage fermentation.

Differences in corn hybrid varieties were also found to impact production of organic acids, although aerobic and anaerobic treatments resulted in inconsistent production and a specific variety was not identified as most or least favourable. However, the indigenous microbial

population was found to produce the highest amount of total volatile organic acids under anaerobic conditions, typical of the silage fermentation. Collection and analysis of silage effluent produced in larger-scale field settings would be important to show whether this agricultural waste product could become an economically viable option for production and supply VOAs.

Acknowledgements

Funding from the Ontario BioCar Initiative is gratefully acknowledged. Rachel Campbell Murdy was a recipient of an NSERC postgraduate scholarship during the course of this work. We thank Mr. Peter Smith for his assistance in finding and supplying the corn samples. The authors are also grateful to Dr. Sarah Meunier for her help with the method development and analysis of the GC-MS results.

Chapter 6

Characterization of Cornstalk Fibres from Three Corn Isoline and Mechanical Properties of Cornstalk-Reinforced Polypropylene Biocomposites

Summary

Plant fibres isolated from three corn isolines were analyzed to determine variability as reinforcement fillers in polypropylene by comparing the physical and mechanical properties of the cornstalk biomass and biocomposites, respectively. Cornstalk material from the *Bt*-Roundup Ready® corn (*Zea mays* L.) isoline was found to have higher cellulose content and a higher onset of degradation than its genetic Roundup Ready® (RR) and conventional counterparts. However, RR cornstalk-reinforced polypropylene exhibited the highest flexural strength and modulus, with a 37 and 94% increase in these properties, respectively. Impact strength of the biocomposites increased 50% on average in comparison to pure polypropylene. As a result of pleiotropic effect from genotypic manipulation and/or crop management practices, plant fibres isolated from different hybrids may result in biocomposites with different mechanical properties, supporting the notion that crop variety may be an important factor when considering fibre source.

Keywords: Polymer-matrix composites (PMCs), mechanical properties, thermal properties, natural fibres

Note: The following chapter entitled "Characterization of Cornstalk Fibres from Three Corn Isoline and Mechanical Properties of Cornstalk-Reinforced Polypropylene Biocomposites" co-authored by Rachel G. Campbell Murdy, Larry Erickson, FrançoisTardif, Leonardo Simon and Raymond L. Legge will be submitted for publication as a refereed journal article.

6.1 Introduction

Increasing pressure for environmental sustainability is leading to an interest in biocomposite materials, which consist of a matrix material reinforced with natural fibres. Plant fibres add strength and stiffness to the material due to their cellulose microfibrils. Cellulose, hemicellulose and lignin content of plant-derived fibres are important parameters when considering their use in biocomposites, as non-cellulose components reportedly decrease the strength of the fibre and make the resulting biocomposite more susceptible to biological, ultraviolet and thermal degradation (Mohanty *et al.*, 2000).

Cornstalks provide a large source of renewable lignocellulosic biomass, offering an inexpensive and renewable source of fibres for textiles and reinforcement applications (Reddy and Yang, 2005). Corn stover, the residue which is left on the field after grain harvest, consists of approximately 50% stalks, 22% leaves, 15% cob and 13% husk (The Carbohydrate Economy, 2002). One thousand kg of corn stover is produced for every 1000 kg of grain corn harvested and between 30 to 50% of this stover could be harvested without detriment to soil nutrient levels, soil moisture or erosion issues (Graham *et al.*, 2007).

Corn stover has been considered for its potential use in textile and reinforcement industries. The overall cellulose, hemicellulose and lignin content of cornstalks were determined to be 38-40, 28 and 7-21%, respectively (Reddy and Yang, 2005) although corn variety was not specified in the study. Genetic transformation of corn with the *Bt* gene has been observed to result in a 33-97% higher lignin content in the corn stems of 10 different *Bt* corn hybrids when compared to their respective non-*Bt* isoline (Saxena and Stotsky, 2001). There is also potential for higher deposition of lignin in Roundup Ready® (RR) varieties, as constitutive expression of the glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (Padgette *et al.*, 1995) could lead to the over-production of phenylalanine via the shikimate pathway. Phenylalanine is a precursor in the phenylpropanoid pathway leading to the formation of the phenolic compounds present in lignin (Bentley, 1990). The glyphosate-tolerant soybean (*Glycine max* (L.) Merr.) variety 40-3-2 was recently reported to have the

same chemical composition when compared to the conventional control Dekabig (Harrigan *et al.*, 2007); however, the two lines studied were not genetic equivalents or isolines.

The genetic events introduced in RR and *Bt* corn varieties could have pleiotropic effects on fibre properties and processing. Potential variability in plant composition and thermostability as well as mechanical properties of cornstalk fibre-reinforced polypropylene was studied with 3 corn isolines representing a conventional, RR and *Bt*-RR varieties with the objective of assessing whether specific isolines might produce biocomposites with better thermal and mechanical properties when used as reinforcement material.

6.2 Materials and Methods

6.2.1 Corn Isolines

Three Pioneer corn isolines were studied: 38N85, 38N86 and 38N87, representing a Roundup Ready® (RR), a conventional and a *Bt*-Roundup Ready® (*Bt*-RR) stacked variety, respectively (Pioneer Hi-Bred International, Inc., Johnston, Iowa). These three isolines were selected due to their immediate availability and proximate growth area, minimizing the variability of environmental impact on plant composition. Cornstalks were collected at or in the vicinity of the Elora Research Station of the University of Guelph (Elora, Ontario) in early-November. Dried cornstalk material from each variety was ground to a 1 mm mesh size with a Brinkman Retsch-Muhle grinder (Retsch, Haan, Germany) and stored at room temperature until further use.

6.2.2 Thermal and Compositional Characterization of Cornstalk Isolines

Thermogravimetric analysis **(**TGA) experiments were performed using an SDT 2960 (TA Instruments, New Castle, DE) with a heating rate of 10^{o}C/min from 30^{o}C to 400^{o}C in a nitrogen environment. TGA plots were analyzed for: (i) three onset points of degradation at

0.5, 1.0 and 2.0 wt%/min; (ii) the maximum rate of degradation and its corresponding peak temperature and (iii) the residue at run termination.

Samples of ground material were analyzed for acid detergent fibre (ADF), neutral detergent fibre (NDF) and lignin content (%) using the filter bag technique (Komarek, 1994; Komarek *et al.*, 1993) with an ANKOM^{2000} Fibre Analyzer (ANKOM Technology, Macedon, New York) by Agri-Food Laboratories (Guelph, Ontario). The NDF fraction contains the hemicellulose, cellulose and lignin components; the ADF contains only the cellulose and lignin. Therefore, the difference between NDF and ADF reflects the hemicellulose content. After digestion with 72% sulfuric acid, the cellulose and lignin can be separated. The lignin content can be determined and subtracted from the ADF to reflect the cellulose content.

6.2.3 Composite Formulation and Testing

Formulations are presented in Table 6.1. The base resin used was a homopolymer polypropylene (PP), grade D180M (MFI 18), manufactured and supplied by Braskem America, Inc. (Houston TX, USA). Irganox 1010 and Irgafos 168 (Ciba Specialty Chemicals, Inc., Mississauga ON, Canada) and a maleic anhydride grafted polypropylene (Fusabond® P grade MD-353D) (DuPont™, Wilmington DE, USA) were included as antioxidants and coupling agent, respectively. The weight ratio of cornstalk to matrix was 30:70 (w/w), which was selected to offer a basis for comparison with published data (Mohan Sharma, 2010; Panthapulakkal and Sain, 2006; Reddy *et al.*, 2010). The weight of the coupling agents and antioxidants were deducted from the matrix content. The ground filler was dried for 1 hour at 110^{o}C at which point moisture content was measured with a Moisture Analyzer MB45 balance (OHAUS Corporation, Parsipann NJ, USA).

Table 6.1 Formulation of the cornstalk-reinforced polypropylene biocomposites.

	Polypropylene (wt%)	Fibre (wt%)	Coupling Agent (wt%)	Antioxidant (wt%)
PP[1]	99.5	-	-	0.25+0.25
PP[1] Non-extruded	100	-	-	-
38N85 (RR)[2]	67.5	30	2	0.25+0.25
38N86 (Conventional)	67.5	30	2	0.25+0.25
38N87 (*Bt*-RR)[3]	67.5	30	2	0.25+0.25

[1]PP – Polypropylene
[2]RR – Roundup Ready
[3]*Bt*-RR – *Bt*-Roundup Ready

6.2.3.1 Extrusion and Injection Moulding

The formulations were hand-mixed and fed into a Haake Minilab Micro-compounder set with a co-rotating twin screw extruder (Thermo Scientific, Waltham MA, USA) at 190^{o}C and 40 rpm. The compounded material was collected, cooled and pelletized. The pellets were injection moulded using a Ray Ran Laboratory Injection Moulding apparatus (Ray Ran Test Equipment Ltd., UK) set at 190^{o}C and a pressure of 100 psi into a mould tool set at a temperature of 50^{o}C to obtain test specimens according to ASTM standards. The test bars were annealed to remove thermal history in a Hewlett Packard 5890A GC oven (Agilent Technologies, Santa Clara CA, USA) with a heating rate of 10^{o}C per minute up to 150±1^{o}C and held for 11 min before ramping down to room temperature.

6.2.3.2 Differential Scanning Calorimetry

A Q2000 Differential Scanning Calorimeter (TA Instruments, New Castle DE, USA) was used to determine melting point, crystallization peaks and enthalpy of phase transition of composite with the following cycling conditions in a nitrogen environment: samples were equilibrated at 35^{o}C, heated to 210^{o}C at a ramp of 20^{o}C/min and held for 5 minutes (cycle 1), cooled to 35^{o}C at a ramp of 10^{o}C/min and held for 5 minutes (cycle 2), and finally ramped to 210^{o}C at a rate of 10^{o}C/min (cycle 3). Cycles 2 and 3 were used to analyse the thermal properties of the composites by plotting the data using the TA Universal Analysis software

(TA Instruments, New Castle DE, USA). The degree of crystallinity of the composites was calculated as:

$$\% = \frac{\Delta H}{\Delta H^o (\text{wt\% filler})} \times 100$$

where ΔH represents the heat of enthalpy required to melt the resin and ΔH^o is the heat of fusion of 209 J/g for 100% crystalline polypropylene (Marinelli *et al.*, 2003).

6.2.3.3 Mechanical Properties

Test specimens were conditioned at a temperature of 23 ± 2^{o}C and relative humidity of 31% for over 48 hours prior to testing. Flexural properties of six specimens per formulation were measured using the Three-Point-Bending test following ASTM D 790 using a Q Series Mechanical Test Machine (Test Resources Inc., Shakoppe MN, USA). Izod impact testing was performed according to ASTM D 256. Six specimens were notched at 45±1^o and a depth of 2.5 mm using a milled notch cutter. Bars were notched prior to the annealing described in 2.3.1. Bars were mounted on a Monitor Impact Tester (Testing Machines Inc., New Castle DE, USA) and izod impact was determined using a swinging pendulum type hammer (Izod Hammer 0-1 X 0.01 FT Lbs, TMI 43-0A-03) at 90^o. All testing was performed at room temperature.

The fiber-matrix interface was characterized using a Zeiss Ultra-Plus field emission scanning electron microscope (SEM) (Carl Zeiss, Oberkochen, Germany). The samples were mounted on metal stubs using double-sided conductive tape and the surface was gold-coated. Images were taken at 10 kV.

6.3 Results and Discussion

6.3.1 Corn Isoline Composition

Analysis of the composition of the cornstalks from the 3 isolines revealed some differences for components analyzed (Table 6.2). Content was found to be close to or within the wide ranges reported by Reddy and Yang (2005) in a broad survey of corn genotypes. Cornstalk material from 38N87 (*Bt*-RR) corn was found to have slightly but significantly higher cellulose content than its RR and conventional counterparts (P-values of 0.078 and 0.0061, respectively). Hemicellulose content did not show significant difference between isolines. Conventional corn showed a significantly higher lignin content than *Bt*-RR corn (P-value of 0.076), which is contrary to the reportedly higher lignin content of *Bt* corn found by Saxena and Stotzky (2001). This might be explained in that the inclusion of the RR gene may have affected the pleiotropic effect associated with the *Bt* gene.

Table 6.2 Per cent weight content of lignin, hemicellulose and cellulose of cornstalks from 3 corn isolines (wt%) (standard deviations, calculated from triplicate analyses, are presented in parentheses).

% Weight	Roundup Ready	Conventional	*Bt*-Roundup Ready
Lignin	6.7 (0.5)	6.7 (0.3)	6.0 (0.1)
Hemicellulose	25.9 (2.0)	25.8 (0.1)	25.1 (0.9)
Cellulose	38.3 (1.4)	37.8 (0.4)	41.6 (0.1)

6.3.2 Thermal Characterization of Cornstalk Fibres

The thermal degradation of cornstalk fibres were comparable overall to those obtained for mechanically processed wheat straw (Panthapulakkal *et al.*, 2006). TGA analysis of the ground unprocessed biomass of the 3 corn isolines showed significant differences with regards to the different onsets of degradation (0.5, 1.0 and 2.0 wt%/min) and peak temperatures (^{o}C), determined from the first derivative plot of the TGA curves (Figure 6.1). Material from the *Bt*-RR corn was found to have the highest 0.5 wt%/min onset of

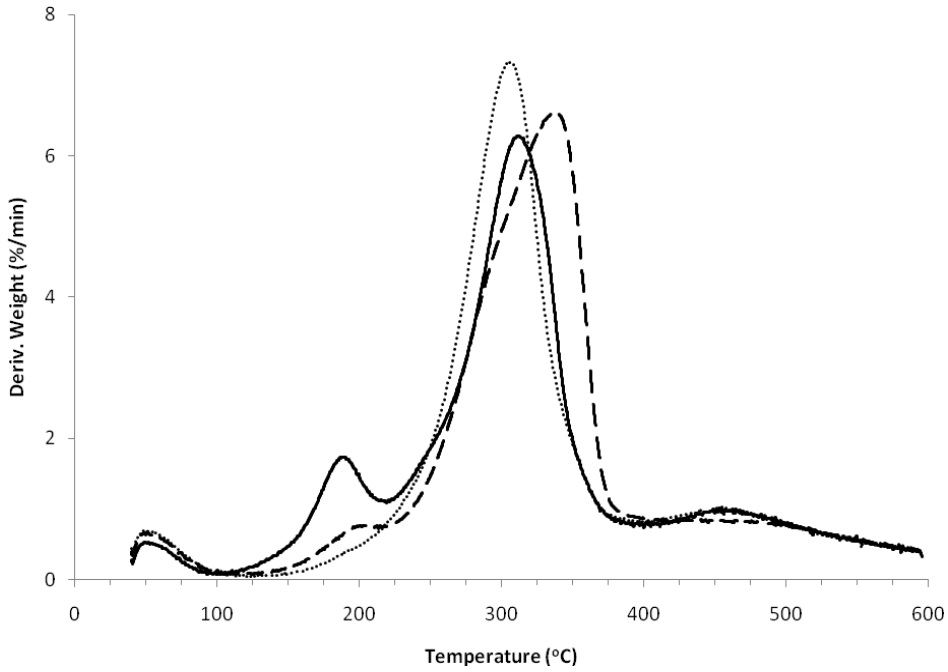


Figure 6.1 Comparison of the first derivative plot of thermogravimetric analysis curves resulting from Roundup Ready (solid line), conventional (dashed line), and *Bt*-Roundup Ready (dotted line) cornstalk material.

degradation and maximum rate of degradation (%/min) at 198 and 7.0, respectively, followed by the conventional and RR isolines (Table 6.3). This observation can possibly be attributed to the higher cellulose content of the *Bt*-RR isoline. However, conventional corn had the highest 1.0 and 2.0 wt%/min onsets of degradation of 237 and 262, respectively, and a significantly higher peak temperature of 342^{o}C. A comparison of the conventional and the *Bt*-RR corn isolines showed no difference in maximum rate of degradation (%/min), although both were significantly different than the RR isoline. Conversely, moisture content and residue at 600^{o}C (%) only showed significant differences between the conventional and the *Bt*-RR corn (data not shown). The higher 1.0 and 2.0 wt%/min onset of degradation of conventional corn may be due to its slightly elevated lignin content, as lignin forms the matrix sheath by cross-linking the spaces between the cellulose and hemicellulose thereby lowering the diffusion of the plant fibre constituents during heating (Grabber, 2005). This observation is further substantiated by the higher peak temperature of conventional corn,

Table 6.3 Cornstalk fibre characteristics determined by thermogravimetric analysis (standard deviations are presented in parentheses).

Corn Line	Onsets of degradation (wt%/min deg.)			Peak temp.	Rate of deg.
	0.5	1.0	2.0	(^{o}C)	(%/min)
RR[1]	153 (0.8)	169 (1.5)	254 (0.8)	312 (0.9)	6.1 (0.26)
Conventional	181 (2.7)	238 (0.8)	262 (1.2)	342 (3.4)	6.7 (0.26)
Bt-RR[2]	198 (3.4)	227 (1.0)	252 (0.6)	305 (0.9)	7.0 (0.17)

[1]RR – Roundup Ready
[2]*Bt*-RR – *Bt*-Roundup Ready

since an increase in peak temperature has been attributed to a reduction in the diffusion of degradation products (Sain and Panthapulakkal, 2006).

6.3.3 Thermal Characterization of Biocomposites by DSC

Addition of 30% cornstalk material to the PP was found to decrease the heat of enthalpy of crystallization and melting point (ΔH_c and ΔH_m) by approximately 35% (Table 6.4). The biocomposites also showed an increase in crystallization temperature (T_c). However, the melting point temperatures and % crystallinity of the biocomposites were not significantly different ($P < 0.05$) from those of the pure PP. Antioxidants present in the extruded PP did not affect the thermal properties of the plastic in comparisons with those of the non-extruded pure PP.

6.3.4 Mechanical Properties of Biocomposites

The properties of PP biocomposites with cornstalk in comparison to pure and non-extruded PP are presented in Figures 6.2-6.4. There were no differences in extruded and non-extruded pure PP in the properties tested. The average flexural strength for the pure PP was found to be 41.4 MPa, significantly lower than the reported 50.0 MPa for homopolymer PP (Mohan Sharma, 2010). Incorporation of cornstalks from the 3 varieties improved the flexural strength, flexural modulus and impact strength of the biocomposites.

Table 6.4 Thermal characterization of cornstalk-reinforced polypropylene biocomposites by dynamic scanning calorimetry.

Composite	T_c (^oC)	ΔH_c (J/g)	T_m (^oC)	ΔH_m (J/g)	$\%C_c$
PP[1]	115.47a	95.67a	159.40	71.13a	46.01
PP Non-extruded	115.43a	96.57a	161.83	72.05a	46.21
38N85[2]	119.92b	62.64b	161.79	45.68b	44.40
38N86[3]	118.28b	62.06b	163.39	45.50b	43.99
38N87[4]	117.59b	63.87b	162.72	47.05b	45.27

Note: lower-case letters denote statistically significant differences ($P < 0.05$)
[1]PP – Polypropylene
[2]38N85 – Roundup Ready
[3]38N86 – Conventional
[4]38N87 – *Bt*-Roundup Ready

Material from the RR isoline resulted in significantly higher flexural strength (Figure 6.2) and flexural modulus (Figure 6.3) than its conventional and *Bt*-RR counterparts. For example, flexural strength increased 37% in biocomposites reinforced with RR cornstalk, but increased only 27% and 26% for in conventional and *Bt*-RR biocomposites, respectively. The flexural strength of the pure PP and biocomposites were found to be within the range reported by Panthapulakkal and Sain (2006) for polypropylene (MFI 12) reinforced with 30% wheat straw. The flexural modulus of the pure PP was 0.961 ± 0.048 GPa, which is lower than the manufacturer's reported 1.31 GPa (Braskem America, Inc., Houston TX, USA). The flexural modulus was closer to that of a homopolymer PP with an MFI of 12, found to be approximately 1.1 GPa by Panthapulakkal and Sain (2006) and Reddy *et al.* (2010). This difference may be due to polymer batch to batch deviations. There was a 94% increase in flexural modulus in biocomposites reinforced with RR plant material, while conventional and *Bt*-RR cornstalks resulted in increases of 89% and 78%, respectively. Impact strength increased 49%, 46% and 56% in PP reinforced with RR, conventional and *Bt*-RR cornstalk material although these differences were not found to be significant between the 3 isolines (Figure 6.4). These results were unexpected given the higher cellulose content and thermal properties of the *Bt*-RR cornstalk material and indicate that composition and

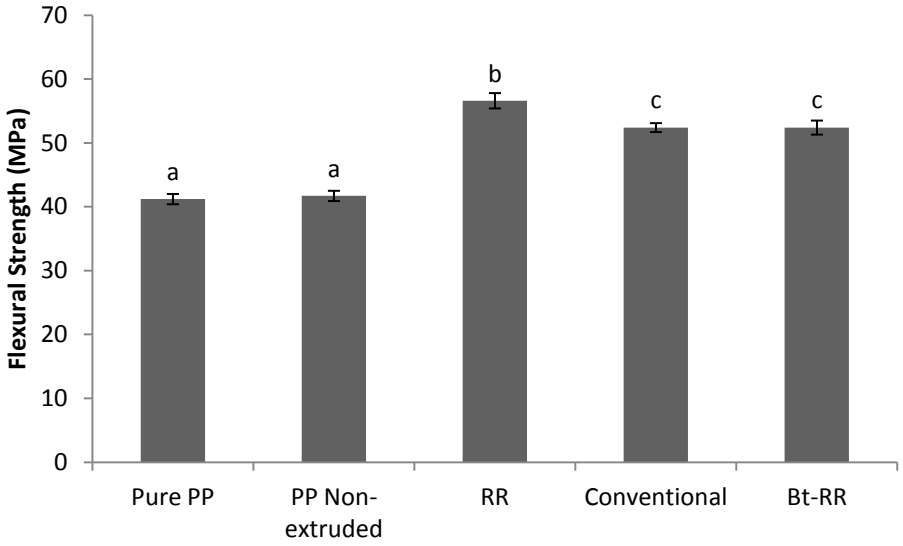


Figure 6.2 Comparison of flexural strength of cornstalk-reinforced-polypropylene biocomposites. A total of 6 replicates were tested. Lower-case letters denote statistically significant differences ($P < 0.05$). (PP – polypropylene; RR – Roundup Ready; *Bt*-RR – *Bt*-Roundup Ready)

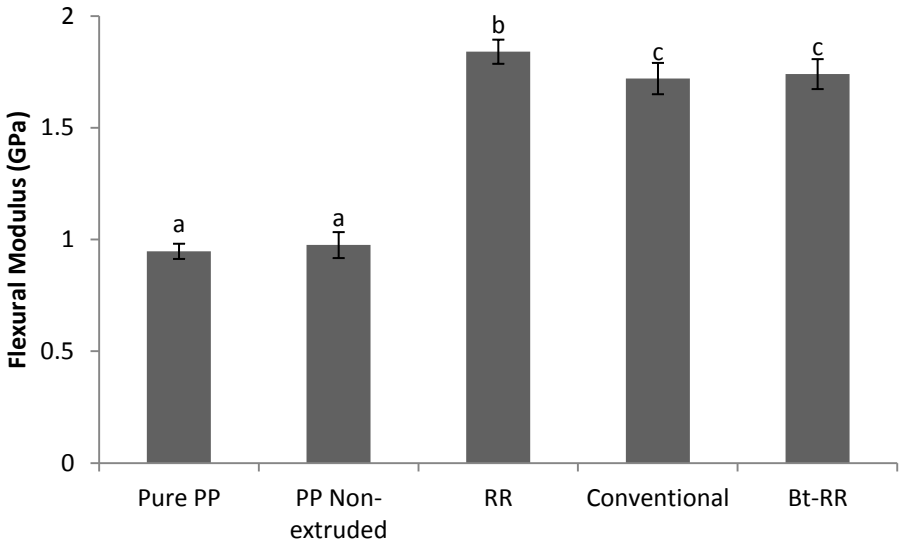


Figure 6.3 Comparison of flexural modulus of cornstalk-reinforced polypropylene biocomposites. A total of 6 replicates were tested. Lower-case letters denote statistically significant differences ($P < 0.05$). (PP – polypropylene; RR – Roundup Ready; *Bt*-RR – *Bt*-Roundup Ready)

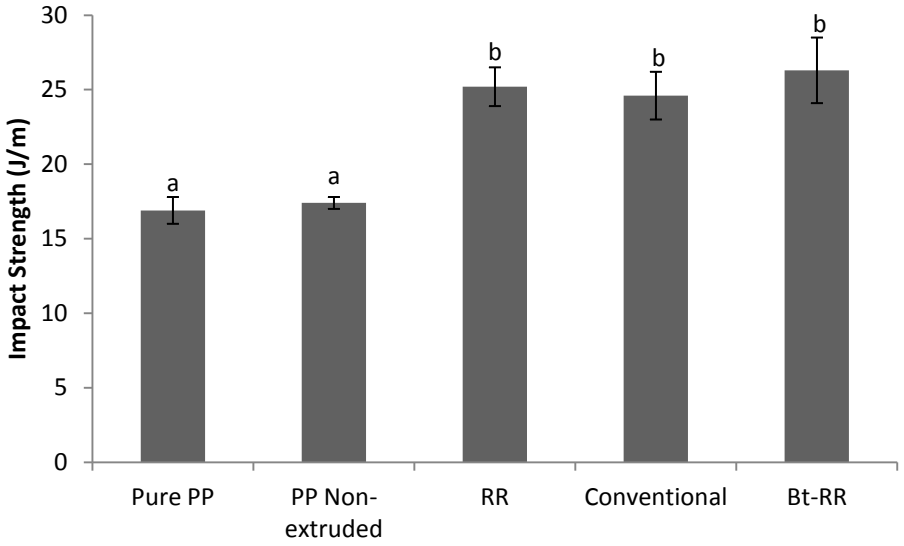


Figure 6.4 Comparison of notched Izod impact strength of cornstalk-reinforced polypropylene biocomposites. A total of 6 replicates were tested. Lower-case letters denote statistically significant differences ($P < 0.05$). (PP – polypropylene; RR – Roundup Ready; *Bt*-RR – *Bt*-Roundup Ready)

thermostability may not necessarily be good predictors of mechanical properties. The differences in mechanical properties of the biocomposites may be attributed to either pleiotropic effect of the RR genetic modification or to crop management practices resulting from the herbicide tolerance. Given the high variability in flexural properties as a result of corn genotype, crop variety may need to be specified for research and industry replication purposes.

Cornstalk material has been reportedly used to reinforce high-density polyethylene (HDPE) (Panthapulakkal and Sain, 2007; Trigui *et al.*, 2012) and linear low-density polyethylene (LLDPE) (Thamae *et al.*, 2008). Cornstalks were not found to improve mechanical properties of the biocomposites in either matrix due to a weak interface. It has been recommended that cornstalk be used as filler rather than as reinforcement (Thamae *et al.*, 2008) or in textiles (Reddy and Yang, 2005). Maleated coupling is frequently used to strengthen composites reinforced with natural fibres by improving the matrix-filler interfacial bonding (Faruk *et al.*,

2012). For example, the use of a coupling agent in wheat-straw biocomposites has been shown to increase mechanical properties interaction (Mohan Sharma, 2010; Panthapulakkal and Sain, 2007; Panthapulakkal *et al.*, 2006; Reddy *et al.*, 2010). The increase in mechanical properties in the cornstalk-reinforced PP reported in flexural and impact strengths may be attributed to the use of the coupling agent Fusabond®. SEM investigation of the biocomposites showed a good interfacial bonding between the PP matrix and corn fibre with no significant voids (Figure 6.5). Use of antioxidants may also have reduced oxidative damage during the processing, leading to the increases in the properties tested.

The significant increase in mechanical properties of the cornstalk-reinforced PP may also be a result of the high cellulose content of the cornstalk. For example, the mechanical properties of whole corn biomass-reinforced PP were investigated under the same conditions and formulations. The per cent cellulose content of corn biomass, which included the leaves, grain and corn cobs, from a 37K84 Pioneer Poncho Roundup Ready variety was 24.2, or 37% lower than cornstalk-only material. When compared to the mechanical properties of pure PP resin, whole corn biomass resulted in increases of 44%, 14% and 5% in flexural modulus, flexural strength and and impact strength, respectively. However, these values represent respective drops of 50% in flexural modulus, 23% in flexural strength, and 44% in impact strength when compared to the mechanical properties imparted by the cornstalk material.

6.4 Conclusions

Plant fibres isolated from three corn isolines were studied to establish their potential use as reinforcement fillers in polypropylene. Composition, thermostability and mechanical properties of the biocomposites were found to vary depending on the variety of corn. Cornstalk material from the *Bt*-RR corn (*Zea mays* L.) isoline was found to have higher cellulose

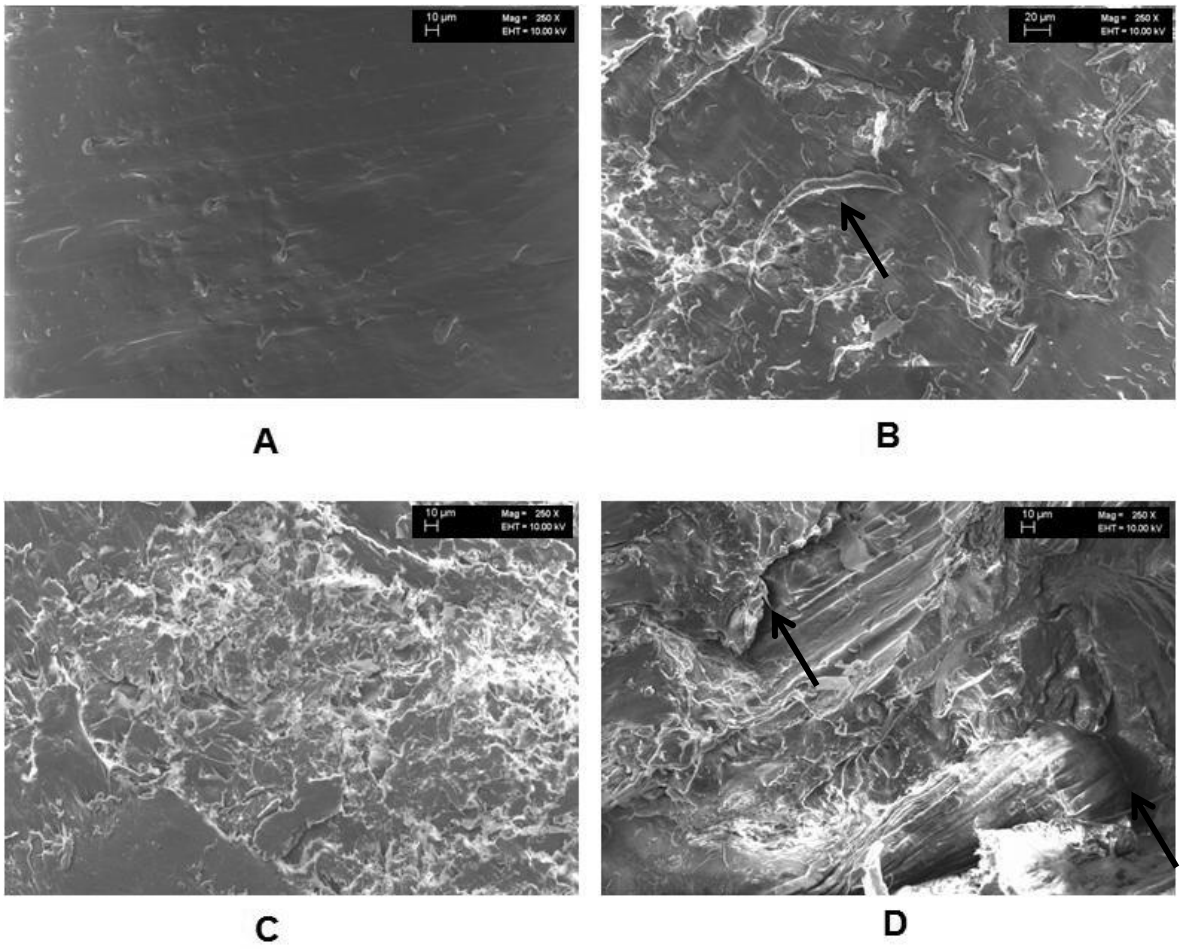


Figure 6.5 Scanning electron microscope images at 250X magnification of the resulting fracture of biocomposites with (A) pure polypropylene (PP), (B) Roundup Ready corn-reinforced PP, (C) conventional corn-reinforced PP, and (D) *Bt*-Roundup Ready corn-reinforced PP, resulting from an impact strength test. Arrows indicate the fibres and the interfacial interaction between the fibre and the matrix.

content than its genetic RR and conventional counterparts, with a corresponding higher onset of thermal degradation. However, polypropylene reinforced with cornstalk from the RR isoline had the highest improvement in properties with a higher flexural strength and modulus. All biocomposites showed higher impact strength than pure polypropylene with no differences between isolines. These results indicate that mechanical properties are not

necessarily proportional to higher cellulose content and thermal degradation. Corn variety was also found to impact the mechanical properties of the reinforced polypropylene potentially due to pleiotropic effect from genotypic manipulation and/or crop management practices, indicating the importance of specifying the plant genotype for industrial replication.

Acknowledgements

Funding from the Ontario BioCar Initiative is gratefully acknowledged. Rachel Campbell Murdy was a recipient of an NSERC postgraduate scholarship during the course of this work. The authors thank Mr. Peter Smith for his assistance in finding and supplying the corn samples. In addition, the authors also thank Dr. Barbara Guettler and Dr. Mohammad Arif for their assistance with composite preparation and testing.

Chapter 7

Effectiveness of Corn Silage Retting as a Biological Fibre Extraction Technique: Field Trial

Summary

A new approach to biological fibre extraction is presented which makes use of silage fermentation as a pre-processing technique for plant biomass. A field scale study of the impact of this solid state fermentation on corn fibres was undertaken with the co-operation of 30 farmers across Southwestern Ontario. The physical properties of the corn fibres pre- and post-ensiling were analyzed and compared. Overall, ensiled corn biomass had a lower 0.5 wt%/min onset of degradation of 20^{o}C, most likely as a result of lower percent cellulose content. Statistical analysis showed that location according to plant hardiness zone, use of inoculant and silage treatment were all factors that had impacts on the thermal behaviour when comparing individual pre- and post-silage samples; however, only the silage fermentation process had an impact on biomass composition. The sample showing the highest variation in thermostability was selected for further processing into biocomposites to determine the potential benefits with regards to mechanical properties. In comparisons to the pure resin, corn biomass-reinforced polypropylene (PP) showed a 15 and 43 % higher flexural strength and modulus, respectively, with no reduction in impact strength. Although biocomposites formulated with pre- and post-silage corn biomass did not show differences in mechanical properties, ensiled corn biomass resulted in an increase in smaller particle sizes with more optimal thermostability and purity. Biocomposites prepared with the less than 0.5 mm size fraction had higher flexural strength and modulus than whole corn-PP biocomposites, especially at higher extrusion temperatures. This biomass fraction composed of highly processed plant material and cell biomass may present better mechanical properties in higher melting thermoplastics and may allow processing conditions with a higher shear stress as a result of its already small aspect ratio.

Keywords: Corn biomass, fibre reinforced biocomposites, mechanical properties, silage fermentation, thermal properties

7.1 Introduction

Increasing pressure for environmental sustainability is giving rise to a new industry in green technology (Faruk *et al.*, 2012). Development of biocomposite materials, which consist of a polymer matrix material reinforced with natural fibres, present an appealing alternative to traditional glass fibre-reinforced composites due to their processing flexibility, high stiffness and low cost (Mohanty *et al.*, 2000). The inherent higher surface polarity of the plant fibres also present incompatibility issues with the hydrophobic polymer matrix, requiring modification by various physical, chemical or enzymatic pre-treatments (Faruk *et al.*, 2012). Plant fibres add strength and stiffness to the material as a result of their cellulose microfibrils. The large production volume of biocomposites is hindered by the large variation in properties and characteristics of the plant, which are influenced by the plant fibre source, growth and processing conditions. In order for plant-derived fibres to be used in biocomposites, the fibres must first be extracted from the surrounding lignin and hemicellulose, as these non-cellulose components decrease the strength of the fibre and make the resulting biocomposite more susceptible to biological, ultraviolet and thermal degradation (Mohanty *et al.* 2000).

Current biological fibre extraction methods are water retting and dew retting, both of which use microorganisms as the plant biomass processors. Water retting involves bacterial degradation of the plant matrix polysaccharides while submerged in water. Clostridia and bacilli are the main water retting agents due to the production of the extracellular enzyme polygalacturonase (Zhang *et al.*, 2000; Akin *et al.*, 2001; Tamburini *et al.*, 2003). Water retting results in pollution, high costs associated with labour and drying, as well as significant waste water production. Dew retting has slowly replaced water retting, where the crops are left out in the field following harvest and exposed to fungal organisms that partly degrade the same polysaccharides releasing the fibres (Meijer *et al.*, 1995; Henriksson *et al.*, 1997; Akin *et al.*, 1998). Disadvantages of dew retting include the need for appropriate climates, variable and inferior fibre quality in comparison to water retting, risks of over retting as well as health effects due to dust and fungal contaminants (Van Sumere, 1992).

A new approach to biological fibre extraction is presented which makes use of solid state fermentation in traditional silage systems of tower, bag or bunker silos. The benefits of exploring silage retting as a pre-processing technique for plant biomass include the development of a process which could make use of existing farm infrastructure while offering contained conditions in a climate where dew retting is precluded. This new microbial processing technique could combine the advantages of water and dew retting while addressing some of the shortcomings. Parallels between silage and current methods of biological fibre extraction involve the significant role of fermentation in both processes. During the silage fermentation, microorganisms found on the plant material rapidly convert the plant polysaccharides into sugars and organic acids, resulting in a rapid decrease in pH. This process may also modify the plant fibre surface, potentially allowing better adhesion between the hydrophilic fibres and hydrophobic matrix when producing a biocomposite.

A field scale study of the impact of the silage fermentation on corn fibres was undertaken with the co-operation of 30 farmers across Southwestern Ontario with the objective of exploring corn silage as a new form of biological fibre extraction technique. Pre- and post-ensiled corn biomass was characterized for its thermal and compositional properties. The mechanical properties of processed corn biomass-reinforced polypropylene were also compared.

7.2 Materials and Methods

7.2.1 Corn Biomass Collection

Corn biomass, which included the cornstalk, leaves, grain and cobs, was collected in woven polypropylene mesh bags at harvest and after at least 4 weeks of fermentation from 30 farms across Southwestern Ontario. Corn variety (if known), location according to global positioning system (GPS) coordinates (confidential), harvest date, silo type (tower, bunker or bag) and use of inoculant (type included if volunteered) were noted for each sample (Table

7.1, 7.2, Figure 7.1). Bags were dried for at least 7 days in an oven at 80^{o}C. Dried material was processed with a Brinkman Retsch-Muhle grinder (Retsch, Haan, Germany) to a 6 mm mesh size to maintain the highest aspect ratio possible and stored at room temperature until further use. Particle size analysis was determined by light microscopy (Leica Microsystems GmbH, Wetzlar, Germany) using image analysis software (ImageJ, National Institutes of Health, Bethesda MD, USA). Ten randomly selected samples from the pre- and post-silage treatments were also size fractionated using 1 mm and 0.5 mm sieves to determine the size fractions by weight%.

7.2.2 Characterization of Pre- and Post-Silage Corn Biomass

7.2.2.1 Thermal Characterization of Pre- and Post-Ensiled Corn Biomass

Pre- and post-ensiled samples from each treatment listed in Table 7.1 were analyzed for their thermal stability. Thermogravimetric analysis **(**TGA) experiments were performed using an SDT 2960 (TA Instruments, New Castle, DE) with a heating rate of 10^{o}C/min in a nitrogen environment from 40^{o}C to 600^{o}C. TGA plots were analyzed for (i) 5 and 10% weight loss temperatures (T5 and T10, respectively), (ii) three onset points of degradation at 0.5, 1.0 and 2.0 wt%/min, and (iii) the maximum rate of degradation and its corresponding peak temperature (T_{max}). The 10 randomly selected size fractionated sample fractions were also analyzed by TGA using the same conditions.

7.2.2.2 Compositional Analysis of Pre- and Post-Ensiled Corn Biomass

Pre- and post-ensiled samples of ground material from each treatment listed in Table 7.1 were analyzed for crude protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrients, lignin content (%), minerals including calcium, phosphorus, potassium, magnesium and sodium, starch, total ash, and crude fat by near infrared spectroscopy (NIR) by A&L Canada Laboratories Inc. (London, Ontario). The NDF fraction contains the hemicellulose, cellulose and lignin components; the ADF contains only the

Table 7.1 Corn biomass collection sites across Southwestern Ontario. Inoculants are described in Table 7.2.

	Variety	Location	Date Collected (Harvested)	Silo Type	Inoculant
1	*Bt*-RR mix	Elora	Sept 16, 2011	Bunker	Sila-Bac® Brand 11C33
2	A6564G3 + K293	Erin	Sept 16, 2011	Bunker	Sil-All$^{4X4®}$ Dry Inoculant
3	37K84 Pioneer Poncho RR	Woodstock	Sept 20, 2011	Tower	BioMax®
4	P8906HR	Arthur	Sept 24, 2011	Bag	Yes
5	Mix mostly RR	Rockton	Sept 26, 2011	Tower	No
6	NK N53W	Embro (Zorra)	Sept 27, 2011	Tower	Anhydrous ammonia
7	F2F569	Woodstock	Sept 27, 2011	Bag	No
8	Mycogen TMF 2N494	Elmira	Sept 28, 2011	Tower	Yes
9	Masters Choice MC 515	Elmira	Sept 28, 2011	Tower	Yes
10	DKC41-20	Guelph	Sept 28, 2011	Bunker	No
11	Pioneer 38N85	Elora	Sept 29, 2011	Bunker	No
12	Hyland CVR54	Rockton	Sept 29, 2011	Tower	No
13	Mix *Bt*-RR	Rockwood	Sept 29, 2011	Tower	Sil-All$^{4X4®}$ WS
14	Mycogen	Rockwood	Sept 29, 2011	Tower	Yes
15	F2F 387 BMR and TMF 2L 418 (Mycogen)	Elmira	Sept 30, 2011	Tower	Yes
16	Hyland	Elmira	Sept 30, 2011	Bunker	Yes
17	Pioneer *Bt*	Fergus	Oct 2, 3011	Bunker	Yes
18	Pioneer 38N86	Guelph/Eramosa	Oct 4, 2011	Tower	No
19	Pride A5891 RWR EDF	Tavistock	Oct 5, 2011	Tower	AgMaster
20	38N88	Guelph	Oct 5, 2011	Bag	No
21	38N88	Guelph	Oct 5, 2011	Tower	No
22	34A85	Embro (Zorra)	Oct 10, 2011	Tower	Sila-Bac® Brand 11Cft
23	Hyland	Guelph	Oct 12, 2011	Tower	Yes
24	DeDell "Del-licious" 2	Elmira	Oct 12, 2011	Tower	No
25	MastersChoice MC-490	Elmira	Oct 12, 2011	Tower	Yes
26	BMR	Elmira	Oct 12, 2011	Tower	Yes
27	MC 490	Elmira	Oct 12, 2011	Tower	Yes
28	MZ4245 CBR	Embro	Oct 18, 2011	Tower	Yes
29	Pioneer 38B14	Elmira	Oct 21, 2011	Tower	Yes
30	NK19G	Rockwood	Oct 23, 2011	Tower	No

Table 7.2 Description of inoculants used in corn silage field trial.

Inoculant	Manufacturer	Bacteria	Enzymes
Sila-Bac® Brand 11C33[a]	Pioneer	*Lactobacillus buchneri*, *Lactobacillus plantarum* and *Enterococcus faecium*	
Sil-All[4X4®] Dry Inoculant[b]	Hubbard Feeds	*L. plantarum*, *E. faecium*, *Pediococcus acidilactici*, *Bacillus pumilus*	Amylase, cellulase, hemicellulase and pentosanase
BioMax®[c]	Pioneer	*L. plantarum* Strains PA-28 and K-270	
Sil-All[4X4®] WS[d] (Water Soluble)	Hubbard Feeds	Proprietary (4 strains)	Proprietary (4)
AgMaster[e]	King's AgriSeeds Inc.	*Pediococcus acidilactici* and *Lactobacillus xylosus*	
Sila-Bac® Brand 11Cft[f]	Pioneer	*L. buchneri* and *Lactobacillus casei*	

[a]http://www.pioneer.com/CMRoot/Pioneer/Canada_en/products/inoculants/pdfs/11C33_eng.pdf
[b]http://www.hubbardfeeds.com/dairy/ProdInfo/silallgranular.aspx?menu=Dry%20Cow&item=Dry%20Cow%20Health%20%26%20Performance%20Products
[c]http://www.svfeeds.com/data/forage/Biomax_5.pdf
[d]http://www.hubbardfeeds.com/dairy/ProdInfo/silallliquid.aspx?m..
[e]http://www.kingsagriseeds.com/forage-preservation/silage-baleage-haylage.php
[f]https://www.pioneer.com/CMRoot/Pioneer/Canada_en/products/inoculants/pdfs/11CFT_eng.pdf

Figure 7.1 Map indicating locations of farms participating in the corn silage retting trial across Southwestern Ontario. Plant hardiness zones 5a and 5b are indicated (according to the Natural Resources Canada).

cellulose and lignin. Therefore, difference between the NDF and ADF reflects the hemicellulose content. After digestion with 72% sulfuric acid, the cellulose and lignin can be separated. The lignin content can be determined and subtracted from the ADF to reflect the cellulose content. The NIR samples were run on a near infrared diffuse reflectance spectrometer (Unity SpectraStar 2500 (Unity Scientific, Columbia MD, USA)). The scanning range used was from 1100 to 2500 nm. All calibration properties were developed from standard methods of analysis. The crude fat content was determined by extraction with petroleum ether according to ANKOM Technology, Method 2, AOCS Official Procedure AM 5-04. Elemental composition was determined by the inductively coupled plasma spectroscopic method (AOAC Official Method 985.01) and the atomic absorption spectrophotometric method (AOAC Official Method 975.03). Starch content was analyzed using the amyloglucosidase-α-amylase method (AOAC Official Method 996.11), and ash content was determined using AOAC Official Method 942.05, with 2 g of test portion heated at 600^{o}C for 2 hours. NIR results for ADF, NDF and lignin were confirmed by wet chemistry by the same location using an Ankom 200 Fibre Analyzer (Ankom Technology, Macedon, NY). The ADF, which is the residue after digestion with sulfuric acid and cetyl trimethylammonium bromide (CTAB), was determined using the Filter Bag Technique for Acid Detergent Fibre in Feeds (for A200), Method 5 (ANKOM Technology, 2011). The NDF, which is the residue remaining after digestion in a detergent solution, was determined using the Neutral Detergent Fiber in Feeds Filter Bag Technique (ANKOM Technology), Method 6 (ANKOM Technology, 2011). The lignin content was quantified by the Acid Detergent Lignin in Beakers (ANKOM Technology, FSA72). All method descriptions are available at ANKOM Technology at http://www.ankom.com/procedures.aspx.

7.2.2.3 Statistical Analysis

Treatments were analyzed as complete 2^3 and 2^4 factorial designs with Design Expert 6.0.6 software (Stat-Ease, Inc., Minneapolis MN, USA) to determine which factors (regional zone, use of inoculants, silo type and treatment) were significant and their effect on composition

(cellulose, hemicelluloses, lignin, starch, ash, crude protein and crude fat) and thermostability (temperature at 5% weight loss or T5, temperature at 10% weight loss or T10, T_{max}, peak rate, 0.5, 1.0 and 2.0 onsets of degradation).

7.2.3 Composite Formulation and Testing

Formulations are presented in Table 7.3. The base resin used was a homopolymer polypropylene (PP), grade D180M (MFI 18), manufactured and supplied by Braskem America, Inc. (Houston TX, USA). Irganox 1010 and Irgafos 168 (Ciba Specialty Chemicals, Inc., Mississauga ON, Canada) and a maleic anhydride grafted polypropylene (grade MD-353D) (DuPont™, Wilmington DE, USA) were included as antioxidants and coupling agent, respectively. The weight ratio of corn biomass to matrix was 30:70 (w/w), which was selected to offer a basis for comparison with published data (Mohan Sharma, 2010; Panthapulakkal and Sain, 2006; Reddy *et al.*, 2010). The weight of the coupling agents and antioxidants were deducted from the matrix content. The ground corn biomass was dried for 1 hour at 110^{o}C at which point moisture content was measured with a Moisture Analyzer MB45 balance (OHAUS Corporation, Parsipann NJ, USA).

Table 7.3 Formulation of the pre- and post-ensiled corn biomass-reinforced polypropylene (PP) biocomposites.

	Polypropylene[1] (wt%)	Fibre (wt%)	Coupling Agent[2] (wt%)	Antioxidant[3] (wt%)
PP	99.5	-	-	0.25+0.25
PP Non-extruded	100	-	-	-
PP_Pre[4]	67.5	30	2	0.25+0.25
PP_Post[5]	67.5	30	2	0.25+0.25
PP_0.5mm_Post	67.5	30	2	0.25+0.25

[1]Polypropylene (PP): MFI 18
[2]Coupling Agent (CA): Fusabond
[3]Antioxidant (AO): Irganox 1010 + Irgafos 168
[4]Pre-ensiled corn biomass
[5]Post-ensiled corn biomass

7.2.3.1 Extrusion and Injection Moulding

The formulations were hand-mixed and fed into a Haake Minilab Micro-compounder set with a co-rotating twin screw extruder (Thermo Scientific, Waltham MA, USA) at 190^{o}C or 220^{o}C (as a heat stress treatment) and 40 rpm. The compounded material was collected, cooled and pelletized. The pellets were injection moulded using a Ray Ran Laboratory Injection Moulding apparatus (Ray Ran Test Equipment Ltd., UK) set at 190^{o}C and a pressure of 100 psi into a mold tool set at a temperature of 50^{o}C to obtain test specimens according to ASTM standards D790 (flexural testing) and D256 (notched Izod impact testing). Non-extruded polypropylene was included as a control to ensure that extrusion conditions and the presence of antioxidants did not have a significant effect on the mechanical properties of the matrix. The test bars were annealed to remove the thermal history in a Hewlett Packard 5890A GC oven (Agilent Technologies, Santa Clara CA, USA) with a heating rate of 10^{o}C per minute up to 150±1^{o}C and held for 11 min before ramping down to room temperature.

7.2.3.2 Differential Scanning Calorimetry

A Q2000 Differential Scanning Calorimeter (TA Instruments, New Castle DE, USA) was used to determine melting point, crystallization peaks and enthalpy of the phase transition of the composite with the following cycling conditions in a nitrogen environment: samples were equilibrated at 35^{o}C, heated to 210^{o}C at a ramp of 20^{o}C/min and held for 5 minutes (cycle 1), cooled to 35^{o}C at a ramp of 10^{o}C/min and held for 5 minutes (cycle 2), and finally ramped to 210^{o}C at a rate of 10^{o}C/min (cycle 3). Cycles 2 and 3 were used to analyze the thermal properties of the composites by plotting the data using the TA Universal Analysis software (TA Instruments, New Castle DE, USA). The degree of crystallinity of the composites was calculated as:

$$\% = \frac{\Delta H}{\Delta H^{o}(\text{wt\% filler})} \times 100$$

where ΔH represents the heat of enthalpy required to melt the resin and ΔH^o is the heat of fusion of 209 J/g for 100% crystalline polypropylene (Marinelli and Bretas, 2003).

7.2.3.3 Mechanical Properties

Test specimens were conditioned at a temperature of 23±2^{o}C and relative humidity of 31% for over 48 hours prior to testing. Flexural properties of six specimens per formulation were measured using the Three-Point-Bending test following ASTM D790 using a Q Series Mechanical Test Machine (Test Resources Inc., Shakoppe MN, USA). Notched Izod impact testing was performed according to ASTM D256. Six specimens were notched at 45±1^o and a depth of 2.5 mm using a milled notch cutter. Bars were notched prior to the annealing described in 7.2.3.1. Bars were mounted on a Monitor Impact Tester (Testing Machines Inc., New Castle DE, USA) and notched Izod impact was determined using a swinging pendulum type hammer (Izod Hammer 0-1 X 0.01 FT Lbs, TMI 43-0A-03) at 90^o. All testing was performed at room temperature.

The fiber-matrix interface was characterized using a Zeiss Ultra-Plus field emission scanning electron microscope (SEM) (Carl Zeiss, Oberkochen, Germany). The samples were mounted on metal stubs using double-sided conductive tape and the surface was gold-coated. Images were taken at 10 kV.

7.3 Results and Discussion

7.3.1 Characterization of Pre- and Post-Silage Corn Biomass

Freshly harvested and ensiled corn biomass was dried and ground before thermal and compositional characterization. During processing, post-silage corn was observed to have a different overall particle size distribution than its non-ensiled counterpart. A preliminary particle size analysis by light microscopy using ImageJ indicated that, in fact, the aspect ratios (ratio of length versus width) of the pre- and post-silage corn biomass showed

variation. The main differences were found to be at both ends of the size spectrum. In the aspect ratio range of over 5.0, non-processed corn had close to double the frequency of occurrence than post-silage corn; however, the ensiled corn had a higher quantity of particles in the range of 1.0 to 2.0 (Figure 7.2). This qualitative observation was confirmed by size fractionation of 10 randomly selected pre- and post-silage samples (raw data available in Appendix I). By sieving the biomass material, the weight percentages of the > 1.0 mm, 0.5 to 1.0 mm, and < 0.5 mm fractions were determined. Every pre-silage corn sample tested had a higher percent content of particles > 1.0 mm ($P = 0.00204$) (Figure 7.3). Post-silage corn had a higher content of particles between 0.5 and 1.0 mm ($P = 0.0346$), and < 0.5 mm ($P = 0.00112$). Particle size of corn silage can be affected by chop length, hybrid, maturity, and equipment processing (Johnson *et al.*, 2002). The variation in particle size distributions may be also a result from the combination of an increase in microbial biomass from the silage fermentation as well as a decrease in plant biomass from the processing activities of the bacterial and fungal populations.

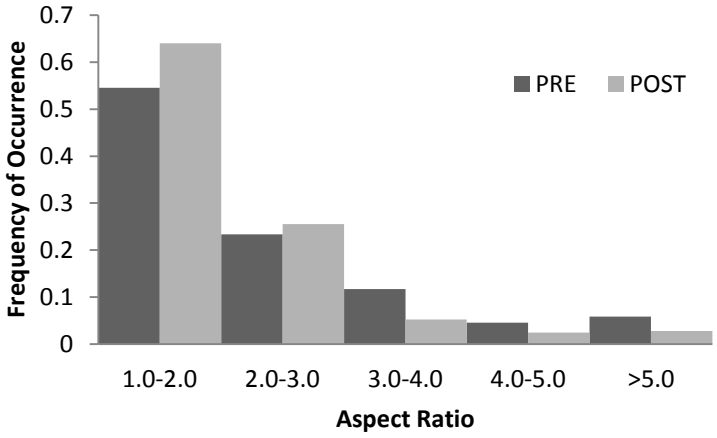


Figure 7.2 Particle size distribution analysis based on the aspect ratios of particles from one representative pre- and post-silage corn biomass sample.

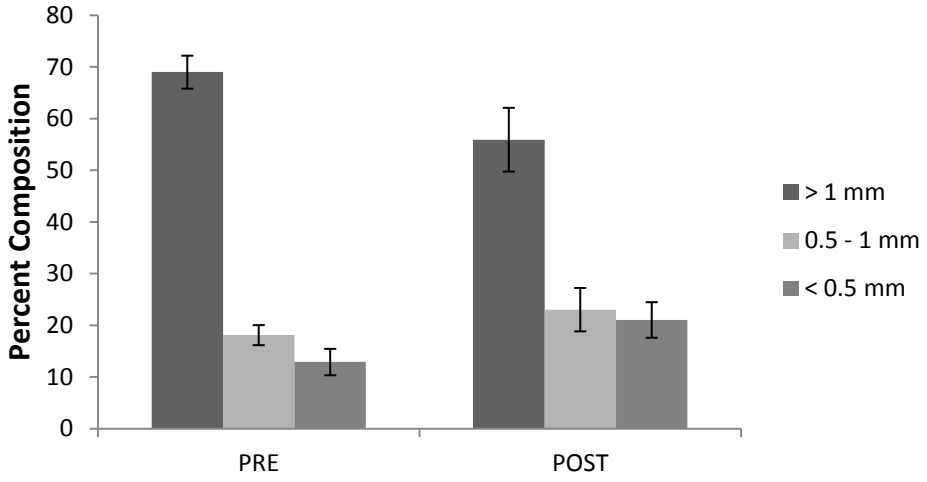


Figure 7.3 Average size distributions of 10 randomly selected and size fractionated pre- and post-silage corn biomass by weight percent.

7.3.2 Thermal Characterization of Pre- and Post-Ensiled Corn Biomass

An overall comparison of the thermal properties of the corn biomass is presented in Table 7.4 (the raw data is included in Appendix F). A 2-tailed t-test revealed significant differences between 0.5 and 2 wt%/min onsets of degradation. However, a statistical analysis based on a

Table 7.4 Overall comparisons of the thermal characteristics of pre- and post-silage corn determined by thermogravimetric analysis.

Treatment		T5 (°C)	T10 (°C)	Onset of degradation (wt%/min) 0.5	1	2	Peak temperature (°C)	Max. rate of degradation (wt%/min)
Pre	Average	190.04	252.22	199.26	236.34	258.64	311.72	7.92
	St. dev.	35.25	12.47	15.09	9.48	3.10	9.01	0.98
Post	Average	201.34	252.61	179.69	233.84	255.67	310.74	7.83
	St. dev.	25.03	10.70	25.51	12.19	4.17	8.03	0.94
	P-values	0.181	0.901	0.001	0.404	0.005	0.677	0.745

2^3 factorial design showed that location according to plant hardiness zone (5a (-1) →5b (1)), inoculant use (No (-1) →Yes (1)) and treatment (Pre (-1) → Post (1)), were factors that had impacts when comparing individual pre- and post-silage samples (the data analysis is included in Appendix H). For example, zone 5b showed an increase T10 temperature (temperature at 10% weight loss) (T10 = 254.17 + 3.64 * Zone). Maximum rate of degradation and 2.0 wt%/min onset of degradation were both influenced by an interaction between zone and inoculant use, although the maximum rate of degradation did not vary significantly with individual terms, represented respectively by the following models:

- Peak rate = 8.06 + 0.19 * Zone - 0.15 * Inoculant use + 0.33 * Zone * Inoculant use
- 2.0 wt%/min Onset Temp = 257.91 + 0.82 * Zone - 0.38 * Inoculant used - 1.48 * Treatment + 1.39 * Zone * Inoculant used

As indicated from the overall statistical analysis, fermentation had a significant impact on 0.5 and 2 wt%/min onsets of degradation:

- 0.5 wt%/min Onset Temp = 193.13 + 8.10 * Zone + 0.95 * Inoculant used - 11.60 * Treatment + 6.99 * Inoculant used * Treatment
- 2.0 wt%/min Onset Temp = +257.90690 + 0.82424 * Zone - 0.37527 * Inoculant used - 1.48 * Treatment + 1.39 * Zone * Inoculant used

When including silo type (Bunker (-1) → Tower (1)) and performing a 2^4 factorial design analysis, where significant, material collected from tower silos outperformed that of bunker silos. For example, corn from the tower silo had higher T5 and T10 temperatures than corn from the bunker silos:

- T5 = 185.41 - 0.83 * Zone + 10.70 * Treatment + 16.51 * Bunker vs Tower + 11.85 * Zone * Treatment
- T10 = 247.24 + 8.13 * Bunker vs Tower

Based on the lower onsets of degradation of microbially processed corn biomass, silage fermentation did not appear to improve the thermal stability of the treated corn. The sample showing the largest variation in thermal properties between the pre- and post-silage treatment was selected as the filler material for biocomposite production and testing. It was expected that this marked variation would contain the potential gamut of mechanical properties that would result from using pre- and post-silage corn biomass as filler material in polypropylene. Based on its thermal behaviour, sample number 3 (37K84 Pioneer Poncho RR, Woodstock, Tower silo, Pioneer BioMax inoculant) was selected for further processing. This sample's thermal characteristics are presented in Table 7.5.

Table 7.5 Thermal characteristics of sample 3 (37K84 Pioneer Poncho RR, Woodstock, Tower silo, Pioneer BioMax inoculant) pre- and post-silage, selected for further processing.

			Onset of degradation (wt%/min)			Peak temperature	Max. rate of degradation
Treatment	T5 (^{o}C)	T10 (^{o}C)	0.5	1	2	(^{o}C)	(wt%/min)
Pre	133.20	237.10	190.85	93.03	245.64	308.45	62.47
Post	248.80	273.20	227.36	96.61	246.51	302.62	69.24

When comparing the thermogravimetric plots of the size fractions, material from the < 0.5 mm size range showed higher thermal stability in the pre- and post-silage corn (Figure 7.4). This size fraction, most likely representing highly processed plant and microbial biomass resulting from bacterial division during the fermentation, was also included in the composite formulation and testing given its optimal thermal behaviour.

7.3.3 Compositional Analysis of Pre- and Post-Ensiled Corn Biomass

Composition of ensiled corn biomass had significant differences ($P < 0.05$) in numerous components tested, including an increase in soluble crude protein, total digestible nutrients, calcium, potassium, relative feed value, starch, total ash and crude fat (Table 7.6) (the raw

data is presented in Appendix G). Ensiled samples had significant decreases in phosphorus and sodium, as well as the plant cell wall components cellulose and hemicellulose. Lignin (1.65 wt%) and starch (33.90 wt%) showed no differences between pre- and post-silage corn. Statistical analysis by factorial design quantified treatment effect (Pre (-1) → Post (1)) on the composition:

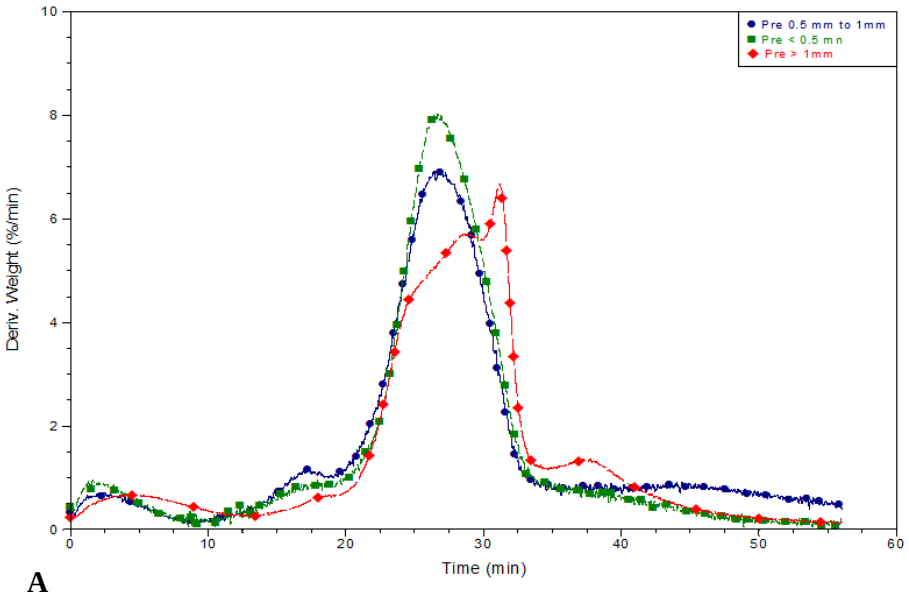
- Cellulose content (wt%) = 17.59 - 1.66 * Treatment
- Hemicellulose content (wt%) = 18.72 - 1.76 * Treatment
- Ash content (wt%) = 3.85 + 0.50 * Treatment
- Crude fat (wt%) = 2.13 +0.31 * Treatment

There were no differences attributed to zone or use of inoculants.

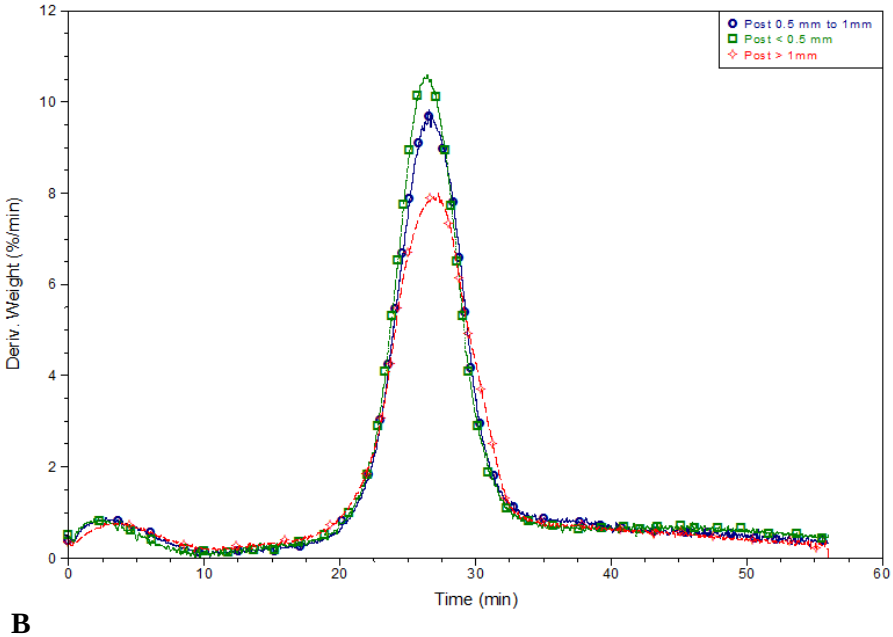
The lower cellulose and hemicellulose content of ensiled corn may have resulted in the lower thermostability of the processed biomass. The activation energy for the thermal degradation of cellulose, hemicellulose and lignin is in the range of 36-60, 15-26 and 13-19 kcal/mol, respectively (Ramiah, 1970).

7.3.4 Thermal Characterization of Biocomposites by DSC

DSC analysis of the homopolymer PP was within results reported for a PP of similar melt flow index (MFI) (Mohan Sharma, 2012). Addition of 30% corn biomass to the PP was found to increase the crystallization temperature (T_c) from an average of 115.26^{o}C to an average of 118.46^{o}C (P = 0.000217) (Table 7.7). PP reinforced with biomass from the <0.5 mm size fraction showed the highest T_c of 119.42^{o}C. This increase in T_c may be attributed to the added crystallinity provided by the crystalline cellulose.



A



B

Figure 7.4 Thermogravimetric plots of size fractions from (A) pre- and (B) post-silage corn biomass (♦/✧ > 1 mm, ●/○ 0.5 to 1 mm, ■/□ < 0.5 mm).

Table 7.6 Percent weight composition of all 30 pre- and post-silage corn biomass samples collected were determined by near infrared spectroscopy. Shaded P-values show significant differences ($P < 0.05$).

Plant Component % Weight		Pre-Silage	Post-Silage	P-value
Cellulose		19.26(3.22)	15.93(2.16)	3.83E-05
Hemicellulose		20.48(3.85)	16.96(1.83)	9.15E-05
Lignin		1.64(0.45)	1.66(0.52)	0.934
Starch		32.00(5.89)	35.81(5.46)	0.015
Total Ash		3.35(0.86)	4.35(0.76)	2.42E-05
Crude Fat		1.82(0.53)	2.44(0.38)	6.44E-06
Crude Protein		8.90(0.95)	8.61(0.55)	0.168
Soluble Crude Protein		37.75(5.98)	45.27(4.33)	2.00E-06
Minerals				
	Calcium	0.32(0.05)	0.36(0.03)	2.82E-03
	Phosphorus	0.22(0.01)	0.20(0.03)	2.12E-03
	Potassium	0.66(0.12)	0.41(0.17)	1.35E-06
	Magnesium	0.16(0.02)	0.16(0.03)	0.975
	Sodium	0.04(0.04)	0.03(0.01)	0.037

Table 7.7 Thermal characterization of pre- and post-silage corn biomass-reinforced polypropylene (PP) biocomposites by dynamic scanning calorimetry.

Composite	T_c (^oC)	ΔH_c (J/g)	T_m (^oC)	ΔH_m (J/g)	$\%C_c$
PP	115.47	95.67	159.40	71.13	46.01
PP Non-extruded	115.43	96.57	161.83	72.05	46.21
PP (220^{o}C)	114.87	97.13	159.67	80.92	46.71
Pre (190^{o}C)	118.40	59.53	160.74	49.41	42.20
Pre (220^{o}C)	116.85	71.57	160.82	59.30	50.73
Post (190^{o}C)	117.99	71.23	161.92	59.30	50.49
Post (220^{o}C)	118.67	69.25	160.86	57.95	49.09
Post <0.5 mm (190^{o}C)	119.64	65.89	161.20	54.40	46.71
Post <0.5 mm (220^{o}C)	119.19	67.16	161.75	55.94	47.61

The corn biomass yielded an overall decrease in the heat of enthalpy of crystallization and melting point (ΔH_c and ΔH_m). This decrease was most pronounced in material produced with pre-silage corn biomass processed at 190^{o}C. However, PP reinforced with this material showed a10^{o}C increase in both ΔH_c and ΔH_m, reaching levels of the biocomposites reinforced with the post-silage biomass. Material reinforced with the post-silage corn biomass showed the highest overall % crystallinity. This increase may have been a result of the addition of a coupling agent (Kim *et al.*, 2007). Antioxidants present in the extruded PP did not affect the thermal properties of the plastic in comparisons with those of the non-extruded pure PP. There was no difference in the melting temperatures of the biocomposites in comparison to those of the pure matrix, indicating that there was minimal bonding occurring at the polymer fibre interface (Yang *et al.*, 2009).

7.3.5 Mechanical Properties of Biocomposites

The flexural strength of biocomposites was determined and compared to the pure PP (Figure 7.5). The average flexural strength for the pure homopolymer PP was found to be 41.4 MPa, significantly lower than the reported 50.0 MPa for PP homopolymer (Mohan Sharma, 2012). There were no differences in the flexural strength of the PP standards extruded at 190^{o}C, 220^{o}C and non-extruded, indicating that extrusion and heat treatment did not impact this mechanical property for the pure PP. It should also be noted that only one operator was involved in the testing to minimize the potential for variability.

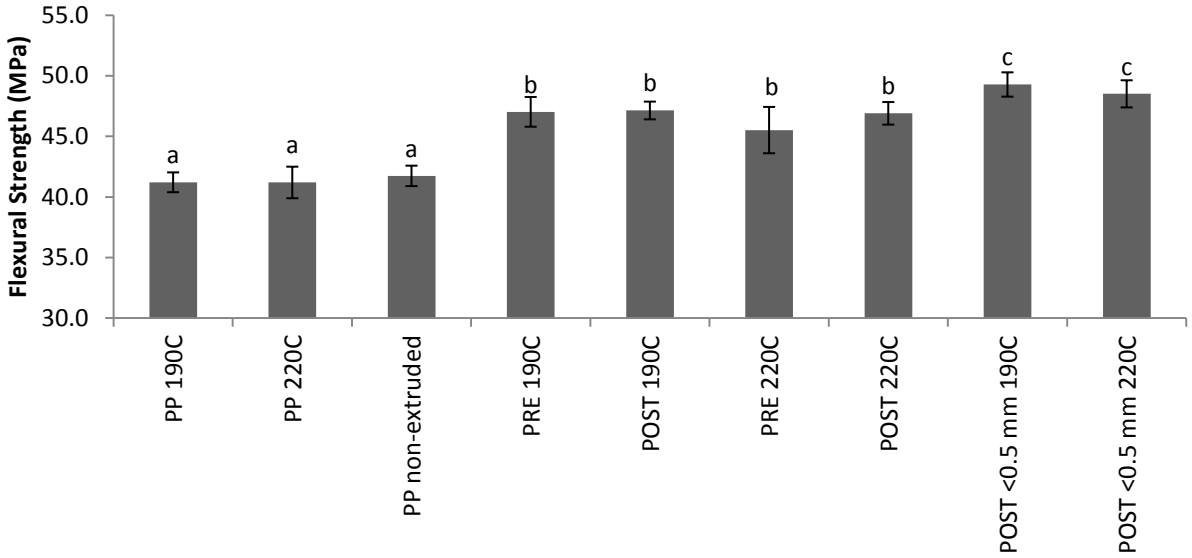


Figure 7.5 Comparison of the flexural strength of pre- and post-silage corn biomass-reinforced polypropylene (PP) biocomposites. Processing temperatures during extrusion (190^{o}C or 220^{o}C) are included. Lower-case letters denote statistically significant differences ($P < 0.05$).

Biocomposites prepared with corn biomass pre- and post-silage showed an increase in comparisons to the pure PP, but did not vary based on treatment or extrusion temperature. Increases ranged from 10.5 to 14.4% for pre-silage and post-silage corn at 190^{o}C, respectively. Formulations prepared with the <0.5 mm size fractions had a higher flexural strength than the pure resins (18.7%) and their whole plant biomass-PP counterparts, potentially as a result of better particle distribution. The overall average of the 30% filler biocomposites was 47.4 MPa, an 8% decrease than the equivalent formulation of 30% wheat straw-homopolymer PP (Mohan Sharma, 2012). However, the flexural strength of the pure resin and biocomposites are found to be within the range reported by Panthapulakkal and Sain (2006) for PP MFI 12 reinforced with 30% wheat straw. Using whole corn biomass as the filler produced biocomposites that had decrease 23% decrease in flexural strength in comparison to those reinforced with 38N85 Roundup Ready cornstalk-only material, indicating that, when available, cornstalk would provide a more suitable reinforcement for

PP. This observation may be attributed to the overall lower cellulose content in whole corn biomass, which was found to be 37% lower than cornstalk-only material.

The flexural modulus of biocomposites was higher than the pure PP standards (Figure 7.6). The average modulus of the pure matrix composite was 0.943 GPa, which is lower than the manufacturer's reported 1.31 GPa (Braskem America, Inc., Houston TX, USA). This difference may be due to batch to batch variation. Material reinforced with pre-silage corn biomass resulted in increases in the flexural modulus ranging from 44.0 to 49.0% when processed at 190^{o}C and 220^{o}C, respectively. The percent increase ranged from 36.6 to 52.7% for post-silage corn when processed at 190^{o}C and 220^{o}C, respectively. The significant increase in flexural modulus of biocomposites processed at 220^{o}C with post-silage corn shows that ensiled material may be better suited for reinforcing thermoplastics with a higher melting temperature than polypropylene. As indicated with the flexural strength, the flexural modulus of biocomposites produced with 38N85 Roundup Ready cornstalk material was 50% higher than those formulated with whole corn biomass. Homopolymer PP reinforced with 30% wheat straw produced biocomposites with a flexural modulus of 1.94 GPa (Mohan Sharma, 2012), significantly higher than the modulus of bars produced with whole corn biomass. These results are again closer to the ranges reported by Panthapulakkal and Sain (2006) for non-reinforced PP MFI 12.

Impact strength of the pure resins determined by the notched Izod impact test showed variation between processing temperatures (Figure 7.7). Given the difference between the impact strength of PP extruded at 190^{o}C and 220^{o}C, the corn biocomposites were compared to their respective standard temperature counterparts. Material prepared with post-silage corn biomass and extruded at 190^{o}C showed an increase in impact strength at 7.6%. There were no statistically significant differences in materials extruded at 220^{o}C or prepared with < 0.5 mm particle size. However, a comparison of the biocomposites reinforced with post-silage processed at 220^{o}C and its < 0.5 mm equivalent yielded an increase of 16.2% ($P = 0.0257$).

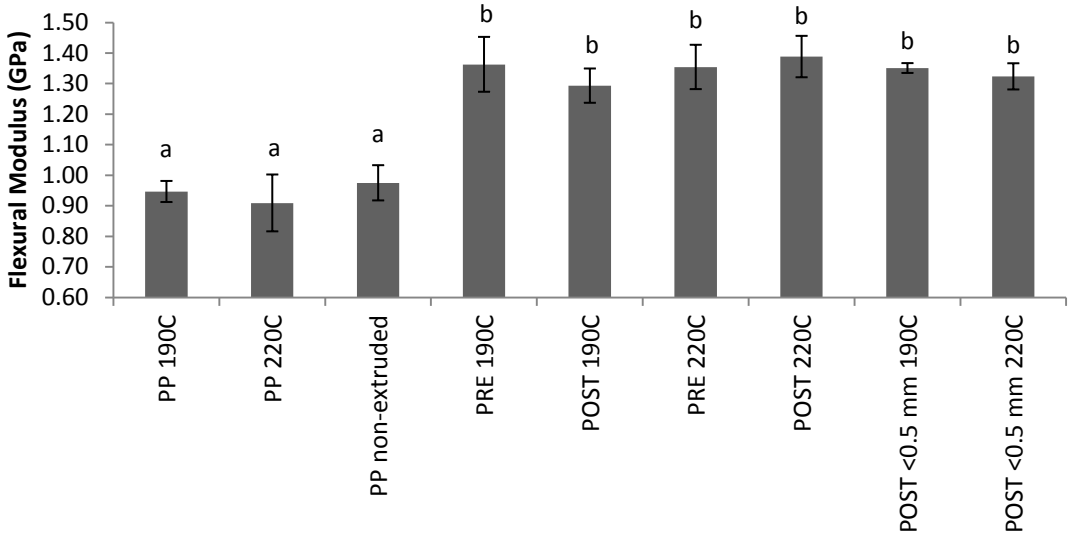


Figure 7.6 Comparison of the flexural modulus of pre- and post-silage corn biomass-reinforced polypropylene (PP) biocomposites. Processing temperatures during extrusion (190^{o}C or 220^{o}C) are included. Lower-case letters denote statistically significant differences ($P < 0.05$).

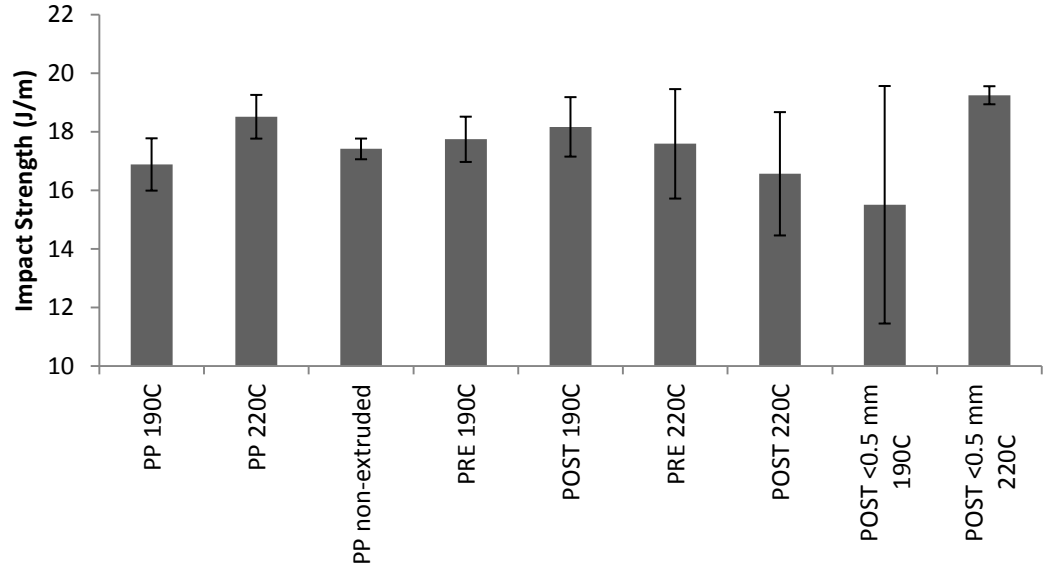


Figure 7.7 Comparison of the impact strength of pre- and post-silage corn biomass-reinforced polypropylene (PP) biocomposites. Processing temperatures during extrusion (190^{o}C or 220^{o}C) are included.

As with the flexural strength and modulus, impact strength of the pure homopolymer PP was found to be lower than that reported by Mohan Sharma (2012), with an average decrease of 22.7%. The reported decrease in impact strength of filler-homopolymer PP biocomposites (Mohan Sharma, 2012) was not observed in corn biomass-homopolymer PP material. These observations may indicate that the filler had a morehomogeneous distribution, perhaps in relation to the wide particle size distribution. Whole corn biomass resulted in a 44% drop in impact strength when compared to a 38N85 Roundup Ready cornstalk-reinforced PP.

In comparison to bast fibre-reinforced polypropylene, corn biomass and cornstalk material produced mechanical properties closest to the range of core hemp fibres (flexural strength of 52 MPa, and notched Izod impact strength of 20 J/m) (Faruk *et al.*, 2012). The higher flexural strength and modulus of corn biomass-reinforced PP indicate the potential attributes of this agro-material, especially as filler material. Ensiled corn may also provide an industrial feedstock throughout the year if supply of other plant materials is waning. Although the silage treatment did not result in an overall improvement of mechanical properties in comparisons with non-ensiled corn biomass, the increase in < 0.5 mm size fraction presents interesting features. This size fraction is thought to include highly processed plant material as well as cell biomass. The microbial spores are especially evident when looking at the SEMs of materials produced with pre-silage and post-silage corn biomass (Figure 7.8). Based on the smaller aspect ratio, biocomposites produced with this fraction were expected have lower mechanical properties. However, the higher thermal stability may have reduced the filler's degradation during processing, especially at high processing temperatures, resulting in its higher flexural and impact strength.

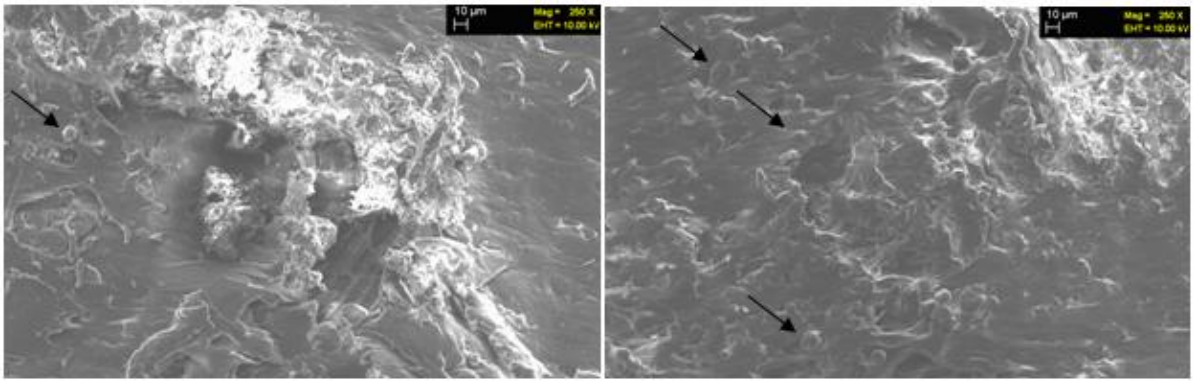


Figure 7.8 Scanning electron microscope images at 250X magnification of (A) pre-silage corn, and (B) post-silage corn biomass-polypropylene biocomposites. Microbial spores are indicated by arrows.

7.4 Conclusions

Thermal behaviour of processed corn biomass is influenced by location according to plant hardiness zone, use of inoculant, and silage treatment when comparing individual pre- and post-silage samples. Silage fermentation is the main factor responsible for variability in biomass composition. Although thermostability and compositional analysis indicated that the silage fermentation process may be detrimental to the corn biomass for use in biocomposites, biocomposites produced with pre- and post-silage corn did not show significant differences in mechanical properties. In fact, ensiled corn resulted in an increase in smaller particle sizes with more optimal thermostability and purity. This highly processed corn and microbial biomass fraction may present better mechanical properties in higher melting thermoplastics and more rigorous processing conditions as a result of its already small aspect ratio. Given the increase in flexural properties without the characteristic decrease in impact strength of corn biomass-reinforced polypropylene, this agro-material may present a potential alternative to other plant fibres, especially as filler material. Cornstalk produced significantly higher flexural strength and modulus as well as impact strength in comparison with whole corn biomass, potentially as a result of decreased overall cellulose content. These results indicate that the lignocellulosic cornstalk material would provide more suitable reinforcement characteristics.

Acknowledgements

Funding from the Ontario BioCar Initiative is gratefully acknowledged. Rachel Campbell Murdy was a recipient of an NSERC postgraduate scholarship during the course of this work. We are especially grateful to the farmers who participated in the study. In addition, the authors also thank Dr. Barbara Guettler and Dr. Mohammad Arif for their assistance with composite preparation and testing.

Chapter 8
Conclusions and Recommendations

8.1 Principle Objectives and their Respective Outcomes

The overall objective of this research was to examine silage fermentation as a potential biological fibre extraction technique. The specific sub-objectives were:

A) To investigate the corn processing ability of the hemp retting agents *Clostridium felsineum* and *Bacillus subtilis*.

B) To determine the changes in microbial populations during silage fermentation using community-level physiological profiling and enzyme production.

C) To explore volatile organic acid production in corn silage retting effluent as a potential value-added source of organic acids.

D) To assess the impact of corn variety on thermal characteristics and composition of plant fibres, in addition to mechanical properties of polypropylene reinforced with 3 corn isolines.

E) To evaluate the potential for large scale applications of ensiled corn biomass as a natural fibre feedstock using farm infrastructure in a field trial across Southwestern Ontario.

8.1.1 *Clostridium felsineum* and *Bacillus subtilis* as Corn Retting Agents (Thesis Objective A)

Chapter 3 summarizes preliminary research showing that *C. felsineum* is an effective corn retting agent, with *B. subtilis* contributing to the retting process by reducing the oxygen content and providing the required anaerobic conditions for clostridial growth. Indigenous

bacteria also showed some retting ability. In addition to bacterial treatment, absence of corn grain also resulted in fibres with a higher cellulose purity and higher onsets of degradation potentially due to a reduction in available sugars.

8.1.2 Community-Level Physiological Profiling and Enzyme Production during Silage Retting (Thesis Objective B)

Chapter 4 describes the potential use of CLPP as an analytical tool to detect differences in silage microbial community dynamics as a result of crop management, plant genotype and presence of spore-forming spoilage organisms. Enzyme activity profiling was also effective in monitoring activity of the silage communities, although CLPP provided a broader understanding of community shifts from a metabolic perspective. Metabolic divergences between microbial communities were time-, region-, and contaminant-sensitive. This regional microbial population variability indicates that the indigenous community in certain regions may out-compete bacterial inoculants and spoilage organisms on cornstalk material. The activity and stability of microbial communities of the phyllosphere before harvest could be monitored by CLPP to increase the predictability of the silage fermentation and produce higher quality silage by determining the resilience of the indigenous populations to introduced organisms.

8.1.3 Volatile Organic Acid Production in Corn Silage Retting Effluent (Thesis Objective C)

Chapter 5 examines the production of volatile organic acids in silage retting effluent as potential value-added by-products. Anaerobic conditions were found to produce the highest levels of acetic and butyric acid. Ensiled corn hybrid varieties were also found to result in the production of different organic acids, although a specific variety was not identified as most or least favourable due to high variability.

8.1.4 Impact of Corn Variety: Characterization of Plant Fibres and Mechanical Properties of Biocomposites (Thesis Objective D)

Chapter 6 describes the thermostability and composition of plant fibres isolated from three corn isolines. Mechanical properties of corn fibre-reinforced polypropylene biocomposites are also outlined. Composition, thermostability and mechanical properties of the biocomposites were all found to vary depending on the variety of corn. Although *Bt*-RR cornstalk material was found to have higher cellulose content and better thermostability than its genetic RR and conventional counterparts, fibres from the RR corn isoline produced biocomposites with the highest flexural strength and modulus. All biocomposites showed higher impact strength than pure polypropylene with no differences between isolines.

8.1.5 Corn Silage Retting as a Biological Fibre Extraction Technique: Field Trial (Thesis Objective E)

Chapter 7 outlines the results of a field trial exploring corn silage retting as a new biological fibre extraction technique. The thermostability of ensiled corn biomass was found to be influenced by region, use of inoculants and silage treatment. However, silage fermentation accounted for most of the variability in corn biomass composition. Given that ensiled corn material had lower thermostability and less cellulose content, its potential use as a reinforcement material was questioned. Yet biocomposites produced with pre- and post-silage corn did not show significant differences in mechanical properties. In fact, ensiled corn resulted in an increase in smaller particle sizes with more optimal thermostability and purity. This material's already small aspect ratio could result in better mechanical properties in thermoplastics with a higher melting temperature or composites requiring higher shear for mixing. Whole corn biomass presents a potential alternative to other plant fibres, especially as filler material.

8.1.6 Contributions to Knowledge

This thesis presents newfindings in the inter-disciplinary fields of microbiology, plant science, chemical engineering and composite materials. Retting as a biological fibre extraction technique has so far been limited to bast fibres, mostly from hemp and flax. This is the first research of its kind to demonstrate the effectiveness of retting agents in separating the vascular bundles containing valuable fibres from the pith of cornstalks. The results of this research indicate that bacteria, both introduced as inoculants or present as indigenous microorganisms, may offer plant fibre processing activities in non-traditional settings.

There is limited information on the properties of cornstalk- and corn biomass-reinforced biocomposites, especially in the area of transgenic hybrids. This thesis presents research that indentifies the potential influence of pleitropic effects or crop management practices in producing plant fibres with significant variability. This could have serious implications for the current and future use of plant fibres as reinforcement or filler material in industrial applications, particularly for replication purposes.

Finally, mechanical testing of cornstalk- and corn biomass-reinforced polypropylene biocomposites indicates this feedstock's potential use as reinforcement material. Research to date has mostly shown that corn could be used as filler in biocomposites, imparting little to no improvement in mechanical properties. Cornstalk, especially, was found to result in a significant enhancement of the flexural strength, flexural modulus and impact strength in comparisons to polypropylene composites. Whether this increase in properties was attributed to the nature of the fibres or the use of a coupling agent during processing, cornstalk material warrants a closer investigation as a potential source of reinforcement in biocomposites manufacturing.

8.2 Recommendations

This thesis describes the effectiveness of *C. felsineum* and *B. subtilis* as corn biomass pre-processing agents. The retting ability of these two bacteria was dependent on (1) hybrid variety either as a result of crop management practices or pleiotropic effect of genetic alterations, and (2) region based on the ability of the introduced organisms to out-compete the native microflora. The processing activity of the bacteria was characterized by analyzing the thermostability and the composition of the plant material. Cornstalk-only and whole corn biomass were used as filler/reinforcement in the production of polypropylene biocomposites. Due to its higher lignocellulosic content, cornstalk was found to produce higher strength materials.

Recommendations for future work include:

1) Ensiling cornstalk-only plant biomass to study the impact of the silage fermentation on this lignocellulosic material. Given that *C. felsineum* and *B. subtilis* were found to have higher retting ability on plant material absent of grain, it would be interesting to determine the microorganisms' effect at a larger scale.

2) Examine the properties of plant fibres from a greater diversity of corn isolines from a larger geographic area. The *Bt*-RR corn isoline was found to have better thermostability and composition characteristics, although its RR genetic equivalent was found to produce biocomposites with more flexural strength. Further research exploring other isolines would be useful to determine whether these observations were representative of hybrid varieties in general and to predict the variability that could be expected when using cornstalk as an industrial reinforcement material.

3) Investigate the mechanical properties of cornstalk and whole corn biomass biocomposites in a larger variety of thermoplastics to determine whether polymers with higher melting temperatures could also be reinforced with corn fibres.

4) Explore the use of microbial cell biomass as potential filler/reinforcement material in biocomposites given its apparent high purity and thermostability, in addition to its potential processing range (high temperature, high shear).

References

Adapa, P.K., Tabil, L.G., Schoenau, G.J., Canam, T., and Dumonceaux, T. 2011. Quantitative analysis of lignocellulosic components of non-treated and steam exploded barley, canola, oat and wheat straw using Fourier transform infrared spectroscopy. J. Agric. Sci. Technol. B 1:177-188.

Aehle, W. 2004. Enzyme in industry: production and application, "industrial enzymes". Weinheim: Wiley-VCH verlag.

Akin, D.E., Condon, B., Sohn, M., Foulk, J.A., Dodd, R.B., and Rigsby, L.L. 2007. Optimization for enzyme-retting of flax with pectate lyase. Ind. Crop Prod. 25:136-146.

Akin, D.E., Foulk, J.A., Dodd, R.B., and McAlister III, D.D. 2001. Enzyme-retting of flax and characterization of processed fibers. J. Biotechnol. 89:193-203.

Akin, D.E., Rigsby, L.L., Henriksson, G., and Eriksson, K.-E.L. 1998. Structural effects on flax stems of three potential retting fungi. Textile Res. J. 68:515-519.

Akin, D.E., Slomczynski, D., Rigsby, L.L., and Eriksson, K.-E.L. 2002. Retting flax with endopolygalacturonase from *Rhizopus oryzae*. Textile Res. J. 72:27-34.

Alves, C., Ferrão, P.M.C., Silva, A.J., Reis, L.G., Freitas, M., Rodrigues, L.B., and Alves, D.E. 2010. Ecodesign of automotive components making use of natural jute fiber composites. J. Clean. Prod. 18:313-327.

Amador J.A., and Görres, J.H. 2007. Microbiological characterization of the structures built by earthworms and ants in an agricultural field. Soil Biol. Biochem. 39:2070-2077.

Anderson, T. 2003. Microbial eco-physiological indicators to assess soil quality. Agric. Ecosyst. Environ. 98:285-293.

Araújo, W.L., Marcon, J., Maccheroni Jr., W., Van Elsas, J.D., Van Vuurde, J.W.L., and Azevedo, J.L. 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl. Environ. Microbiol. 68:4906-4914.

AUTO21. 2011. AUTO21: Innovation through research excellence. Accessed March 4, 2013. Available at http://www.auto21.ca/en/

Banik, S., Basak, M.K., Paul, D., Nayak, P., Sardar, P., Sil, S.C., Sanpui, B.C., and Gosh, A. 2003. Ribbon retting of jute A prospective and ecofriendly method for improvement of fibre quality. Ind. Crop Prod. 17:183-190.

Bentley, R. 1990. The shikimate pathway - a metabolic tree with many branches. Crit. Rev. Biochem. Mol. Biol. 25:307-383.

Bernfeld, P. 1955. Amylases α and β, in: Colowick, S.P., Kaplan, N.O. (Eds.), Methods in Enzymology. Vol. 1., Academic Press, New York, pp. 40–58.

BioAuto Council. 2011. Ontario BioAuto Council: Investing in bio-based materials and manufacturing. Accessed March 4, 2013. Available at http://www.bioautocouncil.com/default.aspx

BioCar. 2011. About the Initiative. Accessed March 4, 2013. Available at http://www.bioproductsatguelph.ca/biocar/about/

Bisanda, E.T.N. 2000. The effect of alkali treatment on the adhesion characteristics of sisal fibres. Appl. Compos. Mater. 7:331-339.

Bisanda, E.T.N., and Ansell, M.P. 1991. The effect of silane treatment on the mechanical and physical properties of sisal-epoxy composites. Compos. Sci. Technol. 41:165-178.

Bismarck, A., Mishra, S., and Lampke, T. 2005 Plant fibers as reinforcement for green composites, in: Mohanty, A.K., Misra, M., and Drzal, L.T. (Eds), Natural Fibers, Bio-polymers and their Composites. CRC Press, Boca Raton, Florida.

Blackwood, C.B., and Buyer, J.S. 2004. Soil microbial communities associated with Bt and Non-Bt corn in three soils. J. Environ. Qual. 33:832-836.

Blais, P., Carlsson, D.J., and Wiles, D.M. 1971. Effecst of corona treatment on composite formation. Adhesion between incompatible polymers. J. Appl. Polym. Sci. 15:129-143.

Bledzki, A.K., Faruk, O., and Sperber, V.E. 2006. Cars from bio-fibres. Macromol. Mater. Eng. 291:449-457.

Bledzki, A.K., Mamun, A.A., Jaszkiewicz, A., and Erdmann, K. 2010. Polypropylene composites with enzyme modified abaca fibre. Compos. Sci. Technol. 70:854-860.

Bledzki, A.K., Mamun, A.A., Lucka-Gabor, M., and Gutowski, V.S. 2008. The effects of acetylation on properties of flax fibre and its polypropylene composites. eXPRESS Polym. Lett. 2:413-422.

Bledzki, A.K., Reihmane, S., and Gassan, J. 1996. Properties and modification methods for vegetable fibers for natural fiber composites. J. Appl. Polym. Sci. 59:1329-1336.

Bolsen, K.K., Lin, C., and Brent, B.E. 1992. Effect of silage additives on the microbial succession and fermentation process of alfalfa and corn silage. J. Dairy Sci. 75:381-389.

Brotz, P.G., and Schaefer, D.M. 1987. Simultaneous determination of lactic and volatile fatty acids in microbial fermentation extracts by gas-liquid chromatography. J. Microbiol. Methods 6:139-144.

Brown, A.E., and Sharma, H.S.S. 1984. Production of polysaccharide-degrading enzymes by saprophytic fungi from glyphosate treated flax and their involvement in retting. Ann. Appl. Biol. 105:65-74.

Bucher, A.E., and Lanyon, L.E. 2005. Evaluating soil management with microbial community-level physiological profiles. Appl. Soil. Ecol. 29:59-71.

Callister, W.D. 2001, Fundamentals of materials science and engineering: an interactive e.Text, 5th edition, John Wiley & Sons Inc., NY.

Canale, A., Valente, M.E., and Ciotti, A. 1984. Determination of volatile carboxylic acids (C_1-C_{5i}) and lactic acid in aqueous acid extracts of silage by high performance liquid chromatography. J. Sci. Food Agric. 35:1178-1182.

Castaldini, M., Turrini, A., Sbrana, C., Benedetti, A., Marchionni, M., Mocali, S., Fabiani, A., Landi, S., Santomassimo, F., Pietrangeli, B., Nuti, M.P., Miclaus, N., Giovannetti, M., 2005. Impact of Bt corn on rhizospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. Appl. Environ. Microbiol. 71:6719-6729.

Chesson, A. 1978. The maceration of linen flax under anaerobic conditions. J. Appl. Bacteriol. 45:219-230.

Clarke, E.G.C., and Humphreys, D.J. 1971. Toxic factors in pea haulm silage effluent – The factors toxic to fish. J. Sci. Food Agric. 22:205-207.

Clarke, S., and Stone, R.P. 2004. How to handle seepage from farm silos. OMAFRA Factsheet, Edition 04-031. Ontario Ministry of Agriculture, Food and Rural Affairs.

Cochran, M.J., Windham, T.E., and Moore, B. 2000. Feasibility of industrial hemp production in Arkansas.

Der Bedrosian, M.C., Nestor Jr., K.E., Kung Jr., L. 2012. The effects of hybrid, maturity, and length of storage on the composition and nutritive value of corn silage. J. Dairy Sci. 95:5115-5126.

Di Candilo, M., Bonatti, P.M., Guidetti, C., Focher, B., Grippo, C., Tamburini, E., and Mastromei, G. 2010. Effects of selected pectinolytic bacterial strains on water-retting of hemp and fibre properties. J. Appl. Microbiol. 108:194-203.

Di Candilo, M., Ranalli, P., Bozzi, C., Focher, B., and Mastromei, G. 2000. Preliminary results of tests facing with the controlled retting of hemp. Ind. Crop. Pro. 11:197-203.

Directive 2000/53/EC of the European Parliament and of the Council of 18 September 2000 on end-of life vehicles. 2000. 34 pages.

Donaghy, J.A., Levett, P.N., and Haylock, R.W. 1990. Changes in microbial populations during anaerobic flax retting. J. Appl. Microbiol. 69:634-641.

Eichhorn, S.J., Baillie, C.A., Zafeiropoulos, N., Mwaikambo, L.Y., Ansell, M.P., Dufresne, A., Entwistle, K.M., Herrera-Franco, P.J., Escamilla, G.C., Groom, L., Hughes, M., Hill, C., Rials, T.G., and Wild, P.M. 2001. Review: Current international research into cellulosic fibres and composites. J. Mater. Sci. 36:2107-2131.

Elsas, J.D., Trevors, J.T., and Wellington, E.M.H. 1997. Modern Soil Microbiology, Marcel Dekker, New York, NY.

Faruk, O., Bledzki, A.K., Fink, H.-P., and Sain, M. 2012. Biocomposites reinforced with natural fibers: 2000-2010. Prog. Pol. Sci. 37:1552-1596.

Fernyhough, A., and Markotsis, M. 2011. Chapter 19: Long biofibers and engineered pulps for high performance bioplastics and biocomposites. Handbook of Bioplastics and

Biocomposites Engineering Application. Pilla, S. (Ed.), Scrivener Publishing LLC. Salem, Massachusetts.

Fischer, J.M., Buchanan-Smith, J.G., Campbell, C., Grieve, D.G., and Allen, O.B. 1994. Effects of forage particle size and long hay for cows fed total mixed rations based on alfalfa and corn. J. Dairy Sci. 77:217-229.

Fond, O., Matta-Ammouri, G., Petitdemange, H., and Engasser, J.M. 1985. The role of acids on the production of acetone and butanol by *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 22:195-200.

Friedrich, J., Cimerman, A., Steiner, W. 1989. Submerged production of pectolytic enzymes by *Aspergillus niger*: Effect of different aeration/agitation regimes. Appl. Microbiol. Biotechnol. 31:490–494.

Gadgil, N.J., Daginawala, H.F., Chakrabarti, T., and Khanna, P. 1995. Enhanced cellulase production by a mutant of *Trichoderma reesei*. Enz. Microbial. Technol. 17:942-946.

Galanos, E., Gray, K.R., Biddlestone, A.J., and Thayanithy, K. 1995. The aerobic treatment of silage effluent: Effluent characterization and fermentation. J. Agric. Engng. Res. 62:271-279.

Garcia-Jaldon, C. 1997. Caracterisation morphologique et chimique du chanvre (*Cannabis sativa*). Pretraitment a la Vapeur et Valorisation. These Universite Joseph Fourier-Grenoble I (Specialite: Chimie).

Garland, J.L. 1996. Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol. Biochem. 28:213-221.

Garland J.L., and Mills, A.L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. 57:2351-2359.

Garland, J.L., Mackowiak, C.L., and Zabaloy, M.C. 2010. Organic waste amendment effects on soil microbial activity in a corn-rye rotation: Application of a new approach to community-level physiological profiling. Appl. Soil Ecol. 44:262-269.

George, J., Sreekala, M.S., and Thomas, S. 2001. A review on interface modification and characterization of natural fiber reinforced plastic composites. Pol. Eng. Sci. 41:1471-1485.

Gibson, T., Stirling A.C., Keddie, R.M., and Rosenberger, R.F. 1958. Bacteriological changes in silage made at controlled temperatures. J. Gen. Microbiol. 19:112-129.

Goda, K., Sreekala, M.S., Gomes, A., Kaji, T., and Ohgi, J. 2006. Improvement of plant based natural fibers for toughening green composites-effect of load application during mercerization or ramie fibers. Composites Part A 37:2213-2220.

Grabber, J.H. 2005. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci. 45:820-831.

Graham, R.L., Nelson, R., Sheehan, J., Perlack, R.D., and Wright, L.L. 2007. Current and potential U.S. corn stover supplies. Agron. J. 99:1-11.

Green, S.J., Inbar, E., Michel Jr., F.C., Hadar, Y., and Minz, D. 2006. Succession of bacterial communities during early plant development: Transition from seed to root and effect of compost amendment. Appl. Environ. Microbiol. 72:3975-3983.

Harrigan, G.G., Ridley, W.P., Riordan, S.G., Nemeth, M.A., Sorbet, R., Trujillo, W.A., Breeze, M.L., and Schneider, R.W. 2007. Chemical composition of glyphosate-tolerant soybean 40-3-2 grown in Europe remains equivalent with that of conventional soybean (*Glycine max* L.) J. Agric. Food Chem. 55:6160-6168.

Hatfield, R.D., Grabber, J., Ralph, J., and Brei, K. 1999. Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes. J. Agric. Food Chem. 47:628-632.

He, X., Su, Y., Liang, Y., Chen, X., Zhu, H., and Wang, K. 2012. Land reclamation and short-term cultivation change soil microbial communities and bacterial metabolic profiles. J. Sci. Food Agric. 92:1103-1111.

Henriksson, G., Akin, D.E., Hanlin, R.T., Rodriguez, C., Archibald, D.D., Rigsby, L.L., and Eriksson, K.L. 1997. Identification and retting efficiencies of fungi isolated from dew-retted flax in the United States and Europe. Appl. Environ. Microbiol. 63:3950-3956.

Heuer, H., and Smalla, K. 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities, in: van Elsas, JD, Trevors, JT, Wellington EMH, (Eds), Modern Soil Microbiology. Marcel Dekker, New York. p. 353-373.

Hill, C.A.S., and Abdul Khalil, H.P.S. 2000. Effect of fiber treatments on mechanical properties of coir or oil palm fiber reinforced polyester composites. J. Appl. Polym. Sci. 78:1685-1697.

Hill, K., Swiecki, B., and Cregger, J. 2012. The bio-based materials automotive value chain. Center for Automotive Research. 92 pages.

Himmelsbach, D.S., Khalili, S., and Akin, D.E. 1998. FT-IR microspectroscopic imaging of flax (*Linum usitatissimum* L) stems. Cell Mol. Biol. (Noisy-Le-Grand) 44:99-108.

Himmelsbach, D.S., Khalili, S., and Akin, D.E. 2002. The use of FT-IR microspectroscopic mapping to study the effects of enzymatic retting of flax (*Linum usitatissimum* L) stems. J. Sci. Food Agric. 82:685-696.

Huda, M.S., Drzal, L.T., Mohanty, A.K., and Misra, M. 2008. Effect of chemical modifications of the pineapple leaf fiber surfaces on the interfacial and mechanical properties of laminated biocomposites. Compos. Interfaces 15:169-191.

Ikeda, S., Ytow, N., Ezura, H., Minamisawa, K., and Fujimura, T. 2006. Soil microbial community analysis in the environmental risk assessment of transgenic plants. Plant Biotechnol. 23:137-151.

Jofriet, J.C., and Daynard, T.B. 1988. Tower silo capacities, OMAFRA Factsheet, Edition 88-033. Ontario Ministry of Agriculture, Food and Rural Affairs.

Johnson, D.B., Moore, W.E., and Zank, L.C. 1961. The spectrophotometric determination of lignin in small wood samples. Tappi 44:793-789.

Johnson, L.M., Harrison, J.H., Davidson, D., Robutti, J.L., Swift, M., Mahanna, W.C., and Shinners, K. 2002. Corn silage management I: Effects of hybrid, maturity, and mechanical processing on chemical and physical characteristics. J. Dairy Sci. 85:833-853.

Johnston, V.J., Chen, L., Kimmich, B.F., Chapman, J.T., and Zink, J.H. 2008. Direct and selective production of ethanol from acetic acid utilizing a platinum/tin catalyst. Patent Publication Number US 7,863,489 B2.

Juska, C., Gearhart, J., and Griffith, C. 2006. 2006 Automotive Plastics Report Card – The policies and practices of eight leading automakers. Ecology Center.

Karus, M., Ortmann, S., and Vogt, D. 2004. Use of natural fibres in composites in the German automotive production 1996 till 2003. Nova Institut für Ökologie und Innovation.

Kent, A.D., and Triplett, E.W. 2002 Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu. Rev. Microbiol. 56:211-236.

Khalil, H.P.S.A., Ismail, H., Rozman, H.D., and Ahmad, M.N. 2001. Effect of acetylation on interfacial shear strength between plant fibres and various matrices. Eur. Polym. J. 37:1037-1045.

Kim, H.-S., Lee, B.-H., Choi, S.-W., Kim, S., and Kim, H.-J. 2007. The effect of types of maleic anhydride-grafted polypropylene (MAPP) on the interfacial adhesion properties of bio-flour-filled polypropylene composites. Composites Part A 38:1473-1482.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H., and Trevors, J.T. 2004. Methods of studying soil microbial diversity. J. Microbiol. Methods 58:169-188.

Kleinschmit, D.H., and Kung Jr., L. 2006. The effects of *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* R1094 on the fermentation of corn silage during various stages of ensiling. J. Dairy Sci. 89:3999-4004.

Knauth, S., Hurek, T., Brar, D., and Reinhold-Hurek, B. 2005. Influence of different *Oryza* cultivars on expression of *nifH* gene pools in roots of rice. Environ. Microbiol. 7:725-1733.

Komarek, A.R. 1994. A comparison of methods for determining ADF using the filter bag technique versus conventional filtration. J. Anim. Sci. 72(Suppl. 1):114.

Komarek, A.R., Robertson, J.B., and Van Soest, P.J. 1993. A comparison of methods for determining ADF using the filter bag technique versus conventional filtration. J. Dairy. Sci. 77(Suppl. 1):1.

Legendre, P., and Legendre, L. 1998. Numerical Cology, 2nd edition. Elsevier B.V., Amsterdam, Netherlands.

Lehman, R.M., Colwell, F.S., Ringelberg, D.B., and White, D.C. 1995. Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. J. Microbiol. Methods 22:263-281.

Leidmann, P., Fischer, K., Bieniek, D., Nüβlein, F., and Kettup, A. 1993. Removal of heavy metals from polluted soil with grass silage juice. Chemosphere 28:383-390.

Lesperance, B., and Anglehart, J. 2008. Silage waste recovery process, facility, system, and product. Patent Publication Number US 2010/0317079 A1.

Liljegren, S. 2010. Phloroglucinol stain for lignin. Cold Spring Harbor Laboratory Press 5:1-2.

Lin, C., Bolsen, K.K., Brent, B.E., Hart, R.A., Dickerson, J.T., Feyerherm, A.M., and Aimutis, W.R. 1992. Epiphytic microflora on alfalfa and whole-plant corn. J. Dairy Sci. 75:2484-2493.

Maier, C., and Calafut, T. 1998. Polypropylene: The definitive user's guide and databook, Plastics Design Library, Norwich, NY.

Malik, S., Beer, M., Megharaj, M., and Naidu, R. 2008. The use of molecular techniques to characterize the microbial communities in contaminated soil and water. Environ. Int. 34:265-276.

Marinelli, A.L., and Bretas, R.E. 2003. Blends of polypropylene resins with a liquid crystalline polymer. I. Isothermal crystallization. J. Appl. Polym. Sci. 87:916-930.

Marschner, P., and Baumann, K. 2003. Changes in bacterial community structure induced by mycorrhizal colonisation in split-root maize. Plant Soil 251:279-289.

Marschner, P., Crowley, D., and Lieberei, R. 2001. Arbuscular mycorrhizal infection changes the bacterial 16 S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11:297-302.

Marschner, P., Crowley, D., and Yang, C.H. 2004. Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant Soil 261:199-208.

McDonald, P., Henderson, A.R., and Heron, S.J.E. 1991. The biochemistry of silage, 2nd Edition. Chalcombe Publications, Great Britain.

Meijer, W.J.M., Vertregt, N., Rutgers, B., and van de Waart, M. 1995. The pectin content as a measure of the retting and rettability of flax. Ind. Crop. Pro. 4:273-284.

Merriman R.J. 1988. Water pollution by silage effluent, in: Stark, B.A., and Wilkinson, J.M. (Eds) Silage Effluent. pp. 5–6. Marlow, Bucks: Chalcombe Publications.

Miller, J.H. 1972. Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mohan Sharma, A. 2012. Mechanical behavior, water absorption and morphology of wheat straw, talc, mica and wollastonite filled polypropylene composites. M.Sc. Thesis, University of Waterloo, Waterloo, Ontario, Canada.

Mohanty, A.K., Misra, M., and Drzal, L.T. 2002. Sustainable bio-composites from renewable resources: Opportunities and challenges in the green materials world. J. Polym. Environ. 10:19-26.

Mohanty, A.K., Misra, M., and Hinrichsen, G. 2000. Biofibres, biodegradable polymers and biocomposites: an overview. Macromol. Mater. Eng. 276/277:1-24.

Mohd-Setapar, S.H., Abd-Talib, N., and Aziz, R. 2012. Review on crucial parameters of silage quality. APCBEE Procedia 3:99-103.

Morrison III.,W.H., Archibald, D.D, Sharma, H.S.S, and Akin, D.E. 2000. Chemical and physical characterization of water- and dew-retted flax fibres. Ind. Crop. Pro. 12:39-46.

Mwaikamboo, L.Y., and Ansell, M.P. 2006. Mechanical properties of alkali treated plant fibres and their potential as reinforcement materials II. Sisal fibres. J. Mat. Sci. 41:2497-2508.

Al-Shorgani, N.K.N., Ali, E., Kalil, M.S., and Yusoff, W.M.W. 2012. Bioconversion of butyric acid to butanol by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) in a limited nutrient medium. Bioenerg. Res. 5:287-293.

Otani, T. 2008. Toyota plans to replace 20% of plastics with bioplastics. Tech On! Accessed March 1, 2013. Available at
http://techon.nikkeibp.co.jp/english/NEWS_EN/20081021/159844/

Padgette, S.R., Kolacz, K.H., Delannay, X., Re, D.B., LaVallee, B.J., Tinius, C.N., Rhodes, W.K., Otero, Y.I., Barry, G.F., Eichholtz, D.A., Peschke, V.M., Nida, D.L., Taylor, N.B., and Kishore, G.M. 1995. Development, identification, and characterization of glyphosate-tolerant soybean line. Crop Sci. 35:1451-1461.

Pancher, M., Ceol, M., Corneo, P.E., Oliveira Longa, C.M., Yousaf, S., Pertot, I., Campisano, A. 2012. Fungal endophytic communities in grapevines (*Vitis vinifera* L.) respond to crop management. Appl. Environ. Microbiol. 78:4308-4317.

Pandey, K.K., and Pitman, A.J. 2003. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. Int. Biodeterior. Biodegrad. 52:151-160.

Panthapulakkal, S., and Sain, M. 2007. Agro-residue reinforced high-density polyethylene composites: Fiber characterization and analysis of composite properties. Composites Part A 38:1445-1454.

Panthapulakkal, S., Zereshkian, A., and Sain, M. 2006. Preparation and characterization of wheat straw fibers for reinforcing application in injection molded thermoplastic composites. Bioresour. Technol. 97:265-272.

Pasebani, M., Yaakub, H., Sijam, K., and Alimon, A.R. 2010. Isolation and identification of epiphytic lactic acid bacteria from guinea grass (*Panicum maximum*). Am. J. Anim. Vet. Sci. 5:146-150.

Peijs, T. 2000. Natural fiber based composite. Mater. Technol. 4:281-285.

Pioneer Hi-Bred Limited. 2008. Pioneer® brand Products & Services 2008-2009.

Preiss, J., and Ashwell, G. 1963. Polygalacturonic acid metabolism in bacteria. I. Enzymatic formation of 4-deoxy-L-threo-5-hexocellulose uronic acid. J. Biol. Chem. 283:1571-1576.

Ramey, D., and Yang, S.-T. 2004. Production of butyric acid and butanol from biomass: final report. Accessed March 4, 2013. Available at

http://www.afdc.energy.gov/pdfs/843183.pdf

Ramiah, M.V. 1970. Thermogravimetric and differential thermal analysis of cellulose, hemicelluloses, and lignin. J. Appl. Pol. Sci. 14:1323-1337.

Ramsey, P.W., Rillig, M.C., Feris, K.P., Holben, W.E., and Gannon, J.E. 2006. Coice of methods for soil microbial community analysis: PLFA maximizes power compared to CLPP and PCR-based approaches. Pedobiologia 50:275-280.

Rau, J.H. 1963. Die infrarot-spektren der Textilfasern II/Zellulosefasern. Meilliand Text. 44:1197.

Ray, D., Sarkar, B.K., Rana, A.K., and Bose, N.R. 2001. The mechanical properties of vinylester resin matrix composites reinforced with alkali-treated jute fibres. Composites Part A 32:119-127.

Reddy, C.R., Sardashti, A., and Simon, L.C. 2010. Preparation and characterization of polypropylene-wheat straw-clay composites. Compos. Sci. Technol. 70:1674-1680.

Reddy, N., and Yang, Y. 2005. Biofibers from agricultural byproducts for industrial applications. Trends Biotechnol. 23:22-27.

Rome. 1998. Improved retting and extraction of jute project findings and recommendations international jute organization fao/government cooperative programmed. Food and Agriculture Organization of the United Nations.

Rudin, A. 1999. Elements of Polymer Science and Engineering, 2nd Edition. Academic Press, San Diego, CA.

Sain, M., and Panthapulakkal, S. 2006. Bioprocess preparation of wheat straw fibers and their characterization. Ind. Crop. Pro. 23:1-8.

Saito, A., Ikeda, S., Ezura, H., and Minamisawa, K. 2007. Microbial community analysis of the phytosphere using culture-independent methodologies. Microbes Environ. 22:93-105.

Sano, K., Uchida, H., and Wakabayashi, S. 1999. A new process for acetic acid production by direct oxidation of ethylene. Catalysis Surveys from Japan 3:55-60.

Saxena, D., and Stotsky, G. 2001. *Bt* corn has a higher lignin content than non-*Bt* corn. Amer. J. Bot. 88:1704-1706.

Scherübl, B. 2005. Application of natural fibre reinforced plastics for automotive exterior parts, with a focus on underfloor systems. Proceedings of the 8th international AVK-TV conference, Baden-Baden, Germany (2005) p. D5/1–8.

Schroeder JW. 2004. Silage fermentation and preservation. North Dakota State University, Fargo, ND.

Seena, J., Koshy, P., and Thomas, S. 2005. The role of interfacial interactions on the mechanical properties of banana fibre reinforced phenol formaldehyde composites. Compos. Interfaces 12:581-600.

Seghers, D., Wittebolle, L., Top, E.M., Verstraete, W., and Siciliano, S.D. 2004. Impact of agricultural practices on the *Zea mays* L. endophytic community. Appl. Environ. Microbiol. 70:1475-1482.

Sgriccia, N., Hawley, M.C., and Misra, M. 2008. Characterization of natural fiber surfaces and natural fiber composites. Composites Part A 39:1632-1637.

Sharma, H.S.S. (1986a) An alternative method of flax retting during dry weather. Ann. Appl. Biol. 109:605-611.

Sharma, H.S.S. (1986b) The role of bacteria in retting of desiccated flax during damp weather. Appl. Microbiol. Biotechnol. 24:463-467.

Sharma, H.S.S., Lefevre, J., and Boucaud, J. 1992. Role of microbial enzymes during retting and their affects on fiber characteristics, in: Sharma, H.S.S., and Van Sumere, C.F. (Eds.), The Biology and Processing of Flax. M Publications, Belfast, UK.

Sharma, H.S.S., Faughey, G., and Lyons, G. 1999. Comparison of physical, chemical, and thermal characteristics of water-, dew-, and enzyme-retted flax fibers. J. Appl. Pol. Sci. 74:139-143.

Sharma, H.S.S., and Faughey, G.J. 1999. Comparison of subjective and objective methods to assess flax straw cultivars and fibre quality after dew-retting. Ann. Appl. Biol. 135:495-501.

Shuler, M.L., and Kargi, F. 1997. Bioprocess Engineering; Basic Concepts. Prentice-Hall, Inc. Upper Saddle River, New Jersey.

Söderberg, K.H., Probanza, A., Jumpponen, A., and Bååth, E. 2004. The microbial community in the rhizosphere determined by community-level physiological profiles (CLPP) and direct soil- and cfu-PLFA techniques. Appl. Soil Ecol. 25:135-145.

Soleimani, M., Tabil, L., Panigrahi, S., and Oguocha, I. 2012. Crystallization and thermal properties of biofiber-polypropylene composites, in: Thermoplastics – Composite Materials. El-Sonbati, A.Z. (Ed.) InTech, Croatia.

Stephens, S.K., Tothill, I.E., Warner, P.J., and Turner, A.P.F. 1997. Detection of silage effluent pollution in river water using biosensors. Wat. Res. 31:41-48.

Sun, R., Tomkinson, J., Wang, S., and Zhu, W. 2000. Characterization of lignins from wheat straw by alkaline peroxide treatment. Polym. Degrad. Stabil. 67:101-109.

Tahir, P.M., Ahmed, A.B., SaifulAzry, S.O.A., and Ahmed, Z. 2011. Retting effect of some bast plant fibres and its effect on fibre quality: a review. Bioresources 6:5260-5281.

Tamburini, E., Len, A.G., Perito, B. and Mastromei, G. 2003. Characterization of bacterial pectinolytic strains involved in the water retting process. Environ. Microbiol. 5:730-736.

Tan, Z., Hurek, T., and Reinhold-Hurek, B. 2003. Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in roots of rice. Environ. Microbiol. 5:1009-1015.

Taylor, L.R. 1961. Aggregation, variance and the mean. Nature 189:732-735.

Te Giffel, M.C., Wagendorp, A., Herrewegh, A., and Driehuis, F. 2002. Bacterial spores in silage and raw milk. Anton. Leeuw. Int. J. G. 81:625-630.

Thamae, T., Marien, R., Chong, L., Wu, C., and Baillie, C. 2008. Developing and characterizing new materials based on waste plastic and agro-fibre. J. Mater. Sci. 43:4057-4068.

The Carbohydrate Economy. Talking About Corn Stover with Jim Hettenhaus. 2002;4(2).

Trigui, A., Karkri, M., Peña, L., Boudaya, C., Candau, Y., Bouffi, S., and Vilaseca F. 2012. Thermal and mechanical properties of maize fibres-high density polyethylene biocomposites. J. Compos. Mater. doi:10.1177/0021998312447648.

Trove, J.P. 1996. Fontaine-caney, Flax breeder. Producing for the Market, 4th Workshop of the FAO Network on Flax, Rouen.

Tserki, V., Zafeiropoulos, N.E., Simon, F., and Panayiotou, C. 2005. A study of the effect of acetylation and propionylation surface treatments on natural fibres. Composites Part A 36: 1110-1118.

Van Sumere, C.F. 1992. Retting of flax with special reference to enzyme-retting, in: Sharma, H.S.S., and Van Sumere, S.F. (Eds.), The biology and processing of flax. M Publications, Belfast, Northern Ireland.

Vlaswinkel, A.G.H. 1996. Falx breeding at van de Bilt Breeding stations. Producing for the Market, 4th Workshop of the FAO Network on Flax, Rouen.

Weber, K.P., Grove, J.A., Gehder, M., Anderson, W.A., and Legge, R.L. 2007. Data transformations in the analysis of community-level substrate utilization data from microplates. J. Microbiol. Methods 69:461-469.

Weber, K.P., Gehder, M., and Legge, R.L. 2008. Assessment of changes in the microbial community of constructed wetland mesocosms in response to acid mine drainage exposure. Water. Res. 42:180-188.

Weber, K.P., and Legge, R.L. 2009. One-dimensional metric for tracking bacterial community divergence using sole carbon source utilization patterns. J. Microbiol. Methods 79:55-61.

Weber, K.P., and Legge, R.L. 2010. Community-level physiological profiling. Methods Mol. Biol. 599:263-281.

Weber, K.P., and Legge, R.L. 2011. Dynamics in the bacterial community-level physiological profiles and hydrological characteristics of constructed wetland mesocosms during start-up. Ecol. Eng. 37:666-677.

Widmer, F., Fließbach, A., Laczo, E., Schulze-Aurich, J., and Zeyer., J. 2001. Assessing soil biological characteristics: A comparison of bulk soil community DNA-, PLFA-, and Biolog-analysis. Soil Biol. Biochem. 33:1029–1036.

Woolford, M.K. 1984. The silage fermentation, Marcel Dekker, Inc. New York, NY.

Yang, C., and Crowley, D.E. 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Appl. Environ. Microbiol. 66:345-351.

Yang, C., Crowley, D.E., Borneman, J., and Keen, N.T. 2001. Microbial phyllosphere populations are more complex than previously realized. Proc. Natl. Acad. Sci. USA 98:3889-3894.

Yang, H.-S., Gardner, D.J., and Kim, H.-J. 2009. Viscoelastic and thermal analysis of lignocellulosic material filled polypropylene bio-composites. J. Therm. Anal. Calorim. 98:553-558.

Yang, M-H., and Choong, Y-M. 2001. A rapid gas chromatographic method for direct determination of short-chain (C2-C12) volatile organic acids in foods. Food Chem. 75:101-108.

Yilmaz, N.D. 2012. Effects of enzymatic treatments on the mechanical properties of corn husk fibers. J. Text. I. DOI:10.1080/00405000.2012.736707

Yu, H., and Yu, C. 2007. Study on microbe retting of kenaf fiber. Enzyme Microb. Technol. 40:1806-1809.

Zak, J.C., Willig, M.R., Moorhead, D.L., and Wildman, H.G. 1994. Functional diversity of microbial communities: A quantitative approach. Soil Biol. Biochem. 26:1101-1108.

Zhang, J., Henriksson, G., and Johansson, G. 2000 Polygalacturonase is the key component in enzymatic retting of flax. J. Biotechnol. 91:85-89.

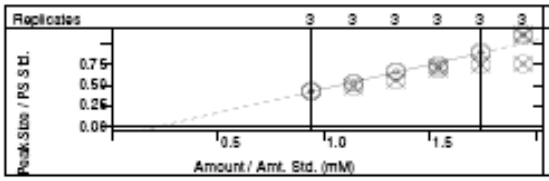
Appendix A

GC-MS calibration curves for organic acid analysis

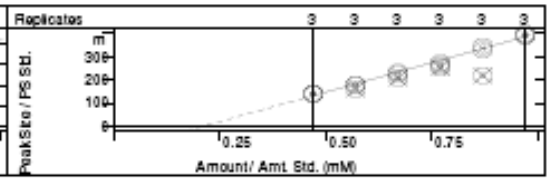
Calibration Curves Report

Method:	...ta files\rachel campbell\methods\fatty acid analysis.mth		
Recalc Method:	...d analysis.mth	Last Calibration:	1/15/2009 12:15 PM
Sample List:	N/A	Cmpd. Table Updated:	1/15/2009 12:25 PM
Sequence:	N/A	Detector:	2000 Mass Spec
MS Workstation		Workstation Version:	Version 6.40
Peak Measurement:	Area	Calibration Type:	Internal Standard Analysis

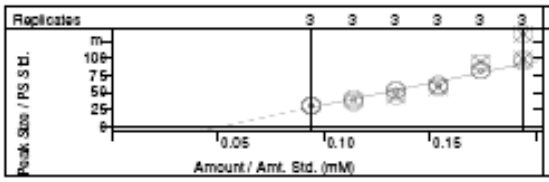
2C
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 5.869%, Coeff. Det.(r2): 0.992668
y = +0.5911x - 0.1357



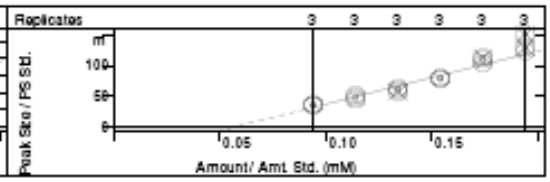
3C
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 12.27%, Coeff. Det.(r2): 0.989179
y = +0.5107x - 0.1083



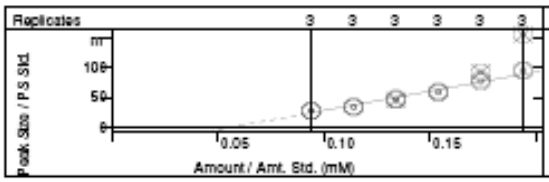
I4C
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 16.10%, Coeff. Det.(r2): 0.974709
y = +0.6440x - 0.0327



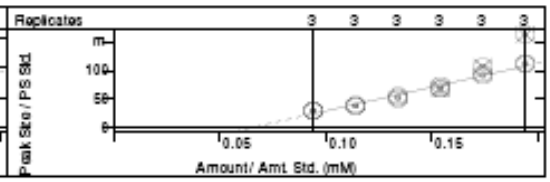
4C
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 19.09%, Coeff. Det.(r2): 0.972925
y = +0.8784x - 0.0507



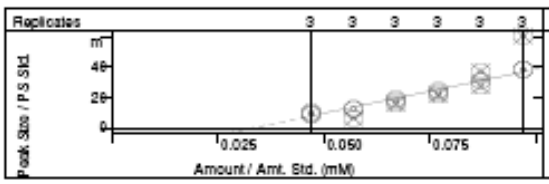
I5C
Curve Fit: Linear, Origin: Ignore, Weight: 1/nX2
Resp. Fact. RSD: 20.78%, Coeff. Det.(r2): 0.972125
y = +0.6539x - 0.0375



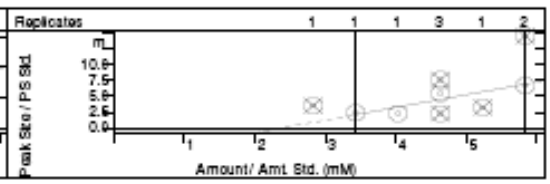
5C
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 24.67%, Coeff. Det.(r2): 0.976481
y = +0.8523x - 0.0568



6C
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 26.02%, Coeff. Det.(r2): 0.978878
y = +0.5733x - 0.0188



Lac
Curve Fit: Linear, Origin: Ignore, Weight: None
Resp. Fact. RSD: 33.93%, Coeff. Det.(r2): 0.863065
y = +0.0020x - 0.0047



Appendix B

Carbon Source Legend of the BIOLOG™ ECOplate

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Appendix C

Sample time point determination for community-level physiological profiling

Table C.1 Numerical analysis of 38A56 Non Inoculated (Day 3)

Incubation Time (hr)	AWCD	values >2	Standard Deviation
0	0.011226	0	0.010375
4	0.077677	0	0.041141
12	0.038677	0	0.045167
20	0.279387	0	0.312632
24	0.459484	0	0.498994
28	0.629645	2	0.611903
36	0.929129	2	0.732418

Table C.2 Numerical analysis of 38A56 Non Inoculated (Day 6)

Incubation Time (hr)	AWCD	values >2	Standard Deviation
0	0.013839	0	0.017588
8	0.062903	0	0.06455
16	0.266968	0	0.224205
20	0.535484	0	0.488732
24	0.806968	1	0.639161
32	1.187871	6	0.723641
40	1.42371	7	0.684698

Table C.3 Numerical analysis of 38N87 Inoculated (Day 3)

Incubation Time (hr)	AWCD	values >2	Standard Deviation
0	0.014355	0	0.012183
2	0.012065	0	0.009567
10	0.036161	0	0.024951
18	0.126452	0	0.111296
22	0.215548	0	0.207915
26	0.394129	1	0.446393
34	0.750419	2	0.720174

Appendix D

Sample statistical constraint diagnostic results for community-level physiological profiling

Table D.1 Statistical diagnostic of 38N85-87

Diagnostic Test	Pretreated (No Trans)	Taylor Transform	LN transform
Average Kurtosis z-value	4.52	2.38	3.17
# Significant Kurtosis Values	15	24	20
Average Skewness z-value	3.23	2.11	2.62
# Significant Skewness Values	12	17	13
# of Linear Correlations	57	67	62
Variance Ratio	243	8.7	27.4

Table D.2: Statistical diagnostic of 38N86 and 38A56

Diagnostic Test	Pretreated (No Trans)	Taylor Transform	LN transform
Average Kurtosis z-value	2.65	2.09	2.25
# Significant Kurtosis Values	23	20	20
Average Skewness z-value	2.26	1.82	2.15
# Significant Skewness Values	19	19	15
# of Linear Correlations	64	58	57
Variance Ratio	322	11.8	46.0

Table D.3 Statistical diagnostic of 38N87 and 38A57

Diagnostic Test	Pretreated (No Trans)	Taylor Transform	LN transform
Average Kurtosis z-value	3.10	1.38	2.08
# Significant Kurtosis Values	18	29	22
Average Skewness z-value	2.70	1.34	1.99
# Significant Skewness Values	15	26	18
# of Linear Correlations	48	58	48
Variance Ratio	28 751	32.0	1904

Appendix E

Material Safety Data Sheets

Ciba Specialty Chemicals
Coating Effects Segment

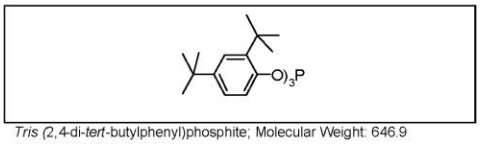
Ciba

Ciba® IRGAFOS® 168 Processing Stabiliser

General

IRGAFOS 168 is a trisarylphosphite processing stabiliser. It is a highly effective, low-volatile and hydrolysis resistant antioxidant for coating resins. It protects the resin polymer against oxidation during resin synthesis, manufacturing of the paint, processing (thermal curing and overbaking) and the designed life-time of the final coating. IRGAFOS 168 provides excellent protection against discolouration and change of physical properties caused by excessive heat exposure.

Chemical Structure



Tris (2,4-di-*tert*-butylphenyl)phosphite; Molecular Weight: 646.9
CAS Number: 31570-04-4

Physical Properties
(typical values)

IRGAFOS 168 is offered in two different product forms:

Appearance :	white powder	IRGAFOS 168
	white free-flowing powder	IRGAFOS 168 FF

Melting point : 185°C

Solubility (g/100g solution) at 20°C :

Acetone	1
Cyclohexane	10
Hexane	11
Ethanol	0.1
Methanol	< 0.01
Ethyl acetate	4
Toluene	30
Water	< 0.01 (degradation)

Applications

IRGAFOS 168 provides outstanding thermal and colour stability to a variety of coating resins upon exposure to high temperatures during synthesis and subsequent processing. It is particularly suited to stabilise alkyd and polyester resin based coating systems that are used in applications such as

- industrial powder coatings (electrical and gas oven heating)
- coil coatings
- other industrial high bake coatings

A special feature of the IRGAFOS 168 phosphite stabiliser is its

Ciba

efficiency in presence of NOx gases and thus its potential to function in critical gas oven curing conditions.

In electrical ovens and gas fired ovens where the coated substrates being exposed to only very low NO_x concentrations, a combination IRGAFOS 168 with hindered phenolic antioxidants such as IRGANOX 1076 or IRGANOX 1010 typically leads to a synergistic performance improvement. In the presence of NO_x the same combination often exhibits adverse discolouration effects, however. In this case phenol-free synergistic antioxidant systems can be obtained by using IRGAFOS 168 in combination with hindered amine light stabilisers (HALS) such as TINUVIN 111 FD, TINUVIN 292 or TINUVIN 622 LD.

For improved performance IRGAFOS 168 may also be combined with other phosphite or phosphonite co-stabilizers such as IRGAFOS 38, IRGAFOS XP 40 and IRGAFOS XP 60 or with thiosynergists such as IRGANOX PS 800.

The amount of IRGAFOS 168 required for optimum performance should be determined in trials covering a concentration range.

Recommended concentration:
(based on resin solids)

• industrial powder coatings • coil coatings • other industrial high bake coatings	**0.2 - 0.8% IRGAFOS 168 alone or in combination with synergists**

Safety and Handling

IRGAFOS 168 is sensitive to moisture. Commercial quantities are offered in special metalised plastic inner packagings to prevent hydrolysis of the product upon storage. To ensure optimum performance, opened packages and small samples should be used up without delay and care taken to avoid exposure to moist environments.

IRGAFOS 168 should be handled in accordance with good industrial practice. Detailed information is provided in the Safety Data Sheet.

Trademark

IRGAFOS and IRGANOX are registered trademarks.

Important Notice

IMPORTANT: The following supersedes Buyer's documents. SELLER MAKES NO REPRESENTATION OR WARRANTY, EXPRESS OR IMPLIED, INCLUDING OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. No statements herein are to be construed as inducements to infringe any relevant patent. Under no circumstances shall Seller be liable for incidental, consequential or indirect damages for alleged negligence, breach of warranty, strict liability, tort or contract arising in connection with the product(s). Buyer's sole remedy and Seller's sole liability for any claims shall be

Ciba

Buyer's purchase price. Data and results are based on controlled or lab work and must be confirmed by Buyer by testing for its intended conditions of use. The product(s) has not been tested for, and is therefore not recommended for, uses for which prolonged contact with mucous membranes, abraded skin, or blood is intended; or for uses for which implantation within the human body is intended.

Ciba Specialty Chemicals

Additives

Polymer Additives

Ciba

®IRGANOX 1010

Phenolic Primary Antioxidant for Processing and Long-Term Thermal Stabilization

Characterization

®IRGANOX 1010 - a sterically hindered phenolic antioxidant - is a highly effective, non discoloring stabilizer for organic substrates such as plastics, synthetic fibers, elastomers, adhesives, waxes, oils and fats. It protects these substrates against thermo-oxidative degradation.

Chemical Name

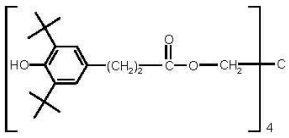
Pentaerythritol Tetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate)

CAS Number

6683-19-8

Structure

®IRGANOX 1010



Molecular weight

1178

Applications

®IRGANOX 1010 can be applied in polyolefins, such as polyethylene, polypropylene, polybutene and olefin copolymers such as ethylene-vinylacetate copolymers. Also, its use is recommended in other polymers such as polyacetals, polyamides and polyurethanes, polyesters, PVC, styrene homo- and copolymers, ABS, elastomers such as butyl rubber (IIR), SBS, SEBS, EPM and EPDM as well as other synthetic rubbers, adhesives, natural and synthetic tackifier resins, and other organic substrates.

Features/ Benefits

®IRGANOX 1010 has good compatibility, high resistance to extraction and low volatility. It is odorless and tasteless.

The product can be used in combination with other additives such as costabilizers (e.g. thioethers, phosphites, phosphonites), light stabilizers and other functional stabilizers. The effectiveness of the blends of ®IRGANOX 1010 with ®IRGAFOS 168 (®IRGANOX B-blends) or with ®IRGAFOS 168 and HP-136 (®IRGANOX HP products) is particularly noteworthy.

Product Forms

Code:	Appearance:
powder	white, free-flowing powder
FF (C)	white, free-flowing granules
DD	white to slightly green pellets

Distributed by
wi

Guidelines for Use

Already 500 ppm - 1000 ppm of ®IRGANOX 1010 provide long-term thermal stability to the polymer. Concentrations up to several percent may be used depending on the substrate and the requirements of the end application.
In polyolefins the concentration levels for ®IRGANOX 1010 range between 0.05% and 0.4% depending on substrate, processing conditions and long-term thermal stability requirements. The optimum level has to be determined application specific.
Concentration levels of ®IRGANOX 1010 in hot melt adhesives range from 0.2% to 1%, in synthetic tackifier resins, ®IRGANOX 1010 concentration ranges between 0.1% and 0.5%.
Extensive performance data of ®IRGANOX 1010 in various organic polymers and applications are available upon request.

Physical Properties

Melting Range (°C)	110-125
Flashpoint (°C)	297
Specific Gravity (20°C)	1.15 g/cm^3
Bulk density	powder:530 - 630 g/l FF (C): 480 - 570 g/l DD: 450 - 550 g/l
Solubility (20°C)	g/100g solution
Acetone	47
Chloroform	71
Ethanol	1.5
Ethylacetate	47
n-Hexane	0.3
Methanol	0.9
Methylene Chloride	63
Toluene	60
Water	<0.01

Handling & Safety

In accordance with good industrial practice, handle with care and prevent contamination of the environment. Avoid dust formation and ignition sources.
For more detailed information please refer to the material safety data sheet.

Registration

®IRGANOX 1010 is listed on the following inventories:

Australia:	AICS	Canada:	DSL	China: First Import
Europe:	EINECS	Japan:	MITI	Korea: ECL
Philippines:	PICCS	USA:	TSCA	

®IRGANOX 1010 is approved in many countries for use in food contact applications.
For detailed information refer to our Positive List or contact your local sales office.

Tuesday, March 05, 2013

Braskem PP D180M

Braskem America Inc. -
Polypropylene
Homopolymer

Units English

Action **Legend (Open)**

General Information

Product Description

Sub-group
Homopolymer

Description
Low Gas Fade

Applications
Suggested Uses Include BCF Multi-Filiments, High Tenacity Continuous Filament Yarn, Fine Denier Staple Fibers

General

Material Status	• Commercial: Active		
Availability	• North America		
Features	• Food Contact Acceptable	• Gas-fading Resistant	• Homopolymer
Uses	• BCF Yarn • Fibers	• Filaments • High Tenacity Flat Yarn	• Nonwovens • Staple Fibers
Agency Ratings	• FDA 21 CFR 177.1520		
Forms	• Pellets		
Processing Method	• Fiber (Spinning) Extrusion		

ASTM & ISO Properties [1]

Physical	Nominal Value	Unit	Test Method
Melt Mass-Flow Rate (MFR) (230°C/2.16 kg)	18	g/10 min	ASTM D1238
Mechanical	**Nominal Value**	**Unit**	**Test Method**
Tensile Strength [2] (Yield, Injection Molded)	5100	psi	ASTM D638
Tensile Elongation [2] (Yield, Injection Molded)	9.0	%	ASTM D638
Flexural Modulus - 1% Secant [3] (Injection Molded)	190000	psi	ASTM D790A
Impact	**Nominal Value**	**Unit**	**Test Method**
Notched Izod Impact (73°F, Injection Molded)	0.50	ft·lb/in	ASTM D256A
Hardness	**Nominal Value**	**Unit**	**Test Method**
Rockwell Hardness (R-Scale, Injection Molded)	104		ASTM D785
Additional Information	**Nominal Value**	**Unit**	**Test Method**
Elongation of Fibers [4]	93	%	ASTM D2256
Takeup Roll Speed	109.4	ft/sec	Internal Method
Tenacity of Fibers [4]	2.90	g/denier	ASTM D2256

Notes

[1] Typical properties: these are not to be construed as specifications.

[2] 2.0 in/min

[3] 0.050 in/min

[4] 3.3 draw ratio, 1,250 m/min roll speed, 225°C spin temperature, D1000/68

DuPont Fusabond® MD353D Random Copolymer Polypropylene

Categories: Other Engineering Material; Additive/Filler for Polymer; Polymer; Thermoplastic; Polypropylene

Material Notes: Typical Use:

- Coupling agent
- Long glass filled polypropylene
- Coupling agent for nonhalogen, flame retarded wire and cable compounds containing magnesium hydroxide
- Adhesion promoter
- Natural fiber wood-plastic compounds

Availability: North America

Information provided by DuPont.

Vendors: No vendors are listed for this material. Please click here if you are a supplier and would like information on how to add your listing to this material.

Physical Properties	Original Value	Comments
Melt Flow	450 g/10 min @Load 2.16 kg, Temperature 190 °C	

Thermal Properties	Original Value	Comments
Melting Point	136 °C	

Descriptive Properties		
MAH Graft Level, wt%	Very High	FTIR (DuPont)

Some of the values displayed above may have been converted from their original units and/or rounded in order to display the information in a consistent format. Users requiring more precise data for scientific or engineering calculations can click on the property value to see the original value as well as raw conversions to equivalent units. We advise that you only use the original value or one of its raw conversions in your calculations to minimize rounding error. We also ask that you refer to MatWeb's terms of use regarding this information. Click here to go back to viewing the property data in MatWeb's normal format.

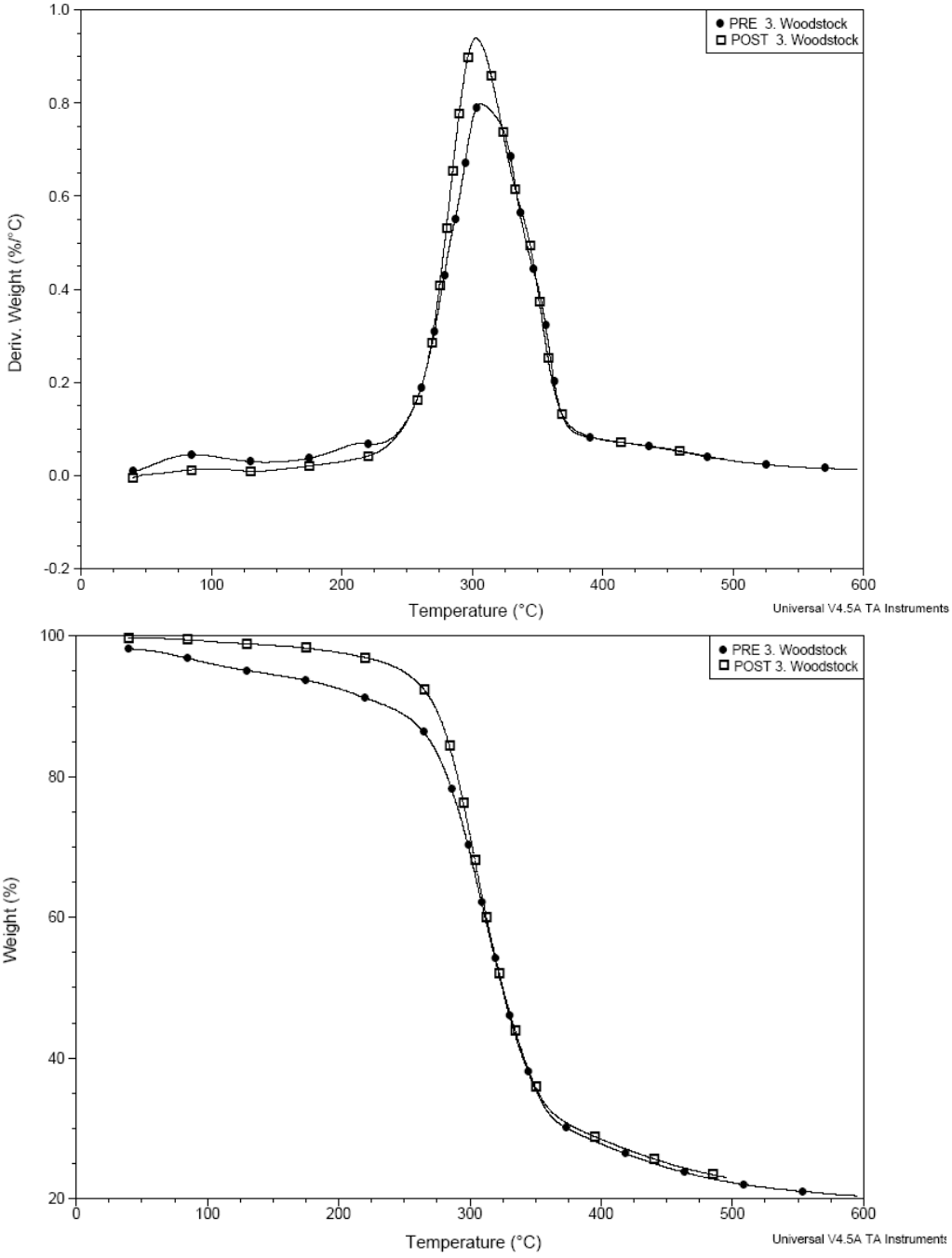
Appendix F

Raw data and TGA plot from the thermal analysis of pre- and post-ensiled corn

				Peak			0.5 wt%/min onset of degradation		1 wt%/min onset of degradation		2 wt%/min onset of degradation		5 wt%/min onset of degradation	
Pre-Silage		T5	T10	Temp. (°C)	Weight %	Peak Rate	Temp. (°C)	Weight %	Temp. (°C)	Weight %	Temp. (°C)	Weight %	Temp. (°C)	Weight %
2	Erin	170.5	236.9	300.94	67.13	6.70	183.36	94.49	232.16	90.51	256.81	87.08	282.05	78.62
3	Woodstock	133.2	237.1	308.45	62.47	7.98	190.85	93.03	245.64	89.26	262.30	86.87	282.73	79.95
4	Arthur	148.5	246.7	300.78	65.13	8.90	197.76	93.47	241.66	90.53	260.40	87.91	277.64	82.29
6	Embro	146.4	258.4	306.83	63.71	9.17	226.14	92.92	247.22	91.43	263.54	89.09	281.06	83.22
7	Woodstock	182.3	253.7	320.64	55.17	7.51	210.29	94.10	237.68	92.25	258.09	89.19	281.57	81.51
8	Elmira	181.7	257.1	299.23	70.96	7.63	218.11	93.71	241.52	92.06	261.09	89.26	280.84	82.79
9	Elmira	246.4	270.3	320.07	57.49	8.30	207.04	98.23	236.77	96.13	258.34	93.03	281.18	85.54
10	Guelph	70.3	207.4	310.37	58.01	8.27	201.12	90.32	243.42	87.53	262.33	84.84	281.51	78.45
11	Elora	183.3	242.8	311.43	60.05	6.90	181.51	95.09	230.48	91.45	255.63	87.86	281.13	79.50
12	Rockton	156.8	250.6	306.09	64.41	8.15	203.12	93.66	241.93	91.04	259.00	88.58	280.93	81.24
13	Rockwood	194.7	253.3	312.53	58.28	8.51	192.13	95.14	239.09	91.78	259.93	88.83	280.18	82.18
14	Rockwood	189.6	241.3	325.03	52.40	6.91	174.46	95.93	224.91	91.88	254.90	87.76	284.63	77.70
15	Elmira	209.1	251.1	317.40	58.05	6.99	187.64	96.49	223.45	93.67	254.77	89.30	283.09	79.95
17	Fergus	197.9	243.4	334.30	48.67	6.61	174.23	96.75	201.12	94.68	253.33	88.30	284.56	77.55
18	Rockwood	215.5	265.7	301.62	68.27	8.97	225.34	94.57	245.41	93.15	262.32	90.74	278.74	85.40
19	Tavistock	207.9	257.0	304.69	64.91	7.95	195.86	95.69	236.41	92.82	258.99	89.62	277.90	83.46
20	Guelph	191.1	258.3	308.15	61.88	8.90	205.18	94.38	244.99	91.75	261.98	89.33	279.08	83.67
21	Guelph	221.7	265.9	311.05	58.44	10.77	218.09	95.19	242.66	93.45	263.06	90.61	279.50	85.16
22	Embro	218.3	257.2	329.29	52.05	7.31	195.36	96.39	232.67	93.80	253.07	90.89	283.23	80.72
23	Guelph	209.7	263.0	312.10	61.15	9.08	207.77	95.12	242.25	93.17	259.98	90.72	278.91	86.42
24	Rockwood	218.5	260.6	310.35	62.94	7.58	210.35	95.45	236.75	93.58	258.31	90.48	280.65	83.23
25	Elmira	216.2	259.0	312.95	61.88	7.25	204.68	95.66	235.99	93.43	257.23	90.37	282.31	81.91
26	Elmira	202.4	249.6	317.19	60.15	6.62	183.20	96.11	226.99	92.98	254.06	89.16	287.13	77.81
27	Elmira	193.9	247.8	316.51	58.19	7.25	174.04	96.29	233.65	91.70	257.89	88.29	283.98	79.48
28	Embro	208.6	262.3	301.85	67.13	8.51	217.33	94.59	243.73	92.78	260.39	90.40	277.72	84.69
29	Elmira	207.1	255.4	300.19	68.25	7.31	200.93	95.33	234.89	92.95	255.30	90.03	278.21	82.42
30	Rockwood	209.5	258.0	316.34	58.94	7.67	194.09	95.91	237.81	92.79	260.13	89.59	282.38	82.28

				Peak			0.5 wt%/min onset of degradation		1 wt%/min onset of degradation		2 wt%/min onset of degradation		5 wt%/min onset of degradation	
Post-Silage		T5	T10	Temp. (°C)	Weight %	Peak Rate	Temp. (°C)	Weight %	Temp. (°C)	Weight %	Temp. (°C)	Weight %	Temp. (°C)	Weight %
2	Erin	160.7	245.1	309.59	63.32	7.52	203.11	93.14	237.66	90.83	260.98	87.49	285.26	79.50
3	Woodstock	248.8	273.2	302.62	69.24	9.38	227.36	96.61	246.51	95.25	262.17	93.01	279.00	87.50
4	Arthur	222.7	260.0	305.80	68.35	6.53	179.55	97.50	234.68	93.98	255.66	90.96	284.65	81.02
6	Embro	193.4	247.0	308.30	62.28	7.55	148.42	98.28	231.69	91.86	255.51	88.55	282.66	79.30
7	Woodstock	205.2	252.9	312.63	60.47	7.47	155.91	98.11	232.35	92.91	253.50	89.88	284.00	79.50
8	Elmira	191.7	243.5	318.96	52.93	7.45	160.08	97.18	226.85	92.07	251.50	88.60	281.58	78.33
9	Elmira	197.6	246.3	325.96	50.32	7.77	168.91	96.74	226.02	92.78	249.84	89.32	286.32	77.26
10	Guelph	194.6	251.8	304.89	66.02	7.47	166.75	96.74	237.11	91.85	259.08	88.70	282.24	81.08
11	Elora	189.0	240.1	305.53	60.49	7.86	148.49	98.36	181.47	95.77	249.75	88.39	279.08	78.42
12	Rockton	226.7	263.2	306.06	64.09	8.63	204.51	96.25	240.14	93.92	256.63	91.57	278.81	83.92
13	Rockwood	217.3	257.1	315.84	57.65	7.76	178.62	97.32	232.08	93.75	254.56	90.53	281.23	81.61
14	Rockwood	206.3	251.2	315.19	59.08	7.12	161.46	97.66	228.74	93.27	251.06	90.04	284.40	78.93
15	Elmira	207.7	251.9	331.47	50.97	6.28	176.57	96.81	229.85	93.24	250.59	90.28	284.58	78.39
17	Fergus	191.6	245.4	324.34	53.92	6.93	150.47	97.84	230.23	91.96	252.22	88.78	288.23	76.65
18	Rockwood	188.6	253.6	308.95	63.48	8.18	167.75	96.19	242.77	91.32	260.50	88.83	283.75	81.03
19	Tavistock	233.5	268.2	306.95	67.28	8.07	219.42	95.84	243.56	94.16	261.39	91.61	282.10	84.76
20	Guelph	169.6	231.4	306.45	60.35	8.03	144.92	96.67	236.68	89.53	254.75	87.02	280.35	78.30
21	Guelph	175.8	242.9	308.22	60.96	7.97	150.59	96.56	236.86	90.65	256.43	87.88	281.50	79.37
22	Embro	214.3	265.3	315.04	57.45	10.05	220.50	94.70	246.98	92.86	261.97	90.74	283.42	83.15
23	Guelph	198.5	253.0	303.72	66.30	7.26	172.83	96.40	235.76	92.35	256.17	89.41	280.75	81.30
24	Rockwood	200.4	254.5	305.05	64.53	7.88	184.48	95.81	236.68	92.54	255.25	89.86	280.25	81.50
25	Elmira	191.1	247.2	307.04	62.83	7.45	169.75	96.28	231.69	91.94	254.01	88.79	281.69	79.48
26	Elmira	137.0	235.1	324.67	51.18	7.44	187.77	93.35	231.40	90.39	253.21	87.26	285.55	76.53
27	Elmira	198.8	248.8	306.64	64.09	6.58	176.99	96.21	229.44	92.69	250.27	89.72	282.24	78.79
28	Embro	225.1	263.2	303.02	67.24	8.24	204.09	96.18	239.90	93.82	257.72	91.27	279.05	84.21
29	Elmira	197.6	253.1	302.68	64.28	8.22	190.51	95.37	236.14	92.37	254.05	89.82	277.37	82.07
30	Rockwood	252.6	275.6	304.51	67.29	10.31	231.75	96.53	250.40	95.23	264.39	93.23	279.22	88.35

TGA plot comparing pre- and post-ensiled corn biomass of sample 3 (Woodstock)



Appendix G

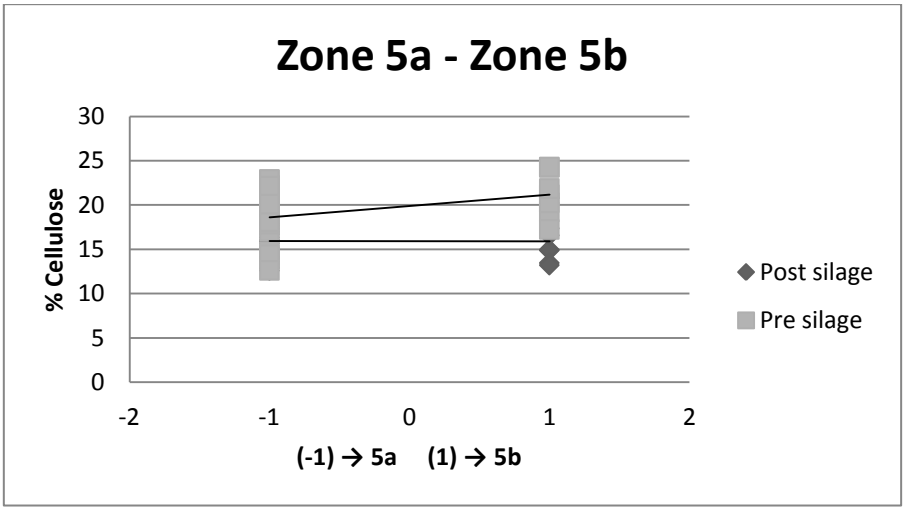
Raw data from the NIR compositional analysis of pre- and post-ensiled corn

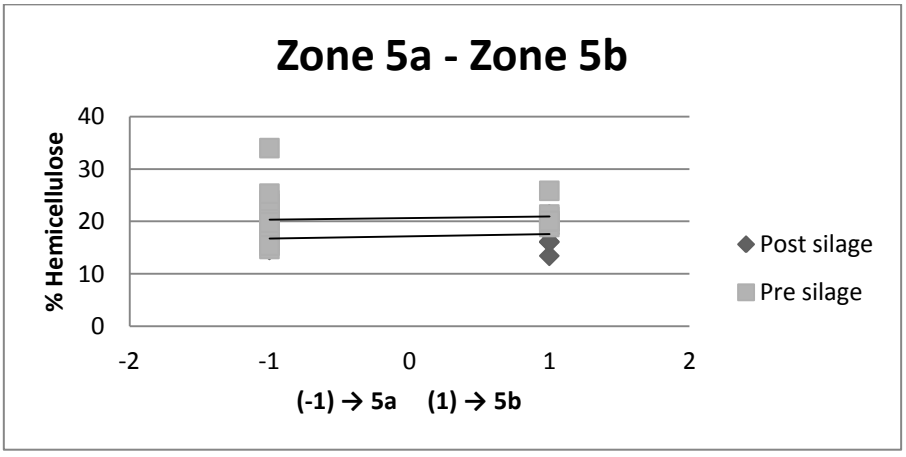
					PROTEIN		MINERALS					OTHER		
Pre-Silage	Location	Cellulose	Hemicellulose	Lignin	Crude (%)	Soluble (% of CP)	Ca	P	K	Mg	Na	Starch	Total Ash	Crude Fat
1	Elora	19.16	18	1.29	7.97	38.87	0.26	0.21	0.85	0.13	0.01	34.61	3.68	2.12
2	Erin	19.98	18.91	1.03	8.3	32.29	0.28	0.21	0.71	0.14	0.01	35.16	3.26	2.22
3	Woodstock	24.24	20.89	1.85	7.47	32.35	0.25	0.2	0.83	0.16	0.01	32.77	3.17	2.09
4	Arthur	19.15	18.48	1.06	7.98	34.57	0.27	0.21	0.77	0.13	0.01	38.27	3.18	2.65
5	Rockton	20.09	18.07	1.79	9.83	36.25	0.34	0.24	0.86	0.17	0.01	26.36	5.11	2.43
6	Embro	19.2	19.4	2.02	8.6	27.26	0.36	0.23	0.6	0.15	0.01	35.88	2.85	1.8
7	Woodstock	21.17	25.85	1.69	8.58	33.77	0.31	0.23	0.69	0.11	0.11	35.93	3.33	1.55
8	Elmira	22.86	22.2	1.58	9.35	27.84	0.39	0.24	0.87	0.14	0.07	32.54	3.8	1.53
9	Elmira	22.23	19.25	1.1	9.71	35.06	0.33	0.23	0.54	0.17	0.03	27.99	4.79	2.43
10	Guelph	22.87	19.63	1.66	8.37	34	0.28	0.22	0.63	0.18	0.01	27.65	3.73	1.7
11	Elora	21.2	18.06	1.21	8.93	36.02	0.28	0.21	0.69	0.18	0.01	31.82	3.99	2.21
12	Rockton	17.24	18.95	1.23	8.03	36.9	0.29	0.22	0.54	0.13	0.02	38.34	3.02	2.13
13	Rockwood	13.99	17.97	1.23	7.8	35.59	0.27	0.22	0.63	0.12	0.01	45.67	2.91	2.44
14	Rockwood	21.24	21.65	1.8	8.97	33.17	0.33	0.22	0.93	0.17	0.03	28.8	4.24	1.79
15	Elmira	17.65	33.99	1.71	12.13	42.56	0.39	0.23	0.7	0.18	0.13	20.46	3.96	1.66
16	Elmira	18.21	24.03	1.8	8.08	38.22	0.33	0.23	0.55	0.16	0.05	30.45	2.45	1.4
17	Fergus	14.09	14.76	1.48	9.45	39.05	0.34	0.22	0.75	0.15	0.03	37.33	4.92	2.57
18	Rockwood	14.73	20.78	1.59	10.09	41.1	0.48	0.22	0.59	0.16	0.05	32.18	3.44	1.24
19	Tavistock	20.26	20.01	1.79	8.81	38.31	0.33	0.23	0.75		0.03	30.22	4.02	1.76
20	Guelph	17.04	16.4	1.2	9.27	38.37	0.32	0.23	0.62	0.16	0.02	34.47	4.47	2.13
21	Guelph	14.38	15.42	1.5	9.23	38.63	0.37	0.23	0.77	0.15	0.02	39.89	3.76	2.36
22	Embro	24.29	21.33	2.42	8.13	34.54	0.31	0.23	0.49	0.17	0.04	26.83	3.28	1.38
23	Guelph	12.62	23.36	1.95	9.27	45.18	0.38	0.21	0.73	0.14	0.04	39.38	3.15	1.64
24	Rockwood	17.85	23.24	1.09	9.22	41.46	0.37	0.23	0.68	0.14	0.07	32.48	2.43	2.04
25	Elmira	21.79	25.31	2.99	10.59	40.5	0.33	0.21	0.62	0.19	0.07	17.51	2.15	0.7
26	Elmira	22.12	20.4	1.44	8.58	52.61	0.3	0.22	0.53	0.16	0.11	28.84	1.42	0.23
27	Elmira	18.66	17.87	1.87	8.74	39.88	0.27	0.21	0.62	0.18	0.01	29.54	3.86	1.95
28	Embro	21.87	20.1	1.59	8.15	35.76	0.3	0.23	0.61	0.17	0.03	29.64	3.33	1.72
29	Elmira	18.14	15.42	2.32	8.32	54.13	0.34	0.19	0.34	0.16	0.11	29.15	1.37	1.67
30	Rockwood	20.1	19.81	1.85	9.12	37.83	0.28	0.22	0.62	0.16	0.04	26.68	3.53	1.9

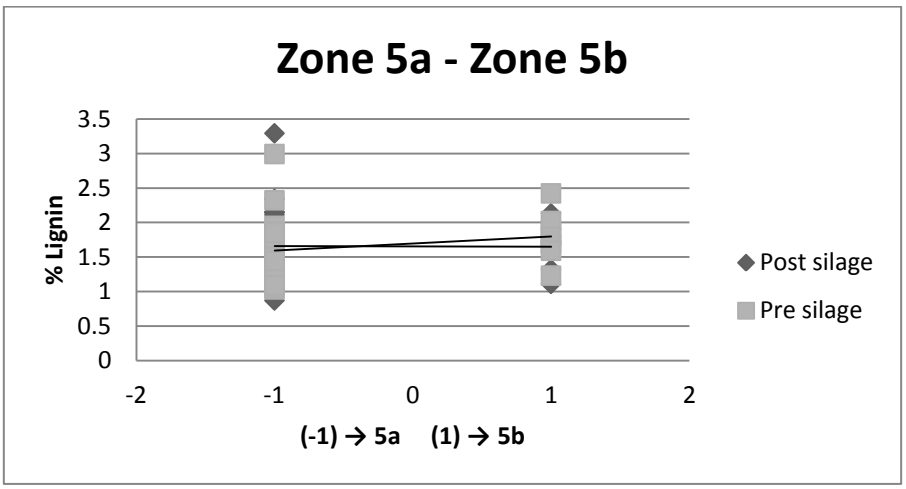
					PROTEIN		MINERALS					OTHER		
Post-Silage	Location	Cellulose	Hemicellulose	Lignin	Crude (%)	Soluble crude (% of CP)	Ca	P	K	Mg	Na	Starch	Total Ash	Crude Fat
2	Erin	17.96	17.72	1.92	9.36	39.38	0.39	0.22	0.81	0.17	0.02	27.6	4.76	2.16
3	Woodstock	20.78	19.93	2.02	8.25	35.4	0.42	0.23	0.64	0.18	0.05	30.42	4.24	2.01
4	Arthur	16.72	17.78	2.15	7.85	46.22	0.36	0.19	0.77	0.2	0.04	27.74	5.8	2.22
6	Embro	14.88	13.45	2.13	9.94	53.31	0.42	0.18	1.22	0.11	0.03	31.69	5.06	2.64
7	Woodstock	14.96	17.68	1.69	8.9	47.32	0.37	0.19	0.82	0.14	0.06	32.64	4.84	1.78
8	Elmira	17.36	15.09	1.34	8.65	48.01	0.32	0.21	1.18	0.18	0	34.77	4.97	2.5
9	Elmira	18.7	15.98	1.92	8.67	42.57	0.34	0.21	0.72	0.19	0.02	32.24	4.96	2.36
10	Guelph	18.93	16.79	1.34	9.37	40.83	0.41	0.2	0.97	0.15	0.02	33.02	4.75	2.51
11	Elora	15.98	15.72	3.29	8.68	51.69	0.35	0.18	1.31	0.19	0.04	28.85	4.47	3.01
12	Rockton	13.46	18.4	1.11	8.27	45.7	0.33	0.21	0.77	0.13	0.02	45.29	3.52	2.43
13	Rockwood	15.95	17.3	1.18	8.77	42	0.41	0.17	0.88	0.16	0.03	33.43	4.81	3.02
14	Rockwood	17.02	17.48	2.11	9.01	46.78	0.33	0.19	1.12	0.2	0.02	30.32	3.67	2.5
15	Elmira	14.92	17.86	1.52	8.82	42.89	0.32	0.26	0.89	0.18	0.02	38.5	4.66	2.18
16	Elmira	14.04	15.81	1.26	7.93	49.11	0.29	0.21	0.74	0.14	0.02	45.53	3.83	2.49
17	Fergus	13.67	15.35	1.13	8.84	44.42	0.38	0.21	0.98	0.15	0.02	39.33	3.87	3.16
18	Rockwood	14.06	14.57	0.87	7.69	48.78	0.35	0.18	0.74	0.13	0.02	43.91	2.96	2.66
19	Tavistock	13.21	21.35	1.32	8.22	45.88	0.35	0.16	0.72	0.11	0.03	43.35	3.02	2.13
20	Guelph	16.35	15.15	2.34	8.22	50.18	0.35	0.2	1.03	0.16	0.05	31.54	5.39	3.15
21	Guelph	14.12	16.34	1.43	8.43	46.45	0.36	0.22	0.94	0.14	0.01	38.15	3.52	3.14
22	Embro	16.57	16.17	1.7	8.23	50.28	0.39	0.18	0.79	0.13	0.04	34.56	5.32	2.19
23	Guelph	16.08	16.58	1.43	8.49	44.63	0.37	0.2	0.87	0.14	0.02	36.59	3.84	2.45
24		17.73	16.31	1.18	8.53	43.29	0.38	0.2	0.87	0.15	0.02	37.43	4.17	2.55
25	Elmira	12.61	16.85	2.15	8.39	51.46	0.34	0.21	0.79	0.15	0.02	44.09	4.02	2.46
26	Elmira	15.68	17.25	1.65	9.61	37.85	0.36	0.25	0.88	0.19	0.01	34.7	4.56	2.02
27	Elmira	19.95	16.41	2.14	8.04	43.5	0.33	0.26	0.87	0.21	0.02	29.78	5.49	2.09
28	Embro	17.36	16.05	1.58	8.33	46.47	0.38	0.18	0.78	0.12	0.04	35.78	4.37	2.04
29	Elmira	14.11	17.88	1.26	8.29	40.84	0.31	0.22	0.72	0.15	0.01	40.95	3.81	2.49
30	Rockwood	12.89	21.65	1.19	9.36	42.24	0.37	0.17	0.66	0.11	0.04	40.45	3.25	2.07

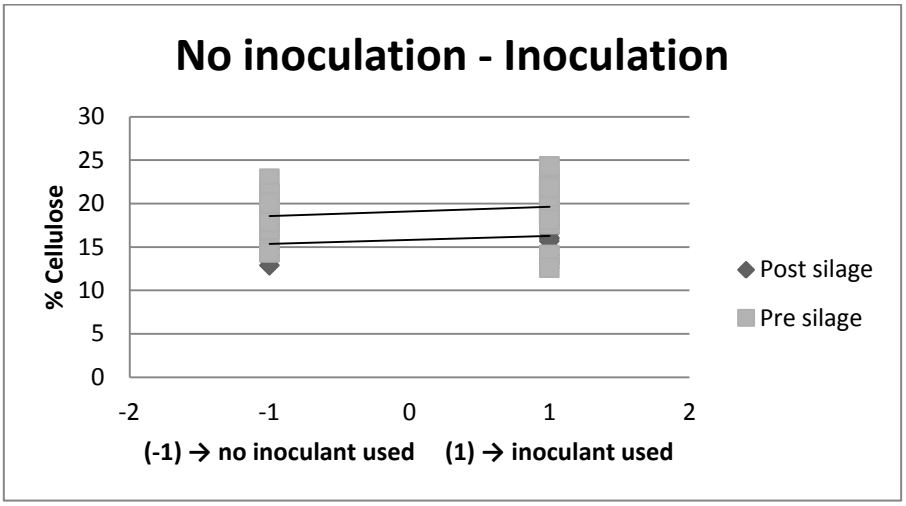
Appendix H

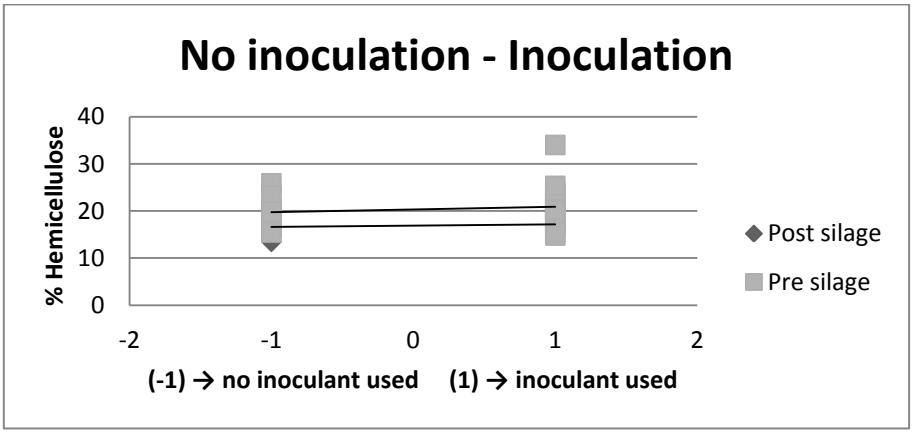
Statistical analysis by factorial design of data collected from corn silage

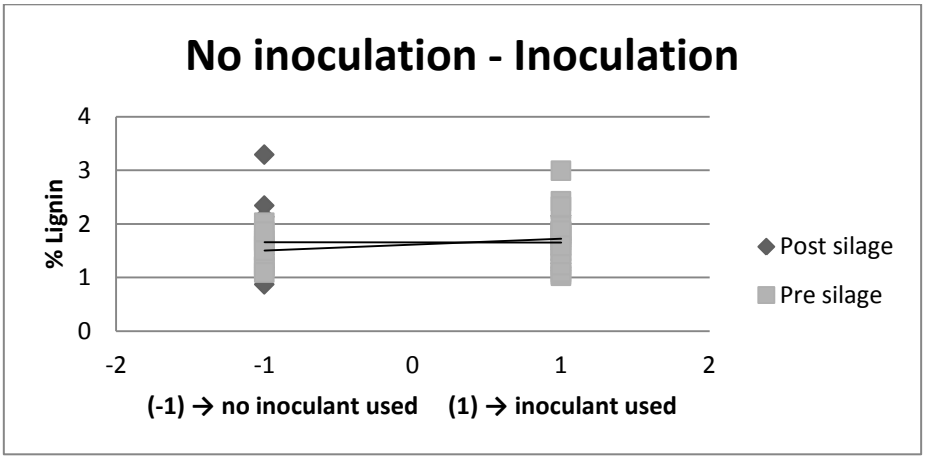


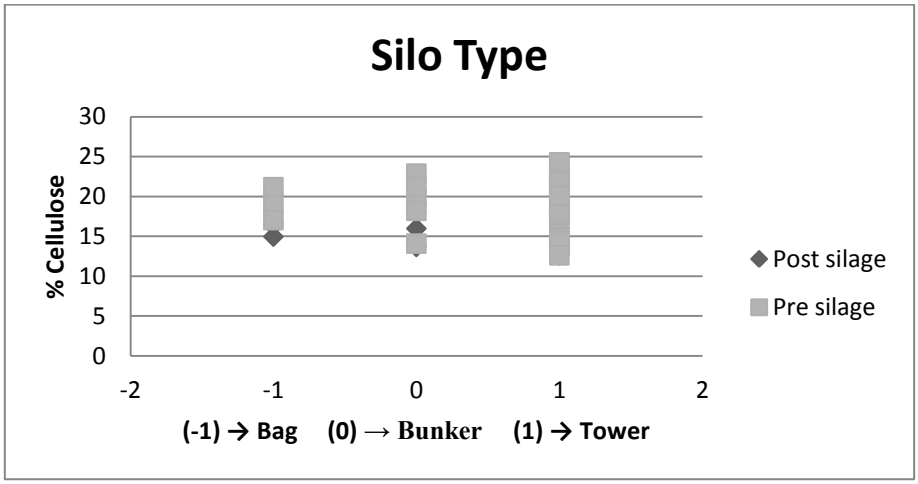


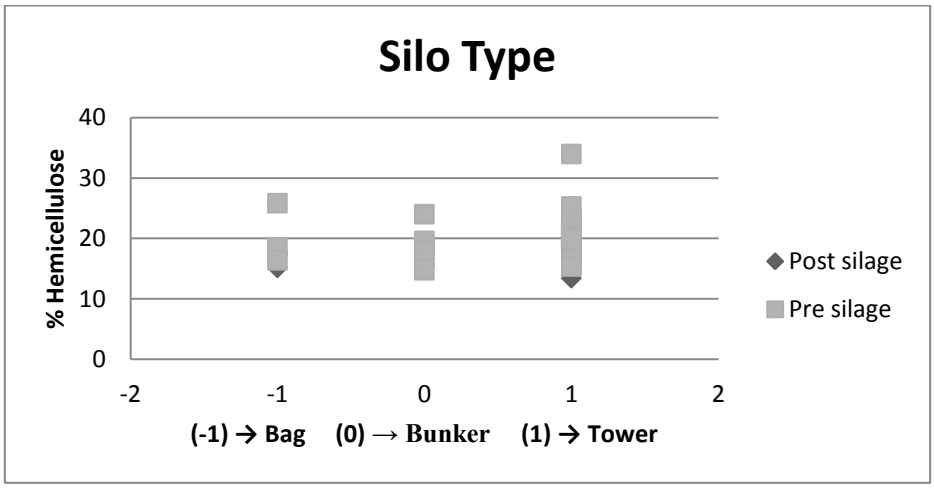


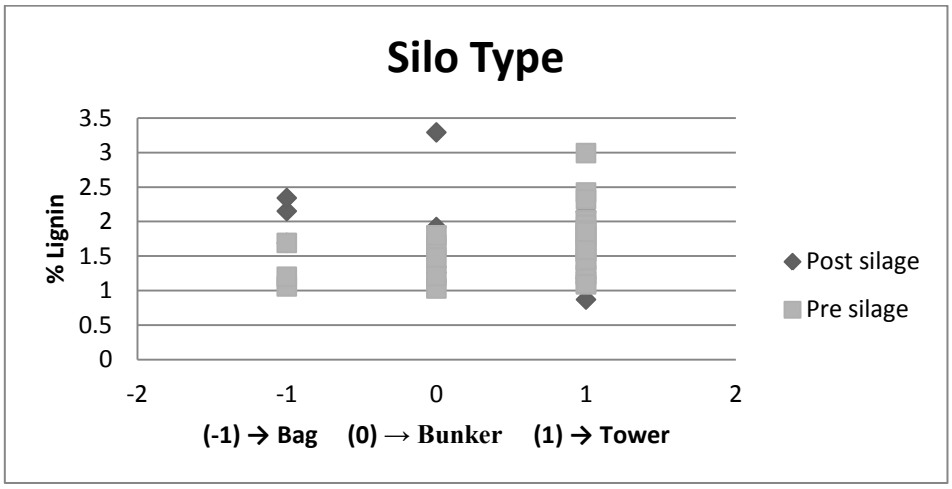

No inoculation - Inoculation
% Cellulose
30
25
20
15
10
5
0
-2
-1
0
1
2
(-1) → no inoculant used
(1) → inoculant used
Post silage
Pre silage


No inoculation - Inoculation
% Hemicellulose
40
30
20
10
0
-2
-1
0
1
2
(-1) → no inoculant used
(1) → inoculant used
Post silage
Pre silage


No inoculation - Inoculation
% Lignin
4
3
2
1
0
-2
-1
0
1
2
(-1) → no inoculant used
(1) → inoculant used
Post silage
Pre silage


Silo Type
% Cellulose
30
25
20
15
10
5
0
-2
-1
0
1
2
(-1) → Bag
(0) → Bunker
(1) → Tower
Post silage
Pre silage


Silo Type
% Hemicellulose
40
30
20
10
0
-2
-1
0
1
2
(-1) → Bag
(0) → Bunker
(1) → Tower
Post silage
Pre silage


Silo Type
% Lignin
3.5
3
2.5
2
1.5
1
0.5
0
-2
-1
0
1
2
(-1) → Bag
(0) → Bunker
(1) → Tower
Post silage
Pre silage

COMPOSITION - ANOVA

INITIAL ANALYSIS (Zone, Inoculant, Treatment)

ANOVA (2^3 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)

Cellulose content = +17.59321 - 1.66286 * Treatment
Hemicellulose content = +18.72089 - 1.76018 * Treatment
Lignin content = +1.65000 (no significant terms)
Starch content = +33.90214(no significant terms)
Ash content = +3.85250 + 0.50214 * Treatment
Crude protein = +8.75732 (no significant terms)
Crude fat = +2.13036 +0.31286 * Treatment

SILO TYPE (BAG VS TOWER)

ANOVA (2^4 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)
D) Silo Type: Bag (-1) → Tower (1)

Cellulose content = +17.26550 + 1.00550 * Inoculant used - 1.68152 * Treatment
Hemicellulose content = +18.94761 - 1.83848 * Treatment
Lignin content = +1.66
Starch content = +33.95
Ash content = +3.81978 + 0.53891 * Treatment
Crude protein = +8.76304
Crude fat = +2.08413 + 0.31065 * Treatment

SILO TYPE (BAG VS BUNKER)

ANOVA (2^4 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)
D) Silo Type: Bag (-1) → Bunker (1)

Cellulose content = + 17.64500
Hemicellulose content = +19.61786 + 2.14714 * Zone - 1.50750 * Treatment
Lignin content = +1.64063
Starch content = + 33.58312
Ash content = + 4.19000
Crude protein = + 8.63188
Crude fat = + 2.30688

SILO TYPE (BUNKER VS TOWER)

ANOVA (2^4 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)
D) Silo Type: Bunker (-1) → Tower (1)

Cellulose content = **+17.56292** - 0.25292 * Inoculant used - **1.67580 * Treatment** - 0.47208 * Silo type +**1.45708 * Inoculant used * Silo type**
Hemicellulose content = +18.74060
Lignin content = +1.64540
Starch content = +33.95860
Ash content = +3.77460 + 0.46140 * Treatment
Crude protein = +8.64748 - 0.27831 * Zone
Crude fat = +2.11640 + 0.33400 * Treatment

TGA - ANOVA

INITIAL ANALYSIS (Zone, Inoculant, Treatment)

ANOVA (2^3 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)

T5 = +195.69074
T10 = +254.16857 + 3.63857 * Zone
Tmax = +311.23104
Tmax weight = +61.15785
Peak rate = +8.06373 + 0.19399 * Zone - 0.14537 * Inoculant used + **0.32522 * Zone * Inoculant used**
0.5 Onset Temp = **+193.12932 + 8.10410 * Zone +** 0.94703 * Inoculant used **- 11.59745 * Treatment + 6.98621 * Inoculant used * Treatment**
0.5 Onset Weight = **+95.68237** + 0.12789 * Inoculant used + 0.88478 * **Treatment - 0.51042 * Inoculant used * Treatment**
1.0 Onset Temp = +236.83531 + 3.62359 * Zone
1.0 Onset Weight = +92.51497
2.0 Onset Temp = +257.90690 + 0.82424 * Zone - 0.37527 * Inoculant used - **1.48200 * Treatment + 1.39421 * Zone * Inoculant used**
2.0 Onset Weight = +89.43772

SILO TYPE (BAG VS TOWER)

ANOVA (2^4 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)
D) Silo Type: Bag (-1) → Tower (1)

T5 = +199.77805 - 1.60513 * Zone + 5.04638 * Inoculant used + 8**.88371 * Treatment + 7.99320 * Zone * Inoculant used + 12.08058 * Zone * Treatment**
T10 = +254.73043
Tmax = +310.98028
Tmax weight = +61.41103
Peak rate = +8.15628 +0.10144 * Zone - 0.19612 * Inoculant used + **0.37597 * Zone * Inoculant used**

0.5 Onset Temp = +194.63400 + 5.48854 * Zone + 2.30700 * Inoculant used **- 10.09014 * Treatment** + **6.41613 * Zone * Inoculant used** + 3.69542 * Zone * Treatment + **9.48101 * Inoculant used * Treatment** + **5.91132 * Zone * Inoculant used * Treatment**
0.5 Onset Weight = +95.78031 + 0.67852 * Treatment
1.0 Onset Temp = +238.60751 + 1.60667 * Zone - 1.19298 * Inoculant used + **2.90607 * Zone * Inoculant used**
1.0 Onset Weight = +92.63571
2.0 Onset Temp = +258.09859 + 0.63255 * Zone - 0.61440 * Inoculant used **- 1.32310 * Treatment** + **1.63334 * Zone * Inoculant used** + 0.60536 * Zone * Treatment + 0.65056 * Inoculant used * Treatment + **1.13016 * Zone * Inoculant used * Treatment**
2.0 Onset Weight = +89.74389

SILO TYPE (BAG VS BUNKER)

ANOVA (2^4 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)
D) Silo Type: Bag (-1) → Bunker (1)

T5 = +176.95000
T10 = +243.99286
Tmax = +311.13003
Tmax weight = +60.64047
Peak rate = +7.54190
0.5 Onset Temp = +179.09211 + 2.32019 * Inoculant used **- 13.22966 * Treatment** + **9.52546 * Inoculant used * Treatment**
0.5 Onset Weight = +95.49860 + 1.41072 * Treatment
1.0 Onset Temp = +230.11898
1.0 Onset Weight = +91.82267
2.0 Onset Temp = +256.75047
2.0 Onset Weight = +88.26499

SILO TYPE (BUNKER VS TOWER)

ANOVA (2^4 factorial design)

A) Zone: 5a (-1) →5b (1)
B) Inoculant use: No (-1) →Yes (1)
C) Treatment: Pre (-1) → Post (1)
D) Silo Type: Bunker (-1) → Tower (1)

T5 = +185.41042 - 0.83333 * Zone + **10.70417 * Treatment** + **16.50625 * Bunker vs Tower** + **11.84583 * Zone * Treatment**
T10 = +247.23875 + 8.12625 * Bunker vs Tower
Tmax = +310.58783 + 2.73894 * Inoculant used
Tmax weight = +61.06612
Peak rate = +8.05231 + 0.36392 * Zone
0.5 Onset Temp = +190.53527 + **7.91815 * Zone** - 1.58625 * Inoculant used - **10.19738 * Treatment** + 6.48864 * Bunker vs Tower + **5.86705 * Inoculant used * Treatment**
0.5 Onset Weight = +95.65991 + 0.17043 * Inoculant used + **0.81101 * Treatment** - **0.53914 * Inoculant used * Treatment**
1.0 Onset Temp = +231.09273 - 0.89509 * Inoculant used

-1.68651	* Treatment
+**6.88886**	*** Bunker vs Tower**
+**5.77934**	*** Inoculant used * Treatment**
-1.98213	* Inoculant used * Bunker vs Tower
+0.90228	* Treatment * Bunker vs Tower
-5.46135	*** Inoculant used * Treatment * Bunker vs Tower**

1.0 Onset Weight = +92.60117
2.0 Onset Temp = +258.11053

+1.10046	* Zone
-0.69205	* Inoculant used
-1.32239	*** Treatment**
+**1.23114**	*** Zone * Inoculant used**

2.0 Onset Weight = +88.76271 + 1.08547 * Bunker vs Tower

Appendix I

Raw data and statistical analysis of particle size-fractionated pre- and post-ensiled corn

		Weight per size fraction (g)			
		> 1mm	0.5 - 1.0mm	< 0.5mm	Total Weight
6	PRE	31.49	9.72	7.72	48.93
	POST	26.19	15.18	9.71	51.16
18	PRE	33.67	7.71	6.73	48.39
	POST	28.15	13.32	12.52	54.08
19	PRE	32.29	8.39	7.21	48.41
	POST	28.81	8.54	8.30	45.99
22	PRE	28.87	6.47	3.64	39.12
	POST	32.43	9.33	8.48	50.05
28	PRE	32.00	8.13	5.92	46.48
	POST	22.79	10.67	10.49	44.11

		% weight per size fraction		
		> 1mm	0.5 - 1.0mm	< 0.5mm
6	PRE	64.36	19.87	15.78
	POST	51.19	29.67	18.98
18	PRE	69.58	15.93	13.91
	POST	52.05	24.63	23.15
19	PRE	66.70	17.33	14.89
	POST	62.64	18.57	18.05
22	PRE	73.80	16.54	9.30
	POST	64.80	18.64	16.94
28	PRE	68.85	17.49	12.74
	POST	51.67	24.19	23.78
Average	PRE	68.66	17.43	13.32
	POST	56.47	23.14	20.18
St Dev	PRE	3.151	1.340	2.251
	POST	5.964	4.174	2.766
T-Test	P-values	0.01096	0.04977	0.00530