BIOCHEMICAL AND MICROBIOLOGICAL CHANGES IN SUGARCANE STALKS DURING A SIMULATED HARVEST-TO-CRUSH DELAY

by

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ABSTRACT

Post-harvest cane deterioration in the South African sugar industry results in significant revenue loss that is estimated to be in the region of ZAR 60 million per annum. Despite these large losses, precise biological data relating to the process of cane deterioration under South African conditions is limited. Severity of deterioration is influenced by a number of factors, including the length of the harvest-to-crush delay (HTCD), ambient temperature and harvesting practices. For example, burning of cane prior to harvest may result in rind splitting, which provides entry for microbes, particularly *Leuconostoc mesenteroides* that may exacerbate deterioration. The effect of these factors on deterioration was examined by quantifying the biochemical and microbiological changes that occur in sugarcane stalks after harvest, with the influence of length of HTCD, degree of *L. mesenteroides* infection and ambient temperature receiving attention. The primary novelty of the work resides in the analysis of deterioration under tightly regulated temperatures, which were designed to reflect diurnal variations typically experienced during summer and winter in the South African sugar belt. In addition, inoculation of mature internodes with a consistent titre of *L. mesenteroides* was used as a means to mimic a consistent level of infection of harvested stalks by the bacterium.

Metabolites selected for analysis were those both native to the stalk and produced as by-products of microbial metabolism, *viz.* sucrose, glucose, fructose, ethanol, lactic acid, dextran and mannitol. Simulated HTCDs under summer temperatures resulted in increasing glucose and fructose levels with time, which contrasted to the approximately constant levels of these hexose sugars under winter conditions. Commonly referred to as ‘purity’ in an industrial context, precise determination of the concentration of these hexoses in cane consignments could potentially indicate the extent of deterioration. Despite the detection of a basal concentration of lactic acid in unspoiled cane, the observed increase in concentration of this organic acid over the simulated summer HTCD suggests that this metabolite could also potentially serve as an indicator for post-harvest deterioration. In contrast, the investigation indicated that ethanol was an unsuitable biochemical marker for deterioration of *L. mesenteroides* infected cane. An inability to detect dextran and mannitol in the samples, combined with consistent sucrose levels and variable mill room data, suggest that extreme proliferation of *L.*

mesenteroides is facilitated primarily by in-field practices, particularly the manner in which cane is prepared prior to harvest and transport to the mill.

Bacterial proliferation and infection by *L. mesenteroides* of inoculated stalks were monitored by standard selective culturing techniques. Despite the limited detection of *L. mesenteroides*-associated metabolites, culture-based analyses revealed that the bacterium was the dominant bacterial species within the samples. A number of other bacterial species were isolated and identified, however the extent to which the total number of microorganisms proliferated was limited to a maximum of 1×10^5 colony forming units per gram of fresh tissue. In conjunction with these analyses, a molecular approach known as Polymerase Chain Reaction-Mediated Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was undertaken to investigate the bacterial diversity patterns associated with deteriorating sugarcane stalks throughout the delay period. In contrast to the results obtained by means of the culture-based assays, PCR-DGGE revealed that *L. mesenteroides* was not the dominant bacterial population, and showed that the level of bacterial diversity was relatively consistent across the differing treatments and with time. The use of complimentary culture-dependent and culture-independent analyses thus permitted the detection of this discrepancy and indicated the utility of PCR-DGGE in the determination of bacterial community structure of post-harvest sugarcane tissue.

The biology of post-harvest deterioration of green sugarcane stalks is highly complex, even under rigorously controlled temperature and infection regimens. The results of this study emphasize the important effects that harvest method and environmental conditions have on post-harvest sugarcane deterioration. Towards the formulation of industry-relevant recommendations for combating post-harvest deterioration, future work will strive to mimic the effects that harsh harvesting and transport practices have on the severity of the problem.

PREFACE

The experimental work described in this dissertation was carried out in the laboratories of the South African Sugarcane Research Institute (SASRI) and the University of KwaZulu-Natal (UKZN, Pietermaritzburg) under the supervision of Dr Derek Watt (SASRI and UKZN) and Mr Charles Hunter (UKZN).

DECLARATION: PLAGIARISM

I, **Lauren Anne Martin**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
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SUPERVISORS' DECLARATION

As Supervisors of the Candidate, we agree to the submission of this dissertation.

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Derek Watt

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Charles Hunter

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LIST OF ABBREVIATIONS

A_{600}	Absorbance at 600 nm
ANOVA	Analysis of variance
APS	ammonium persulfate solution
BHTCD	burn-to-harvest-to-crush delay
BLAST	Basic Local Alignment Search Tool
bp	base pair
BTTD	burn-to-truck delay
cfu	colony forming units
CTS	Cane Testing Services
DAC	Direct Analysis of Cane
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
E-value	expect value
FDA	fluorescein diacetate
FM	fresh mass
GC	guanine–cytosine
HPLC	High Performance Liquid Chromatography
HTCD	harvest-to-crush delay
LTM	long-term mean
MΩ	megaohm
MAU	milli absorbance units
MCA	monoclonal antibody
MHB	Mueller-Hinton broth
NCBI	National Centre for Biotechnology Information
NIR	Near Infrared Spectrometry
ng	nanogram
nm	nanometre
ND	not determined
PAD	Pulsed Amperometric Detection
PCR	Polymerase Chain Reaction

PES	phenylethyl alcohol sucrose
rDNA	ribosomal deoxyribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SA	South Africa
SASRI	South African Sugarcane Research Institute
SAST	South African Sugar Terminal
SASTA	South African Sugar Technologists' Association
SDS	sodium dodecyl sulphate
SMRI	South African Sugar Milling Research Institute
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	N,N,N',N'-tertramethylethylenediamine
Tm	melting point temperature
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TSA	Tryptic Soy Agar
U	units of enzyme activity
VHP	Very High Pol
ZAR	South African Rand

CHAPTER 1

INTRODUCTION

Like many other economically important staple food crops, *Saccharum* spp. are C_4 grasses within the Order Graminae (Barnes, 1974). However, instead of the value of this crop residing in grain starch, as is the case for many members of this Order, sugarcane has been selected by man specifically for the capacity of the plant to store high levels of sucrose in the stalk (Bull, 2000). A number of species of the *Saccharum* genus exist and most modern varieties cultivated for sucrose extraction are inter-specific hybrids of *S. officinarum* L. (the noble canes) and *S. spontaneum* (Brumbley *et al*., 2008). Sugarcane is grown worldwide between the latitudes of 35°N and 35°S (Babu, 1979) and at altitudes ranging from sea level to several thousand metres above sea level (Barnes, 1974). An average of 650 million metric tons of sugar is produced from sugarcane annually, from approximately 60 countries (Naik, 2001), with the South African industry contributing an estimated average of 2.5 million metric tons of sugar per season (Meyer, 2007a). In terms of industrial uses, sugarcane has been recognised as a highly efficient biomass producer and has been used in the co-generation of electricity, and the production of bioethanol (Birch, 2007).

South African sugarcane growers and millers constitute one of the world's leading sugar industries and are renowned for producing cost competitive, high quality cane sugar (Fry, 1998; Meyer, 2007a). The South African sugar industry combines the agricultural activities of sugarcane cultivation with industry-based production of raw and refined sugars, together with syrups, specialised sugars and a range of by-products (SASA, 2008). The combined industry makes major contributions towards the South African economy, particularly towards agricultural and industrial investments; the generation of foreign exchange earnings, and high employment opportunities. In addition to this are the major suppliers, support industries and customers linked directly with the South African sugar industry which contribute considerably to the economy as a whole. In terms of direct income towards the economy the South African sugar industry is responsible for generating an average of ZAR 7 billion per year on sugar sales alone (SASA, 2008).

Commercial sugarcane production worldwide is facing a number of physical challenges, including a decrease in arable land due to the spread of development, increased soil erosion and degradation, and decreased water quantity and quality (Kishore and Shewmaker, 1999). Furthermore, in developing countries, the combined effect of insect pests and pathogens is estimated to reduce crop yields by as much as 50% (Herrera-Estrella, 2000). Harsh environmental conditions, such as drought, floods and extreme temperatures also contribute to high yield losses. Thus, for the South African sugar industry to maintain its position as a leading sucrose producer, substantial investment in agricultural research and development is required, particularly to optimise resource utilisation and the efficiency of processes involved in growing and harvesting sugarcane.

At the South African Sugarcane Research Institute (SASRI), considerable research effort is devoted to the development of cost-effective solutions to the agronomic, bioresource engineering and logistical problems that beset the industry. The issue within this project is post-harvest sugarcane deterioration, which has a negative impact on many aspects of sucrose production, including the loss of sucrose in harvested stalks left in the field for extended periods and increased production costs in the mills that are caused by the accumulation of by-products of the deterioration process (Eggleston and Harper, 2006). Deterioration-associated problems are not limited to the South African sugar industry, and also plague many sugarcane growing regions throughout the world, including Australia, India, United States (Louisiana) and China (Kirby, 1968; Hidi and Staker, 1975; Clowes and Wood, 1978; Bacci and Guichard, 1994; Kulkarni and Warne, 2004; Mao, *et al*., 2005; Foster and Bryan, 2007; Eggleston *et al*., 2008). Financial losses caused by post-harvest cane deterioration in South Africa are calculated to be in the region of ZAR 60 million per season, based on deterioration-associated sucrose loss estimates of between 1.4 and 2.2 kg sucrose per metric ton cane (Ravnö and Purchase, 2005). Hence, knowledge of the deterioration process may lead to recommendations that could potentially save substantial revenue.

The degree of post-harvest degradation of sugarcane prior to crushing is affected by different factors, for example the time between harvesting and crushing (harvest-to-crush delays, HTCD), as well as temperature. Other factors, such as damage to the stalk resulting from mechanical harvesting and burning of the standing crop to remove foliage can also have a negative impact on post-harvest cane deterioration (Eggleston *et al*.,

2001). Damage to the stalk while in the field may provide entry points for microbes that increase the rate and extent of deterioration. Despite the large losses incurred by this process, only limited biological data are available on the subject of post-harvest sugarcane deterioration. This may be due to the complexity of the deterioration process within the sugarcane stalk, namely the difficulty of differentiating among biochemical, chemical and microbial degradation (Eggleston, 2002; Mao *et al*., 2005). However, the availability of this biological information, combined with the parallel analysis of economics, crop husbandry and logistics will allow for the development of effective management practices. This in turn will have a positive impact on factory efficiency due to the milling of better quality cane.

The deterioration process within harvested cane results in sucrose breakdown and the accumulation of a variety of deterioration products. The type and level of these products have been used in the past as a means to determine the level of deterioration, and have also been used to predict and control processing problems at the factory (Eggleston, 2002). Such products include high hexose (invert) concentrations and microbial contamination by-products, such as ethanol, lactic acid and polysaccharides. Heterofermentative lactic acid bacteria, namely those from the genus *Leuconostoc*, form a polysaccharide known as dextran that causes a rise in viscosity of the extracted sugarcane sap and crystal deformation, both of which have a negative impact on factory processing and sugar refining. Hence, the presence of dextran in cane juice and raw sugar is commonly used as a deterioration indicator. Dextrans and other metabolites (e.g. levan, alternan, mannitol, lactic acid, and ethanol) are products of the array of degradation-related enzymes secreted by microbial cells, which results in a cascade effect of enzymatic reactions bringing about the negative impacts mentioned above (Eggleston *et al*., 2004).

At present, the South African sugar industry does not incorporate a deterioration quality parameter into the sugarcane payment formulae, despite refineries penalising factories for excessively high contents of dextran in raw sugar (Eggleston *et al*., 2008). It is suspected that the incorporation of such a parameter into the sugarcane payment mechanism may effectively deter the delivery of overly deteriorated sugarcane, as well as reduce HTCD, improve sugar processing and encourage better overall management (Eggleston *et al*., 2008). The contentious nature of this problem cannot be overlooked,

however, with the practical implication of this proposed solution causing concern for both grower and miller alike. Other issues specific to the South African sugar industry include the wide climatic variability known to exist between sugarcane growing regions (Barnes, 1974) causing certain areas to experience deterioration associated problems more frequently than others (e.g. high frost areas). South Africa also has a unique situation where a large majority of producers are small-scale producers and who are not economically and logistically empowered to reduce HTCDs (Ravnö and Purchase, 2005). Subsidized systems have been set up between small-scale producers, and respective mill rooms to try and relieve this problem. Ideally these systems should allow for a more organised stream of cane harvesting, transportation and hence mill room supply among small-scale farmers to assist in alleviating high costs associated with independent transport systems and consequently lengthy HTCDs (Ridge and Norris, 2000).

Despite this, further investigation of post-harvest cane deterioration is presently being undertaken by SASRI to understand the biological processes that contribute to deterioration-associated sucrose loss and the production of undesirable products. In 2005, SASRI initiated a project entitled ‘Measuring and modelling the factors and effects of post-harvest cane deterioration’ on the basis of results from a meticulously designed and executed field trial undertaken in 2004 to investigate the effect of HTCDs on grower profitability (Lyne and Meyer, 2005). While the trial provided invaluable information to the South African sugar industry, it was concluded that analysis of deterioration under field trial conditions is highly complex, primarily due to climate unpredictability. A study of deterioration under controlled environmental conditions was thus deemed essential for determining the agronomic, biochemical, physiological and microbiological changes that occur in the mature sugarcane stalk after harvest; data which are fundamental to the development of reliable grower support systems. The major project was split into three sub-sections, firstly the core biochemical and microbiological analysis aimed at defining the relationships between quantifiable metabolites and bacterial activity under varying temperature conditions and HTCDs, presented herein. The second aspect of the project was to establish the identity of a metabolite signature pattern that may serve as a reliable and sensitive indicator of severity of cane damage with the use of an electronic olfaction device (Gastrow *et al*., 2007), and finally, with the use of data from the

aforementioned projects, to model the effect of temperature within harvested cane fields and stock piles on grower profitability.

The goal of the project described herein was to quantify the biochemical and microbiological changes that occur in sugarcane stalks after harvest, with the influence of length of HTCD, degree of *Leuconostoc mesenteroides* infection and temperature variation receiving particular attention. Strict temperature control during the post-harvest phase was achieved through the use of a custom-built incubator, which was able to simulate diurnal temperature fluctuations experienced under winter and summer conditions. Mature internodes were inoculated with *L. mesenteroides* to mimic the natural infection that occurs in stalks with rinds that have been damaged during burning and harvesting. The burning of cane immediately prior to harvest serves to remove leaves and is implemented widely in the South African sugar industry to ease harvesting. Stalks inoculated with the bacterium were incubated at regulated temperatures for nine days; a period which is in excess of the average HTCD reported for the South African sugar industry (Davis and Archery, 2007). Mature internodes were harvested at intervals throughout this period and were analysed for the concentrations of selected sugarcane metabolites and by-products of microbial metabolic activity. Parallel shifts in the composition of microbial communities were monitored by means of complementary culture-dependent and culture-independent techniques. In addition, stalks were subjected to analysis by techniques widely applied in South African sugarcane mills to provide industrially relevant data. The specific research questions addressed by this work were: the extent that (1) ambient temperature; (2) *L. mesenteroides* infection; and (3) HTCD have on sucrose loss; the appearance of hexoses, and the accumulation of deleterious microbial metabolites, respectively. Bacterial succession patterns were also analysed over the nine-day delay period to determine the effect that *L. mesenteroides* inoculation; ambient temperature; and HTCD have on endophytic bacterial populations within sugarcane stalks. The ultimate goal of the current and allied investigations is to provide empirical data for the development and validation of mathematical models to quantify losses incurred to the industry through post-harvest deterioration. Knowledge of the effects of ambient temperature and the extent of microbial infection (caused by rind damage) on such losses may facilitate the development of management advice to enable sugarcane growers to limit the negative consequences of deterioration on the profitability of their sugarcane farming activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Cause and effect of post-harvest cane deterioration

2.1.1 A South African perspective

Once sugarcane is harvested it begins to deteriorate, and the capacity of sugarcane to lose sucrose rapidly after harvest continues to be underestimated, and has been for a number of years (Alexander, 1973; Lyne and Meyer, 2005). It has been reported that the delay between harvesting and transporting to the mill room (HTCD) in Australia is kept to below 24 hours (Lionnet and Moodley, 1994), whereas in South Africa the average reported delay is thought to be approximately 68 hours (Giles, 2007). However in the small scale sector, burn HTCDs (B/HTCD) of several weeks are frequently observed (Davis and Archery, 2007). The rate and extent of post-harvest sugarcane deterioration is known to vary considerably, and is dependent on HTCDs, harvesting techniques and weather conditions (Brokensha *et al*., 1975). South Africa's main cane growing areas fall under a 'wet-dry' subtropical climate which is characterized by a strong seasonality and high variability of rainfall (Dyer and Gosnell, 1978; Blume, 1985; Bezuidenhout and Singels, 2007). As a result, the marked environmental variability experienced in South Africa may negatively affect deterioration rates further.

A large majority of South African sugarcane growers burn their crops prior to harvest (Meyer, 2007b). Burning cane is suspected to exacerbate post-harvest deterioration rates, with burnt cane left more exposed and reaching higher deterioration levels compared to green cane (Foster, 1969; Lionnet and Moodley, 1994). Cane that is burnt is thought to be more vulnerable to opportunistic microbial infection due to stalk rind splitting and loss of wax integrity, causing seepage of juice and an increased level of exposure (Lionnet, 1986a). Billeted or chopped cane, although less commonly seen in South Africa due to a low prevalence of mechanical harvesting, is also known to deteriorate more rapidly than whole stalk cane due to the larger surface area exposed to infection (Salassi *et al*., 2002, Eggleston *et al*., 2001). The method of cane loading and logistics may also affect cane deterioration rates, with bruised or damaged cane stalks

appearing to be more prone to deterioration than undamaged cane stalks (Munsamy, 2007).

In South Africa, major efforts have been undertaken to reduce B/HTCD by focusing on supply chain management and the development of effective benchmarking systems (Barnes, 1998; Wynne, 2001). The Sugar Logistics Improvement Programme (SLIP) was developed and implemented in certain regions in 2002 and has been extremely successful in highlighting particular problem areas around communication and information flows between the grower, the haulier and the mill (Perry and Wynne, 2004; Planting, 2005). As a result, regions making use of this system have managed to significantly reduce B/HTCD and best practices have also been identified in several mill regions which could potentially reduce delays further (Planting, 2005; Giles, 2007). The small-scale sector however, is fraught with a number of challenges (Mahlangu and Lewis, 2008), particularly regarding B/HTCDs, with poor coordination between harvesting and logistics negatively affecting supply chain management to the mill. Many small-scale farmers rely on contractors for harvesting and haulage services and thus have limited control over their B/HTCD (Nothard *et al*., 2004a; Nothard *et al*., 2004b). This issue is compounded by lack of disincentive for poor contractor services, who are not directly affected by extended B/HTCD (Nothard *et al*., 2004b). Despite the small percentage of sugarcane supplied by these growers to the mill compared to large-scale operations, the effects of these heavily deteriorated consignments on the milling process could potentially be just as detrimental as the delivery of large consignments of deteriorated cane.

2.1.2 Physiological effects of post-harvest deterioration

Limited published work is available regarding the physiological and chemical changes which occur within the sugarcane stalk after harvest (Mao *et al*., 2005; Watt and Cramer, 2008). Studies have shown that soluble acid invertase, neutral invertase, sucrose synthase and sucrose-phosphate synthase are all key regulators for sugar accumulation and degradation in sugarcane stem storage (Lingle, 1997; Mao *et al*., 2005). Simplistically, living sugarcane parenchyma (or sucrose-storing) cells in young, actively growing internodes will cycle sucrose to fructose and glucose monomers using an invertase enzyme (Lingle, 2004). In mature internodes, where higher levels of sucrose

are found, fructose and glucose are cycled to form sucrose by sucrose-phosphate synthase to be stored within the vacuole (Sehtiya *et al*., 1991). Once a sugarcane stalk is harvested, the stalk is no longer growing and photosynthesis ceases. The storage parenchyma cells within the entire stalk move from a sucrose accumulating function to an energy-producing, sucrose-consuming function, due to the maintenance of physiological integrity after harvest. Such ongoing stalk respiratory activity after harvest results in substantial sugar loss (Watt and Cramer, 2008). Further losses result from sucrose seepage through rind damaged during burning, loading and transport, as well as during cane washing at the mill (Lionnet, 1986b; Corcodel and Mullet, 2007; Munsamy, 2007).

Post-harvest sugar loss from cut stalks is further aggravated by microbial infection and subsequent deterioration. Sucrose is broken down by the microorganisms present endophytically and within the immediate external environment, providing an energy source for microbial growth and proliferation. Many microbially derived by-products also accumulate within the stalk, such as dextran, lactic acid, mannitol and ethanol (Figure 2.1), which often result in problems more deleterious than the simple loss of sucrose (Eggleston *et al*., 2004). The degree of post-harvest deterioration depends on the environmental conditions (Morel du Boil, 2001), with the nature of the microbial infection strongly influencing the type of microbial end-product produced.

Identifying visible symptoms of post-harvest cane deterioration is a challenge, since the effects of deterioration are largely exhibited within the stalk. Stalk dry matter would be the most obvious change observed between fresh and deteriorated cane, accompanied with a sour smell. This odour is derived from the acidity arising from partial degradation of hexoses by *L. mesenteroides* (Tilbury, 1968). At the mill room level, deteriorated cane has previously been detected through the measurement of ethanol and dextran levels prior to processing, and consignments of cane have occasionally been rejected on the basis of unacceptably high dextran levels (Rauh *et al*., 2001; Lionnet and Gooch, 2002; Saska, 2002).

2.1.3 Microbiology of post-harvest cane deterioration

Consideration of sugarcane pathology is of extreme importance in the pursuit of cost-effective production. As sugarcane is essentially a sucrose-producing plant and is widely grown in monoculture, the vulnerability of this crop to pathogenic infection, whether in the field or harvested, is inevitable. Due to its very nature, sugarcane is prone to consumption by anything from mammals to insects, and infection by yeasts, fungi, bacteria and viruses (Hughes and Mungomery, 1965). However, within the scope of this project, only bacteria have been studied.

Microorganisms are known for their ubiquity within diverse environments. An endophytic microorganism is the term given to microbes that can naturally inhabit the growing, inner tissue of a plant, such as within sugarcane stalks and generally causes no actual harm, or may even occur as a mutually beneficial entity (e.g. biological nitrogen fixation by endophytic diazotrophs) (James and Olivares, 1997; Asis *et al*., 2003). Epiphytic microorganisms are those which naturally dominate the surface of a growing plant, including sugarcane stalks, similarly without deleterious consequences (Danhorn and Fuqua, 2007). Epiphytic microorganisms can however cause opportunistic infections, should the surface of the stalk be compromised in a manner permitting their entry into the growing tissue; such microorganisms can then proliferate and hence cause infection (Holzapfel and Schillinger, 1992).

The large majority of known epiphytic microorganisms occurring in sugarcane are lactic acid bacteria, predominantly those from the genus *Leuconostoc* (Mundt *et al*., 1967), with further representation from the genera *Lactobacillus* and *Streptococcus* (Rauh *et al*., 2001). The most common bacterial species associated with post-harvest cane deterioration is *L. mesenteroides* (Holzapfel and Schillinger, 1992). Members from this genus are Gram-positive, slime-producing bacteria (Holzapfel and Schillinger, 1991) and, although known to be coccoid in shape, they can appear as short rods (Garvie, 1986). These microorganisms are heterofermentative and ferment carbohydrates to yield a variety of end products. Lactic acid bacteria are classically fastidious in nature (Whiteside-Carlson and Carlson, 1949; Garvie, 1986), with *Leuconostoc* species being aerotolerant, although proliferation is greater in the absence of oxygen. They utilize carbohydrates, amino acids and peptides, fatty acids, nucleic acids and vitamins, and

prefer a neutral to slightly acidic pH (Holzapfel and Schillinger, 1991). *Leuconostoc mesenteroides* may produce a number of different by-products (Figure 2.1); based on different enzymatic pathways and the formation of these by-products varies with certain metabolic conditions (Eggleston *et al*., 2004).

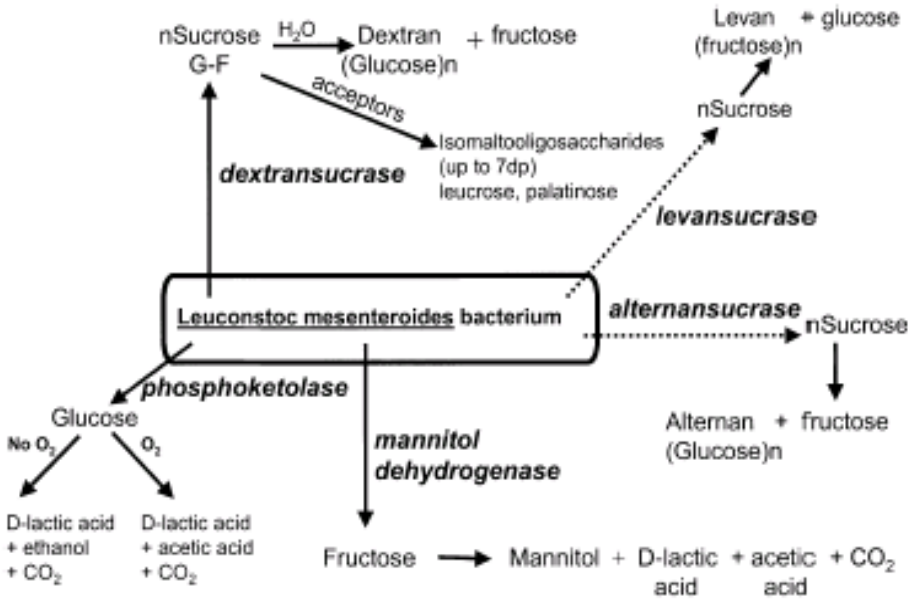


Figure 2.1: *Metabolic and enzymatic pathways of* Leuconostoc mesenteroides *involved in sugarcane deterioration* (Eggleston *et al*., 2004).

Dextran formation, as a direct result of *L. mesenteroides* infection, is considered to be the most detrimental to the milling process due to it being a high viscosity polysaccharide produced in high concentrations. The negative effects of this polysaccharide are exerted from extraction through to exhaustion and crystallisation (Atkins and McCowage, 1984). However, other polymers formed, such as levan, alternan and sarkaran, albeit in lower concentrations, also contribute to the negative effects of post-harvest cane deterioration (Morel du Boil, 2000; Eggleston *et al*., 2004).

It has been reported that, on living undamaged sugarcane tissue, members of the *Leuconostoc* genus appear in relatively low numbers as they have to compete with far less fastidious Gram-negative bacteria and aerobic spore formers (Holzapfel and Shillinger, 1992). Their presence is reportedly associated with the amount of released

nutrients from damaged or decaying plant material (Stirling and Whittenbury, 1963). Thus, when sugarcane is nearing maturity and nutrients become available through the initiation of the harvest season, their numbers increase resulting in the development of opportunistic infections (Holzapfel and Schillinger, 1992). The knowledge of other bacteria contributing to the deteriorative process in harvested sugarcane is fairly limited; however work done by Tilbury (1968) found that the homofermentative organisms *Lactobacillus plantarum* and *Lactobacillus casei* were equally as numerous as *L. mesenteroides* after 10 days storage. Other microorganisms found in first expressed juice at the mill include *Enterobacter aerogenes* and *Enterobacter cloacae*, and from fresh juice various *Bacillus* species have been isolated, together with bacteria from the genera *Achromobacter, Flavobacterium, Micrococcus, Streptomyces* and *Escherichia* (Tilbury, 1968; Bevan and Bond, 1971). Although not included in the present study, yeasts such as those from the genera *Torulopsis, Candida, Rhodotorula, Saccharomyces* and *Pichia* have been found, while fungi such as *Monilia sitophila*, *Aspergillus niger*, *A. flavus*, *Penicillium spp.*, *Rhizopus spp.*, and *Trichoderma viride* have also been obtained (Tilbury, 1968; Bevan and Bond, 1971).

The types of microorganisms associated with the harvested sugarcane stalk, and their respective population sizes, depend on a number of different factors, namely environmental conditions, the particular type of harvesting techniques used, and the time delay between harvesting and crushing. For example, the prevalence of *L. mesenteroides* infection and consequent dextran and mannitol formation is well documented in Louisiana where mechanical harvesting and predominantly wet weather conditions present conditions ideal for proliferation of the bacterium (Eggleston *et al.*, 2008). It is likely that prevailing environmental conditions may ultimately determine which bacterial species will gain pre-eminence.

2.1.4 Bacterial diversity analysis using PCR-DGGE

Microbial analysis of post-harvest sugarcane deterioration has previously been done using cultivation based techniques. Only limited data derived from recently developed molecular technologies are available. Conventional culture-dependent methods using selective enrichment fail to accurately mimic the conditions that particular microorganisms require for proliferation in their natural habitat (Muyzer *et al.*, 1993).

Therefore, culture-independent techniques, such as those provided by molecular technologies, offer an alternative means for the analysis of microbial ecology and genetic diversity. Molecular techniques provide insights into species composition by exploiting sequence variation in rDNA. Such sequence variations permit inference of phylogenetic differences among microbial populations (Muyzer *et al*., 1993).

PCR-DGGE is a method that allows for direct determination of the genetic diversity of complex microbial communities. Unlike most commonly used electrophoretic methods that separate amplified nucleic acid fragments by size, DGGE separates DNA strands of the same length based on their sequence composition (Nakatsu, 2007). The chemical denaturants, urea and formamide, are incorporated into a polyacrylamide gel in a linear gradient. Differential migration between fragments of the same length occurs because an increased concentration of denaturant is required to separate higher guanine (G) - cytosine (C) content as a result of the number of hydrogen bonds between complementary nucleotides (Nakatsu, 2007). As the DNA strands separate with increasing concentration of denaturant, their migration is retarded in the gel. Hence, higher GC content fragments migrate further during polyacrylamide gel electrophoresis, as a result of their higher melting point temperature (Tm). Complete double-stranded DNA separation is prevented by adding a high GC sequence (GC clamp) to the end of one PCR primer, where the choice of PCR primers allows one to selectively amplify the sequence of interest before DGGE is used. The target gene should contain both conserved and variable regions of sequence (Nakatsu, 2007), and within the scope of this study primers corresponding to the variable V3 region of the 16S rDNA are used to amplify all bacterial DNA present within the homogenized sugarcane tissue.

The application of PCR-DGGE in determining bacterial diversity in different environmental niches is well reported (Heuer *et al*., 1997; Yang *et al*., 2001; Sun *et al*., 2004; Das *et al*., 2007). Application in this instance, coupled with culture-dependent analyses, is intended to highlight the major bacterial populations present in harvested sugarcane stalks, and their persistence throughout the deterioration period under typical South African conditions. PCR-DGGE will also allow for comparisons to be drawn between cultivation techniques and molecular techniques, and represents the first of its kind at SASRI.

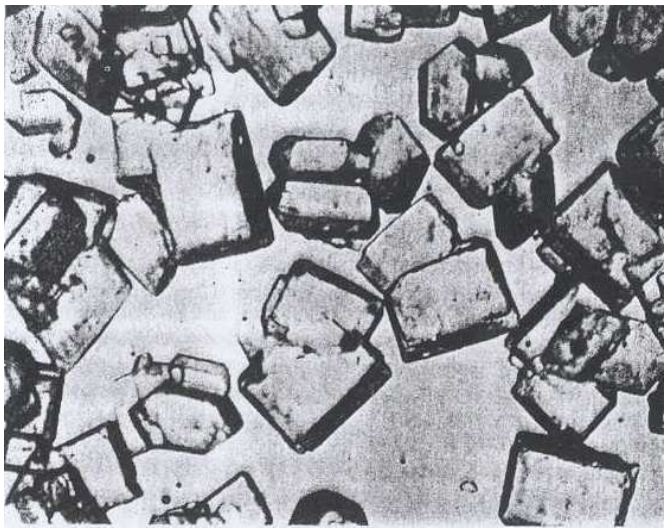
2.1.5 The impact of deterioration on processing and sucrose recovery

Deterioration of harvested sugarcane is characterised by a number of changes in stalk biochemistry, including a decrease in pH, due to lactic acid accumulation, an increase in hexose (invert) concentrations, and the accumulation of oligosaccharides, polysaccharides and sugar alcohols (Lionnet, 1986b; Ravelo *et al*., 1992; Eggleston *et al*., 2001; Saska, 2002). The proliferation of microorganisms, particularly *L. mesenteroides*, within harvested stalks is reported to most negatively affect processing and sucrose recovery (Purchase, 2001; Ravnö, 2001; Eggleston, 2002), primarily due to the production of the bacterial polysaccharide, dextran (Cerutti de Guglielmone *et al*., 2000). Dextran is a polyglucan, with a high percentage of alpha-1-6 linkages and varies in size from very small and soluble to very large and insoluble (Rauh *et al*., 2001). Energy from the glucose-fructose bond in sucrose is used by bacteria and the free glucose remaining is linked into a growing molecular chain or polymer to form dextran (Ravnö and Purchase, 2005).

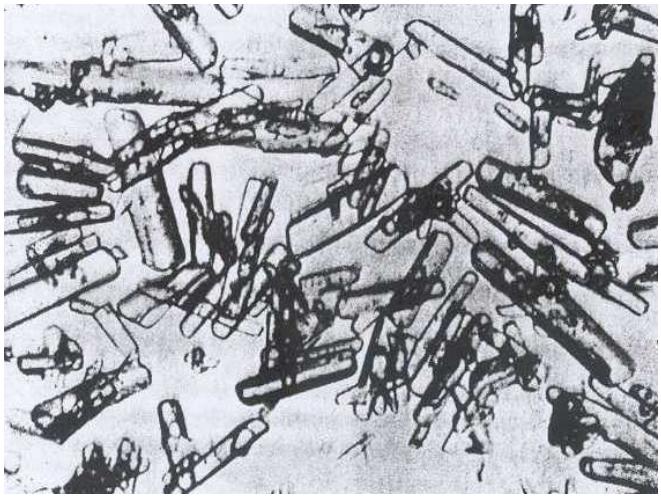
The level of dextran within the factory mixed juice is effectively the sum of dextran accumulated within the stalk, before and after harvest, the dextran formed between the harvest and crushing (harvest-to-crush delay), and the dextran formed during the milling process as a result of unhygienic practice, and or recirculation of infected juice (Rauh *et al*., 2001). Firstly, the presence of dextran within mixed juice indicates that sucrose has been lost, which directly affects sucrose recovery and thus profit margin (Mackintosh, 2000). The lost sugar causes a further reduction in mixed juice purity, thereby reducing further recovery of the remaining sugars (Rauh *et al*., 2001). Dextran increases sugar solution viscosity, decreases clarification efficiency, lowers evaporation rates and reduces heat transfer and thus negatively affects boiling times and centrifugal flushing leading to a decrease in factory capacity (Rauh *et a*l., 2001; Ravnö and Purchase*,* 2005).

The effects of dextran on crystal growth are more problematic, as dextran negatively affects the fastest growing faces on the sugar crystal, slowing the production of correctly-sized crystals and inducing a negative cascade effect downstream of the entire factory process (Morel du Boil, 1991; Koster *et al*., 1992). The visible result is elongated, misshaped crystals (Figure 2.2 B) which are fragile with a tendency to break during

centrifugation with resulting loss through centrifuge screens (Ravnö and Purchase, 2005).



(A)



(B)

Figure 2.2: *Sugar crystals obtained from burnt, mechanically-harvested cane*. (A) No harvest-to-crush delay. (B) Three days delay between harvesting and crushing. Magnification x 20 (Tilbury, 1968).

A rise in viscosity and the changing of crystal growth caused by dextran in juice and consequently in raw sugar leads to poor mill and refinery throughput and contributes to a higher sugar content remaining in the final molasses (Eggleston *et al*., 2006). Combined

with this is the common presence of dextran in the refined sugar product; a situation of which refiners are becoming far less tolerant (Ravnö and Purchase, 2005). The world market for raw sugar is extremely competitive and thus appropriate sugar quality is of paramount importance when securing a favourable price (Fry, 1998). Internationally, raw sugar is sold in classes where South Africa produces VHP sugar ('Very High Pol', or very high polarity), where Pol is defined as the percentage of sucrose present in a mass of raw sugar. VHP sugar requires a Pol of over 99.3% (Mackintosh and Kingston, 2000). South African sugar quality standards need to be strictly controlled to ensure that a favourable export price is maintained.

Possible solutions to the problems associated with post-harvest deterioration have been explored and include the application of anti-bacterial solutions, or disinfectants, either onto the cut ends of harvested stalks (Kulkarni and Warne, 2004), or directly onto the prepared cane before crushing (Ramos *et al*., 1992; Solomon *et al*., 2007). Another widely accepted remedy is the use of dextranase to enzymatically remove dextran from the milling stream to avoid the negative consequences of this polysaccharide on the extraction process (Hidi and Staker, 1975; Morel du Boil and Wienese, 2002; Foster and Bryan, 2007). However, the application of dextranase poses a number of practical challenges, particularly in terms of optimizing enzyme strength, dosage and temperature stability, as well as the obvious cost implication of effectively incorporating this enzyme into the milling process (Morel du Boil and Wienese, 2002; Eggleston *et al*., 2006). As South Africa has not yet introduced a penalty system for the delivery of heavily deteriorated cane to the mill, the likelihood of incorporating costly amendments into the milling process to reduce the effects of deteriorated cane is low.

2.2 Measuring post-harvest cane deterioration

2.2.1 Past to present

The negative effect of bacterial polysaccharides in sugar processing has been recognized for more than 100 years (Rauh *et al*., 2001). However, minimal information is available to elucidate the microbial, enzymatic and chemical reactions that contribute to the post-harvest deterioration process (Eggleston, 2002). Inaccurate purity measurements determined on the basis of optical rotation (or 'Pol') has been used

previously to compare sucrose loss in cane juice samples. Lately, however, the Cane Testing Services (CTS) of the South African Sugar Association have instituted random analytical testing of cane samples to determine deterioration. Such analyses include the measuring of glucose and fructose (invert) concentrations and monitoring levels of deterioration-associated products, such as ethanol and lactic acid (or the associated decrease in pH) (Lionnet and Pillay, 1988; Smith, 1993; Saska, 2002). Such values are routinely measured by a number of factories through implementation of the Direct Analysis of Cane (DAC) methodologies. However, due to time constraints, these measurements are not usually performed prior to the corresponding cane consignment being crushed, thus results are not always immediately available for subjective analysis (Lionnet and Gooch, 2002). In addition, caution should be employed in relating the DAC data directly with deterioration. For example, research has shown that the ethanol content of DAC extracts does not correlate with the more widely recognised dextran content associated with deterioration (Ravnö and Purchase, 2005).

The development of a specific analytical technique to determine dextran levels is reported to be difficult, particularly due to apparent variability in the structure of the polysaccharide (Brown and Inkerman, 1992; Singleton *et al*., 2002). Considerable debate has been generated on this issue, and consensus regarding a standardised analytical protocol is yet to be reached. Consequently, a number of different methods are still in use. For example, the 'Roberts' or 'AOAC' method (Roberts, 1983), previously used at the South African Sugar Terminal (SAST), was originally thought to have advantages of specificity and ability to embrace the entire molecular weight range; however, in practice, it is better suited to measuring high molecular weight dextran as opposed to total dextran (Ravnö and Purchase, 2005). The 'Haze' or 'ICUMSA' method (Nicholson and Horsley, 1959) has been more widely adopted due to its apparent ease of use and precision (Morel du Boil, 2000). Other techniques available include the 'MAU' (milli absorbance units) method (Ravnö and Purchase, 2005), the 'Enzyme – HPEAC' (high performance anion exchange column) method (Morel du Boil, 2000), and the 'MCA', (monoclonal antibody) method (Rauh *et al*., 2003).

2.2.2 Current possibilities

Recently, a number of cane deterioration products have been identified to predict and control processing problems at the factory level (Eggleston, 2002; Lionnet and Gooch, 2002; Eggleston and Harper, 2006; Corcodel and Mullet, 2007). However, reports suggest that a pre-determined cane deterioration product is only useful if it can be related to some negative association with factory processing (Lionnet, 1996). More recently a number of oligosaccharides and sugar alcohols have been targeted as deterioration-indicators, with the formation of total oligosaccharides reported to be higher than that of dextran and ethanol than previously realized. Hence, total oligosaccharide concentration may serve as a more sensitive indicator of cane deterioration than dextran alone (Ravelo *et al*., 1991a). Possible oligosaccharides under investigation include palatinose, leucrose, iso-maltotriose and 1-kestose (Morel du Boil, 1995; Eggleston and Legendre, 2003). More recently, considerable attention has focused on the sugar alcohol, mannitol, which is reported to be a sensitive indicator of sugarcane deterioration that directly affects factory processing and can predict problems from dextran and levan polysaccharides (Eggleston and Harper, 2006).

Due to the uncertainty of the nature, extent and result of post-harvest cane deterioration in South Africa, there is a reluctance to adopt new methods presently being proposed for enhancing factory hygiene, primarily for economic reasons. This uncertainty, coupled with resistance to change, is limiting control of this problem at the factory level. Hence, this aspect of the larger project has been designed to analyse the specific biological factors relating to post-harvest cane deterioration to gain support for more stringent factory control measures.

The second component of the larger project aims to use biological data such as moisture levels, cane weight, Pol % and non-Pol % during controlled deterioration trials to develop a functional-level cane deterioration model. The model outputs will not only be based on the controlled environment data, but will also be evaluated against commercial cane that has been harvested and transported under standard operational conditions. Ultimately, the model will permit assessment of different commercial cane supply scenarios to predict and thus control deterioration-associated losses.

2.3 Controlling post-harvest cane deterioration and its affects

2.3.1 Practical methods

The primary means to reduce post-harvest cane deterioration would be to keep HTCDs to a minimum, particularly where burning of cane prior to harvesting is concerned (BHTCD) (Davis and Archery, 2007). A recent study found that the average BHTCD in SA is between 60 and 70 hours, and over 90% of the total delay occurs between burning, cutting and loading onto the cane delivery system, which is referred to as the burn-to-truck delay (BTTD) (Ravnö and Purchase, 2005). Practical guidelines to reduce BTTD were summarised by Ravnö and Purchase in 2005 and include: the preferred use of green cane harvesting over burning; or if not possible, to burn daily and in the mornings. The next imperative would be to load and remove all harvested cane from the field the same day that it was burnt or cut. Another proposal is to introduce and encourage group harvesting between growers, particularly amongst the small scale grower community and, finally, to facilitate priority processing of accidental fire or severely frosted cane (Ravnö and Purchase, 2005). Simulation modelling of sugarcane harvesting and transport delays has been carried out with reasonable success, highlighting important focus areas for reducing HTCDs further (Barnes, 1998, Barnes *et al*., 2000).

The second means to control the effects of cane deterioration would involve the early detection and rejection at the mill-gate of severely deteriorated cane consignments. However, a rapid and accurate means to measure the extent of deterioration of each cane consignment delivered to the mill remains elusive. Possible methods for such early-warning systems include dextran immuno-assay kits (Ravnö and Purchase, 2005), on-line measurement of ethanol (Lionnet and Gooch, 2002) and electronic olfaction systems (Gastrow *et al*., 2007).

Once deteriorated cane has been introduced into the factory, the presence and build up of dextran becomes inevitable and it is at this stage that the use of dextranase enzymes becomes feasible. The enzymic removal of dextran is possible within the factory setting (Morel du Boil and Wienese, 2002), but is an expensive process. In particular, the lack of thermal stability of dextranase enzymes limits the extent to which they may be applied

within the factory (Eggleston *et al*., 2006). Further research has been recommended within the South African industry to establish the feasibility of applying these enzymes on a production scale (Ravnö and Purchase, 2005).

The stringent control of mill hygiene, in instances where high quality juice may become microbially contaminated from previously crushed deteriorated cane, is a further means by which deterioration may be limited (Amorim *et al*., 2000). Ultimately, however, the responsibility of controlling post-harvest cane deterioration and its negative effects on factory processing falls on the shoulders of growers and the millers alike. Therefore, the cooperation of both partners is essential to solving key issues associated with post-harvest sugarcane deterioration. Until levels of dextran in raw sugar are more strictly controlled, it has been recommended that sugar terminals receiving raw sugar should segregate poor and high quality sugar so as to avoid refinery problems associated with dextran (Ravnö and Purchase, 2005).

2.3.2 Future method for early detection of deterioration at the mill

The third and final component of the larger project involves the proposed use of an electronic olfaction device (or e-nose) to detect deterioration. The e-nose instrument works by capturing volatile compounds in close vicinity and measuring these compounds based on their apparent concentration levels. It is supposed that as cane deteriorates the level of volatiles emitted would be increased or heightened such that one could theoretically relate the level of volatiles to the extent of cane deterioration. This could potentially prove to be a powerful tool whereby mill rooms may have the capacity to detect deterioration levels of cane consignments prior to the cane entering the factory process (Gastrow *et al*., 2007). Such an approach would avoid the negative effects that deteriorated cane has on the mill processing, rendering growers responsible for restricting the extent of post-harvest cane deterioration and millers responsible for the maintenance of exemplary factory hygiene.

CHAPTER 3

MATERIALS AND METHODS

The primary objectives of this project were to elucidate the influence of temperature, time and microbiological presence on sugarcane post-harvest deterioration. Therefore, the experimental design was developed according to these specific intentions, allowing, as far as was reasonably practicable, for each variable to be tested independently. The core experiment involved the two simulated harvest-to-crush delay scenarios, in which harvested stalks were placed in a custom-built incubator that precisely simulated average diurnal temperature variations experienced in the sugar industry during winter and summer. The simulated delays were of nine days duration, with sampling on days 0, 2, 4, 7 and 9. In addition, stalks were inoculated with *L. mesenteroides* at harvest (Day 0) to mimic highly infected cane, while control stalks remained uninoculated. Changes in the concentration of selected sugarcane metabolites and microbial metabolic by-products were assessed over the nine day period. In addition, selected samples were taken for mill room analysis and molecular analysis of bacterial community structures by means of PCR-DGGE.

3.1 Plant material

The sugarcane cultivar *Saccharum* spp hybrid cv. N19 (N19) was selected for this study as it represented approximately ten percent of the total area under sugarcane in the SA Industry for the 2006 – 2007 growing season (commencement date of this study was January 2006) (Davis and Archery, 2007). This variety also represents a large proportion of cane milled in areas where HTCDs have previously been demonstrated to result in processing problems in the mill, for example the high elevation Midlands region. A further criterion underlying the choice of N19 was the early-ripening characteristic of the variety. Early-ripening allowed for experimentation to be conducted earlier in the harvesting cycle than would have been possible with the other variety, *Saccharum* spp hybrid cv. N12 (N12), that is also widely grown in areas of the industry in which post-harvest sugarcane deterioration is severe.

The extreme difficulty in ensuring consistency of temperatures during different cane burning events precluded the use of burned cane in this study, despite the widespread burning of cane immediately prior to harvest in the Midland areas of the industry. The intention of burning is to reduce the difficulty of harvesting through the removal of excess dry leaf material from the stalks, even though the practice is known to exacerbate deterioration during HTCDs. As a result, green (a term used within the industry to designate unburned cane), mature or ripe N19 cane of approximately 18-months of age was used. In this context, the 'mature' or 'ripe' denotes cane in which sucrose concentrations had reached a maximum in the mature internodes. To ensure accuracy of plant age and consistency of growth conditions, the sugarcane used for this project was cultivated on-site at the SASRI Mount Edgecombe site in KwaZulu-Natal, South Africa. This also facilitated rapid turn-around times between harvesting, treatment and incubation, resulting in a maximum sample preparation time of approximately 4 hours (08h00 to 12h00). Due to the limits on the volume of cane that could be accommodated within the incubator, two harvests were conducted, within four weeks of each other, to minimise variation within field material, which may have confounded detection of the simulated seasonal effects.

3.2 Preparation of plant material

On the day of harvest, stalks of similar developmental stage were selected from various sugarcane stools to minimise inter-stalk variation with regards to internode sucrose concentrations. In addition, stalks without obvious superficial damage were selected to avoid microbial contamination. Once harvested, leaves were carefully removed from each stalk (a process referred to as 'trashing'), with the upper part of the stalk being trimmed at the natural breakage point (between internode numbers 3 and 4) (van Dillewijn, 1952) (Figure 3.1). Sterile techniques were employed during subsequent processing of the harvested stalks to reduce external microbial contamination. This involved setting the harvested and trashed stalks onto clean plastic sheets, and with the use of gloves, measuring and trimming the base of the stalk to a final length of approximately 1.4 metres with a measuring tape to ensure stalks would fit into the incubator and to remove the base of the stem possibly contaminated during harvest. The stalks were sprayed down with 70% (v/v) ethanol, and all the instruments used in the field were swabbed with 70% (v/v) ethanol between procedures. All sample processing

was conducted as quickly as possible to reduce the chance of environmental microbial contamination.

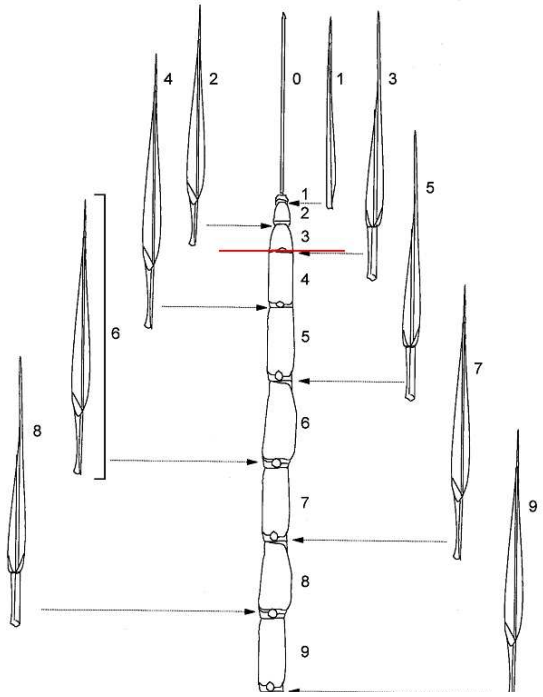


Figure 3.1: *The upper section of a sugarcane stalk showing leaves 1 to 9 and internodes 1 to 9.* The natural breakage point is indicated with the red line. Adapted from van Dillewijn (1952).

3.3 Simulating harvest-to-crush delays: Treatments

3.3.1 Inoculation with L. mesenteroides

Natural *L. mesenteroides* infection levels during harvesting of sugarcane are highly variable and depend on different factors, including the extent of surface damage to the sugarcane stalk. Burning of sugarcane prior to harvesting, which is very common in the SA sugar industry (approximately 90% of SA sugarcane, (Davis and Archery, 2007)), is reported to be another primary cause of *L. mesenteroides* infection. Other plant surface damage may arise from the invasion of stalk borers as well as poor harvesting techniques (manual or mechanical harvesting). The rate and extent of bacterial infection may also increase when damaged sugarcane stalks become exposed to soil and / or dirt. The season in which sugarcane is harvested also plays an important role in the

level of *L. mesenteroides* infection, with warmer, summer months being more favourable for *L. mesenteroides* proliferation, particularly during rainy or heavy dew periods (Ravnö and Purchase, 2005). To consistently and accurately simulate *L. mesenteroides* infected sugarcane stalks on the day of harvest, artificial inoculation of *L. mesenteroides* cells into internode 11 (the mature region) of each experimental stalk was conducted.

a. Isolation

The culture of *L. mesenteroides* used as the inoculum in this project was isolated previously from a fermenting stock-pile of mill room waste at Mount Edgecombe. Isolation was by means of selective culturing on PES media (3.5.1c) followed by confirmatory identification with the Polymerase Chain Reaction and *L. mesenteroides*-specific oligonucleotide primers (3.5.1d). Aliquots of the inoculum were maintained as glycerol stocks (3.5.1b) at -84°C.

b. Culture and maintenance

Modified Mueller-Hinton Broth (MHB), (Atlas, 1997) was used to cultivate the *L. mesenteroides* cells from the glycerol stock (3.3.1a). This broth comprised acid hydrolysate of casein, (17.5 mg ml^{-1}), beef extract, (3 mg ml^{-1}), and dextrose, (1.5 mg ml^{-1}), (pH 7.3). The broth was autoclaved at 121ºC, at 15 psi for only 10 min to prevent denaturation of the beef extract proteins. After growth in MHB overnight at 28ºC with shaking (150 rpm), the purity of the cell suspension was assessed through growth on Tryptic Soy Agar (TSA), consisting of acid hydrolysate of casein (17.0 mg ml^{-1}), NaCl (5.0 mg ml^{-1}), Soytone (3.0 mg ml^{-1}), K_2HPO_4 (2.5 mg ml^{-1}), agar (15.0 mg ml^{-1}) and glucose (2.5 mg ml^{-1}) (Atlas, 1997), supplemented with cyclohexamide (Sigma-Aldrich, Mannheim, Germany) at a final concentration of 0.1 mg ml^{-1} to inhibit fungal growth. Growth on TSA plates was allowed to proceed for approximately 48 hours at 28ºC, and all contaminated plates were discarded.

c. Preparation of inoculum

The concentration of *L. mesenteroides* used to inoculate internodes was based on the work of Daeschel *et al*., (1981), in which the Lactobacilli infection level of sorghum juice shortly after harvest was shown to be approximately: 9 x 10^5 colony forming units per millilitre (cfu ml^{-1}). Determination of average percent wet weight per sugarcane internode, combined with the average mass per internode, was used to calculate the inoculum cell density required to establish a similar degree of infection in the sugarcane internodes. The required infection level was calculated to be approximately 5.4 x 10^7 cfu $internode^{-1}$ and consequently each stalk was inoculated with 100 µl of a *L. mesenteroides* suspension at a density of 5.4 x 10^8 cfu ml^{-1}. The density of *L. mesenteroides* cells in MHB was calculated spectrophotometrically through the measurement of absorbance at a wavelength of 600 nm (A_{600}) (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)) against a standard curve of A_{600} values from a *L. mesenteroides* dilution series. To attain the desired density of *L. mesenteroides*, cells were grown in MHB broth and then concentrated by centrifugation (10000 x *g*) at 4ºC for 2 min. The resulting cell pellet was then resuspended in an appropriate volume of sterile Ringers solution (Merck, Madison, USA) prior to inoculation into the sugarcane stalks. For uniformity and consistency between treatments, control stalks were inoculated with the same volume of sterile Ringers solution.

d. Inoculation

Inoculation was undertaken with a metal coring instrument designed to minimise peripheral damage to the stalk. The diameter of the core produced by the instrument was approximately 4 mm, while the depth was approximately 8 mm. Inoculation was performed in internode 11 of each stalk. Prior to coring, the area to be cored was thoroughly swabbed with 70% (v/v) ethanol. The corer was then used to remove a small plug of tissue from the middle of the internode into which a 100 µl of inoculum or sterile Ringers solution was injected (3.3.1c). Immediately after inoculation, the hole was covered and sealed with tape to avoid cross-contamination or leakage.

3.3.2 Diurnal and seasonal temperature regimens

The incubator used in this experiment was designed and constructed by the School of Bioresources Engineering and Environmental Hydrology (University of KwaZulu-Natal, Pietermaritzburg, KwaZulu-Natal, South Africa). It was specifically designed to consistently simulate and regulate diurnal temperature changes in the field or on the mill room floor that commonly occur during harvest to crush delays in the industry.

3.3.3 *Duration of delay period*

The duration of the harvest to crush delay used during the winter and summer simulations was 9 days (approximately 216 hours). Although a HTCD period of 9 days might be considered to be in excess of those routinely occurring within the industry, there are reports of extended delays, particularly due to unforeseen circumstances such as heavy frosts in the Midlands, prolonged spring rains, and or runaway fires, when large amounts of cane need to be processed simultaneously by the mill (Ravnö and Purchase, 2005). Prolonged HTCD may also be as a result of poor planning with regards to harvesting, sugarcane transport and logistics capacity.

3.4 Sampling and replication

After harvest and processing but prior to placing in the incubator, stalks were bundled into groups of five, with each stalk representing a sample. Samples were removed from the incubator on days 0, 2, 4, 7 and 9 for analysis of sugarcane metabolites, and microbial metabolic by-products. On each sampling day an additional bundle of five uninoculated stalks and a bundle of five inoculated stalks were removed from the incubator specifically for mill room analysis. These samples were transported directly to the mill room and analysed on the basis that five stalks comprising each sample would be pooled together to reflect one measurement per treatment per sampling time. For laboratory analysis a bundle of five uninoculated stalks and a bundle of five inoculated stalks were removed from the incubator. The five stalks per treatment were then unbundled and internodes 10, 11 and 12 were excised from each stalk. Unless otherwise stated these three internodal samples per stalk per treatment were measured independently to allow for 15 biological replicates to represent one sampling day. On

sampling, individual internodes were cut into small pieces, sealed into polyethylene bags and crushed with a mallet. Thereafter the samples were frozen in liquid N_2 and stored at -72ºC. Frozen crushed tissue samples were later reduced to a fine powder using an A11 Basic Analysis Mill (IKA®, Staufen, Germany) and stored in 50 ml polypropylene tubes (Corning®) at -72ºC prior to analysis. For the molecular analyses (3.5.4), sampling was restricted to days 0, 4 and 9 on internode 11 samples (in triplicate) from the winter and summer HTCD experiments, for both uninoculated and inoculated treatments respectively.

3.5 Analyses: Microbiological, biochemical and molecular

3.5.1 Microbiological: Bacteria

a. Cell number determination

Enumeration of bacterial numbers was by standard microbiological plate-counts using TSA as the substrate medium (supplemented with 0.1 mg ml^{-1} cyclohexamide to inhibit fungal growth). For counting, a disc of internode 11 tissue from each of three replicate stalks per treatment was excised and weighed, after which 10 ml of sterile Ringers solution was added. The tissue discs were homogenised (Ultra-Turrax T25, (IKA®, Staufen, Germany)) to disrupt the tissue. The homogenates were subject to serial dilution and 100 µl of each was spread onto the TSA plates and incubated at 28ºC for 48 hours. Two separate colony counts were performed on the TSA plates, firstly that of total bacterial colonies, and secondly those colonies exhibiting the colony morphology characteristics of *L. mesenteroides*, which were later confirmed by selective culturing and PCR (3.5.1c and d).

b. Isolation, storage and maintenance

Bacterial colonies of interest, including those tentatively identified according to colony morphology as *L. mesenteroides,* were isolated from the plates. To obtain a pure isolate, selected individual colonies were subjected to a three-way streak onto TSA plates with subsequent incubation for 48 hours at 28ºC. For long-term storage, glycerol stocks of these isolates were prepared and frozen and subsequently maintained at -84^{o}C. Glycerol

stocks were prepared by combining an aliquot of an overnight culture (MHB) with 60% (v/v) glycerol in a 2:1 ratio in sterilized cryovials, which were then rapidly frozen in liquid N_2.

c. Selective culturing

Phenylethyl alcohol sucrose (PES) growth medium is a selective medium used to isolate *Leuconostoc* (Lee *et. al*, 1997), which was used in this study for the preliminary identification of the bacterial isolates. PES is composed of acid hydrolysate of casein, (5.0 mg ml^{-1}), yeast extract, (0.5 mg ml^{-1}), sucrose, (2.0 mg ml^{-1}), $(NH_4)_2SO_4$, (2.0 mg ml^{-1}), $MgSO_4.7H_2O$, (0.5 mg ml^{-1}), KH_2PO_4, (1.0 mg ml^{-1}), 0.25% (v/v) phenylethyl alcohol and agar, (15.0 mg ml^{-1}), pH 6.8. The bacterial stocks (3.5.1b) were plated out onto PES plates and TSA plates in parallel (TSA plates serving as a positive control for growth), and incubated at 28ºC for approximately 48 hours. PES and TSA plates were then assessed based on the presence or absence of bacterial growth.

d. Identification

To confirm bacterial identity (3.5.1c), PCR was performed using the primers Lmes-f (5'-AACTTAGTGTCGCATGAC-3') and Lmes-r (5'- AGTCGAGTTACAGACTACAA-3'), specifically designed for the rapid identification of *L. mesenteroides* strains (Lee *et al*., 2000), (Inqaba Biotec, South Africa). PCR amplification was performed using the components of the Promega GoTaq® PCR Core System II (Promega, Madison, USA) in a 50 µl reaction mixture containing at a final concentration, 0.5 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1.25 U of GoTaq® DNA polymerase, 1 x GoTaq® Flexi buffer, and 2.5 mM $MgCl_2$. Single bacterial colonies growing on TSA plates (3.5.1c) were used directly as DNA template in the PCR reaction, and were aseptically added by means of a sterile 10 µl pipette tip. Sterile, filter-purified (Acrodisc® PF 0.2 µm pore size membrane filters, PALL Life Sciences, Ann Arbor, Michigan, USA), double-deionised water served as the non-template, negative control and the *L. mesenteroides* source culture growing on PES medium was used as the positive control. Thermal cycling was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, California, USA) temperature cycler, with the following cycling parameters modified from Lee *et al*. (2000); 1 cycle of denaturation for 5 min at 94ºC; 30 cycles consisting of

denaturation at 94ºC for 1 min, annealing at 46ºC for 1 min, and extension at 72ºC for 2 min; with a final extension step at 72ºC for 10 min.

The concentration of the amplicons was determined spectrophotometrically (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)), where absorbency of samples was measured at 260 nm and 280 nm. After quantification, approximately 400 ng of PCR product was size fractionated by means of agarose gel electrophoresis. Agarose gels (1.5% w/v), (Whitehead Scientific, South Africa) were prepared with 1 X TBE buffer (45 mM Tris, 45mM boric acid, 1 mM EDTA; pH 8.0) and ethidium bromide at a final concentration of 0.5 µg ml^{-1}. A set volume of 10 µl of PCR product was mixed with 5 µl of gel loading buffer (0.13% (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol and 20% (w/v) Ficoll® (Pharmacia Inc, supplied by Sigma-Aldrich, Mannheim, Germany) and loaded into the gel wells. Electrophoresis was conducted at 5.0 V cm^{-1} in 1 X TBE buffer. The DNA fragments were visualized by exposure to short wavelength UV light (320 nm) alongside a molecular weight marker (DNA Molecular Weight Marker VI (Roche, Mannheim, Germany)).

In conjunction to the above, the bacterial cultures isolated and established from internodal tissues (3.5.1b), as well as pure *L. mesenteroides* cultures, were grown up overnight (3.3.1b) and applied to the Nucleospin® DNA Extraction kit (as per the manufacturer's instruction) (Nucleospin®, Machery-Nagel, Düren, Germany). Extracted microbial DNA was PCR amplified with primers for conserved regions of the 16S rRNA genes, namely the sequences corresponding to positions 341 (5'-CCTACGGGAGGCAGCAG-3') and 534 (5'-ATTACCGCGGCTGCTGG-3') in the *E. coli* genome as the forward and reverse primers, respectively (Muyzer *et al.*, 1993) (Inqaba Biotec, South Africa). PCR amplification was performed using the components of the Promega GoTaq® PCR Core System II (Promega, Madison, USA) in a 50 µl reaction mixture, according to the manufacturer's instructions. Thermal cycling was conducted in a GeneAmp® PCR System 2400 (Applied Biosystems, California, USA) temperature cycler, with the following cycling parameters; 1 cycle of denaturation for 5 min at 94ºC; 35 cycles consisting of denaturation at 92ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min; with a final extension step at 72ºC for 10 min. PCR product size was assessed and quantified by means of agarose gel electrophoresis (3.5.1d)

against a molecular weight marker (DNA Molecular Weight Marker VI (Roche, Mannheim, Germany)).

The remaining PCR product from each amplification reaction rendering a single PCR product was PCR purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. Purified PCR product was then sequenced using the components of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) according to the manufacturer's instruction and analysed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, California, USA). The resulting nucleotide sequences were submitted to the BLAST (Basic Local Alignment Search Tool) program (Altschul *et al*., 1990) for comparative analysis against the NCBI database, making use of the 'megablast' algorithm.

e. Dextran production

The capacity of the putative *L. mesenteroides* isolate to synthesise dextran in a sucrose-rich environment was used as a further confirmation of identity. The bacterium was inoculated into a sucrose medium (Atlas, 1997), comprised of 150.0 mg ml^{-1} sucrose, 1.0 mg ml^{-1} yeast extract, 5.0 mg ml^{-1} pancreatic digest of casein, 5.0 mg ml^{-1} K_2HPO_4, 2.0 mg ml^{-1} NaCl, and 0.022 mg ml^{-1} $MgSO_4$. Incubation was allowed to proceed at the summer temperatures (cycling from between 21ºC to 31ºC over a 24-hour period, for a total period of 9 days). Samples were removed for analysis of dextran levels (3.5.2e) and *L. mesenteroides* cell number enumeration (3.5.1a) after 0, 1, 2, 3, 4, 7 and 9 days.

3.5.2 Biochemical: Protein, sugars and metabolites

All sample analyses unless otherwise stated were performed in duplicate, and where applicable, standards were performed in triplicate. The corresponding buffer from each analysis acted as the blank, with standardised volumes amongst samples, standards and blanks.

a. Protein

Protein analysis was restricted to internode 11 and was carried out to enable expression of data on a total protein basis. For protein extraction 0.2 g of powdered, frozen tissue was added to 400 µl extraction buffer (300 mM Tris (pH 7.5), 2 mM $MgCl_2$, 5 mM DTT, 2 mM EDTA (pH 8.0) and 0.8% (w/v) protease inhibitor cocktail (Complete®, Roche, Mannheim, Germany). Each sample was briefly homogenised (Ultra Turrax T25, (IKA®, Staufen, Germany)) to ensure thorough disruption of cellular matter. Homogenised samples were clarified by centrifugation (10000 x *g*) for 15 min at 4ºC and the supernatant transferred to fresh tubes. Total protein concentrations were determined by the Bradford method (Bradford, 1975), with γ-globulin (Bio-Rad Laboratories, München, Germany) in the concentration range of 0 – 500 µg ml^{-1} as standard. In a 96-well microtitre plate, 80 µl of sample, or standard was added to 20 µl of undiluted Bradford reagent (Bio-Rad Laboratories, München, Germany), mixed thoroughly and, after approximately 2 min, the absorbance at 595 nm was determined (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)). Data analysis was conducted using KC4 software (Biotek® Instrument, Inc.).

b. Sugars: Sucrose, glucose and fructose

Ten volumes of extraction buffer (30% (v/v) HM-buffer (100 mM HEPES (pH 7.8), 20 mM $MgCl_2$), 70% (v/v) ethanol) were added to one volume of frozen tissue. The tissue suspensions were incubated at 70ºC overnight, mixed well and then approximately 1000 µl of the suspension was syringe filtered through a 0.22 µm filter into a fresh tube. For determination of sucrose, glucose and fructose concentrations by High Performance Liquid Chromatography, samples were diluted 1:100 with ultrapure milliQ water (18.2 $M\Omega.cm^{-1}$) prior to separation on a Waters Associates HPLC system (Millford, USA), equipped with a 1500 series pump, 100 µl injection loop and electrochemical detector (PAD, model number 2456, 50uA). The system is additionally comprised of a Waters 717 plus Autosampler and a CarboPac PA1 column, which uses a mobile phase of vacuum-filtered and degassed NaOH (200 mM) solution. The Breeze software package (Waters Associates, Millford, USA) was used to quantify the eluted sugars on the basis of pre-calibrated sugar standards composed of 80 µg $µl^{-1}$ of analytical grade glucose, fructose and sucrose (Merck, Madison, USA).

c. Lactic acid

L-Lactic acid (or L-lactate) concentrations were determined by means of an Enzymatic BioAnalysis / Food Analysis kit from R – BIOPHARM (Roche, Mannheim, Germany), according to kit specifications. Approximately 0.2 g of tissue was added to 1.0 ml of sterile, distilled water in tubes. The tubes were then vortexed and placed at 60ºC for 45 min with agitation (100 rpm). Samples were centrifuged (10000 x *g*) for 10 min, the supernatant transferred to a fresh tube and centrifuged again to remove any remaining particulate matter. The above-mentioned kit supplied lactic acid standards in the range of 20 – 100 µg ml^{-1}. An aliquot (100 µl) of sample or standard was added to 100 µl of glycylglycine buffer, 20 µl of NAD solution (at a concentration of approximately 35 mg ml^{-1}), and 3 µl of glutamate-pyruvate-transaminase suspension (approximately 5 U per assay). Immediately after mixing, the absorbance at 340 nm was recorded (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)). To initiate the kinetic reaction, 3 µl of L-lactate dehydrogenase suspension (approximately 16 U per assay) were added to each well and the absorbance monitored at 340 nm until an end point (or plateau in absorbance) was reached, at which time a final absorbance reading was recorded. Data was expressed according to kit specification and the analysis was conducted using KC4 software (Biotek® Instrument, Inc.).

d. Ethanol

Samples were processed in the same manner as for the lactic acid assays (3.5.2c). Ethanol concentrations were determined using the Enzymatic BioAnalysis / Food Analysis kit from R – BIOPHARM (Roche, Mannheim, Germany) according to kit specifications, with supplied ethanol standards in the range of 50 – 250 µg ml^{-1}. An aliquot (40 µl) of sample or standard was added to 240 µl of potassium diphosphate buffer containing NAD at a concentration of approximately 1.30 mg ml^{-1} and aldehyde dehydrogenase (0.064 U per assay). Prior to each absorbance measurement the microtitre plate was covered with clear plastic film, as per kit instructions, to prevent adsorption of ethanol from the surrounding air into the assay mixture. Immediately after mixing, the absorbance at 340 nm was recorded (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)). Thereafter, 8 µl of alcohol dehydrogenase suspension (approximately 35 U per assay) was added to each well and

the absorbance monitored at 340 nm until an end point (or plateau in absorbance) was reached, at which time a final absorbance reading was recorded. Data was expressed according to kit specification and the analysis was conducted using KC4 software (Biotek® Instrument, Inc.).

e. Dextran

Approximately 0.2 g of tissue was added to 0.5 ml of sterile, distilled water after which 0.5 ml α–amylase solution (4 mg ml^{-1}, (Sigma-Aldrich, Mannheim, Germany)), to a final concentration of 2 mg ml^{-1}, was supplied. The reaction mixtures were incubated for 1 hour at 55ºC with agitation (100 rpm). Samples were centrifuged (10000 x *g*) for 15 min, and the supernatant transferred to a fresh tube.

Sample preparation for the culture broth samples (3.5.1e) were divided into 1.5 ml aliquots and placed into fresh, sterile tubes containing 0.3 ml of sterile, distilled water. This suspension was then filtered using 0.22 µm bacterial syringe filters, and 3 ml of 99% (v/v) isopropyl alcohol then added to the filtrate, and mixed vigorously. The samples were stored at 4ºC for approximately 20 hours. A volume of 500 µl of sample was added drop-wise to 1.5 ml of 99% isopropyl alcohol, and the mixture centrifuged at approximately 1500 x *g* for 5 min. The supernatant was removed and discarded and the remaining pellet left to dry. The dried pellet was resuspended in 1 ml of potassium phosphate buffer (0.1 M, pH 6.0).

Deteriorated sugarcane tissue samples and culture broth samples were analysed for the presence of dextran using an enzymatic method adapted from Sarkar and Day (1986). A 500 µl volume of sample was added to 1000 µl of absolute ethanol, mixed well and centrifuged at 350 x *g* for 10 min. The supernatant was discarded and the pellet resuspended in 1000 µl 80% (v/v) ethanol and centrifuged again under the same conditions. The supernatant was carefully removed and the pellet left to dry by evaporation. Once dry, the pellet was resuspended in 350 µl potassium phosphate buffer (0.1 M, pH 6.0) (Roskams and Rodgers, 2002).

Two 150 µl aliquots of sample were dispensed into separate tubes, with one tube serving as the sample and the other as the blank. To each of these tubes, 100 µl of α–

glucosidase suspension (6 U in total, (Sigma-Aldrich, Mannheim, Germany)) was added, after which 100 µl of dextranase (10 U in total, (Sigma-Aldrich, Mannheim, Germany)) was added to only the sample tube. To the blank tube, 100 µl of potassium phosphate buffer (0.1 M, pH 6.0) was added. Tubes were mixed well and incubated at 37ºC for 2 hours, with occasional mixing.

The concentration of dextran in the samples was determined on the basis of glucose liberation as a result of enzymatic digestion of dextran. The concentration of glucose was determined colourimetrically by means of the Nelson-Somogyi method (Sarkar and Day, 1986; Green *et al*., 1989). Dextran standards (2500 – 500 µg ml^{-1}, (T2000, Amersham Biosciences, Uppsala, Sweden)) and glucose standards (100 – 20 µg ml^{-1}, (Merck, Mannhein, Germany)) were prepared and included in the alcohol precipitation and enzymatic digestion procedures, as well as the blank, which contained no sugarcane tissue prior to the sample preparation.

Glucose determination involved adding 50 µl of sample to 50 µl of buffer containing 25 mg ml^{-1} Na_2CO_3, 25 mg ml^{-1} Rochelle salt ($C_4H_4KNaO_6.4H_2O$), 20 mg ml^{-1} $NaHCO_3$, 198 mg ml^{-1} Na_2SO_4, and 6 mg ml^{-1} $CuSO_4.5H_2O$, mixing well and heating the tubes on a heating block to 100ºC for 20 min. The tubes were cooled to room temperature by floating in a water bath for 5 min, then vortexed briefly to completely remove remaining CO_2. A volume of 300 µl of chromogenic buffer (8 mg ml^{-1} ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$), 1% v/v H_2SO_4 and 1 mg ml^{-1} $Na_2HAsO_4.7H_2O$) was added to each tube, mixing well on a vortex mixer. The tubes were allowed to stand for 10 min, mixed again and then 200 µl of each sample and / or standard was decanted into the well of a microtitre plate and the absorbance read at 520 nm (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)).

f. Mannitol

Approximately 0.3 g of tissue was added to 1.0 ml sterile glycine buffer (100 mM, pH 8.6) and mixed in a tube. Incubation proceeded at 60ºC for approximately 45 min, with agitation (100 rpm). The samples were then vortexed for 10 sec and subsequently centrifuged (10000 x *g*) for 10 min for clarification. The supernatant was applied to the

QIAshredder (QIAGEN, Hilden, Germany) column and tubes were centrifuged (10000 x *g*) for 2 min for further clarification. The supernatant was then placed into fresh tubes.

Mannitol determination was based on the method described by Eggleston and Harper (2006), which is effective in the detection of mannitol in sugarcane stalk extracts in the range 100 to 500 µg ml^{-1}. An aliquot of 100 µl of sample or standard was added to 100 µl of glycine buffer (100 mM, pH 8.6), followed by the addition of 20 µl of NAD solution (50 mg ml^{-1}, (Roche, Mannheim, Germany)). Immediately after mixing the absorbance at 340 nm was recorded (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)). To initiate the kinetic reaction, 18 µl of mannitol dehydrogenase suspension (0.18 U per assay, (Sigma-Aldrich, Mannheim, Germany)) was added to each well and the absorbance monitored at 340 nm until a plateau in absorbance was reached, at which point a final absorbance reading was recorded. Data analysis was conducted using KC4 software (Biotek® Instrument, Inc.).

g. Statistical analysis

A three-way Analysis of Variance (ANOVA) was used to establish significant differences in sucrose, fructose, glucose, lactic acid and ethanol concentrations with season, time and inoculation as the factors. Data was log-transformed because normality and homogeneity of variance assumption were violated. The all pairwise multiple comparison post hoc test used was the Holm-Sidak test (Keeping *et al.*, 2007).

3.5.3 Mill room analyses

Standard mill room analysis, by means of NIR (Near Infrared Spectrometry) and DAC (Direct Analysis of Cane), was conducted on pooled juice from each of the 5 stalks comprising a single sample. This analysis was done to determine the apparent sucrose in solution (expressed as Pol % cane); all dissolved matter in solution (expressed as Brix % cane and Brix % dry mass); the total Estimated Recoverable Crystal (expressed as ERC % cane); the sample purity (ratio of Pol % cane to Brix % cane); the total dry mass in solution; and total fibre.

3.5.4 Molecular: PCR-DGGE

a. DNA extraction and visualization

DNA extraction from approximately 0,1 g of frozen tissue per sample was achieved by means of a QIAGEN DNeasy® Plant Mini Kit, according to the manufacturer's instructions (QIAGEN, Hilden, Germany). Sample DNA concentration and purity was determined spectrophotometrically (Synergy HT Multi-Detection Microplate Reader (Biotek® Instrument, Inc., Vermont, USA)), where absorbency was measured at 260 nm and 280 nm. Genomic DNA size fractionation was conducted by electrophoresis on agarose gels (0.8% w/v), (Whitehead Scientific, South Africa), which were prepared with 1 X TBE buffer (45 mM Tris, 45mM boric acid, 1 mM EDTA; pH 8.0) and ethidium bromide at a final concentration of 0.5 µg ml^{-1}. A standard volume (2 µl) of extracted DNA, combined with 10 µl of distilled water, was mixed with 3 µl of gel loading buffer (0.13% (w/v) bromophenol blue; 0.05% (w/v) xylene cyanol and 20% (w/v) Ficoll® (Pharmacia Inc, supplied by Sigma-Aldrich, Mannheim, Germany) and loaded into the gel wells. Electrophoresis was conducted at 5.0 $V.cm^{-1}$ in 1 X TBE buffer. The DNA fragments were visualized by exposure to short wavelength UV light (320 nm) alongside a molecular weight marker (DNA Molecular weight marker III (Roche, Mannheim, Germany)).

b. PCR using universal bacterial primers

The variable V3 region of all 16S rDNA present in the samples was amplified using PCR with primers for conserved regions of the 16S rRNA genes, as in 3.5.1d, namely the sequences corresponding to positions 341 (5'-CCTACGGGAGGCAGCAG-3') and 534 (5'-ATTACCGCGGCTGCTGG-3') in the *E. coli* genome as the forward and reverse primers, respectively (Muyzer *et al*., 1993) (Inqaba Biotec, South Africa). The forward primer had at the 5' end an additional 40-nucleotide GC-rich sequence (GC clamp); to ensure the formation of stable, partially melted DNA strands during PCR product analysis by DGGE (Muyzer *et al*., 1993). PCR amplification was performed using the components of the Promega GoTaq® PCR Core System II (Promega, Madison, USA) in a 50 µl reaction mixture, according to the manufacturer's instructions. Thermal cycling was conducted in a GeneAmp® PCR System 2400 (Applied Biosystems, California,

USA) temperature cycler, with the following cycling parameters; 1 cycle of denaturation for 5 min at 94ºC; 35 cycles consisting of denaturation at 92ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min; with a final extension step at 72ºC for 10 min. PCR product size was assessed and quantified by means of agarose gel electrophoresis (section 3.5.1d) against a molecular weight marker (DNA Molecular Weight Marker VI (Roche, Mannheim, Germany)).

c. Production of a culture-dependent DGGE marker

The bacterial cultures isolated and established from internodal tissues (section 3.5.1b), as well as pure *L. mesenteroides* cultures, were grown up overnight (section 3.3.1b) and applied to the Nucleospin® DNA Extraction kit (as per the manufacturer's instruction) (Nucleospin®, Machery-Nagel, Düren, Germany). Extracted microbial DNA was PCR amplified and visualized (section 3.5.4b). The PCR products resulting from this amplification, including the DNA from the various bacteria isolated from the deteriorating stalk tissue, were combined in an equimolar ratio, mixed well, diluted, and then used as a template for a subsequent PCR reaction. This process ensured the availability of a sufficient quantity of microbial DGGE-marker for subsequent analyses.

d. DGGE

The PCR products were profiled by means of the DCode Universal Mutation Detection System and associated apparatus (Bio-Rad Laboratories, München, Germany). Preparation of glass plates for DGGE involved rigorous washing with a sponge and detergent, thorough rinsing, and the drying of glass plates in a vertical orientation with minimal glass surface contact; a process which was repeated to ensure cleanliness of plates. Once dry, the glass plates were rinsed thoroughly with absolute ethanol, left to dry vertically, and then rinsed off with water. Once again the plates were left to dry standing vertically, and rinsing with water and drying was repeated. Once the plates were dry, they were assembled into a sandwich configuration according to the manufacturer's instruction, using the appropriate spacers, glass plate assembly unit and spacer card after which they were locked into a gel casting stand.

A 10% (w/v) bis-acrylamide solution (Sigma-Aldrich, Mannheim, Germany) formed the basis of the denaturing gradient gels. In preparation for gel casting, a 0% denaturant solution (12.5 ml of 40% bis-acrylamide and 1 ml 50 X TAE buffer (Roskams and Rodgers, 2002) made up to a final volume of 50 ml with distilled water) and a 100% denaturant solution (12.5 ml 40% bis-acrylamide, 1 ml 50 X TAE buffer, 20 ml deionized formamide (Sigma-Aldrich, Mannheim, Germany) and 21 g urea (Promega, Madison, USA) made up to a final volume of 50 ml with distilled water) were prepared and allowed to degas at 4ºC overnight. Approximately an hour prior to casting the DGGE gel, the 100% denaturant solution was placed in an incubator at 37ºC to remove crystals. To prevent leakage from the base during gel casting a 100% denaturant gel 'plug' was prepared. This was done by combining 1 ml 100% denaturant solution with 7 µl freshly prepared 10% ammonium persulfate solution (APS) and 1 µl N,N,N',N'-tertramethylethylenediamine (TEMED, Sigma-Aldrich, Mannheim, Germany), mixing and rapidly pouring this solution between the glass plates. Approximately 10 min was allowed for polymerization, or setting of the gel plug.

The DGGE gel was prepared at a relatively wide denaturing concentration gradient of between 30 and 70% denaturant. The 30% denaturant (low density solution) was prepared by combining 7.7 ml 0% denaturant solution with 3.3 ml 100% denaturant solution, and the 70% denaturant (high density solution) was prepared by combining 3.85 ml 0% denaturant solution with 7.15 ml 100% denaturant solution, in glass beakers. At this point the gradient mixer (Watson-Marlow 202 (Watson-Marlow Limited, Cornwall, UK)) and connected tubing were checked for blockages or leaks, and the gel dispensing needle (connected to the gradient mixer) was placed strategically in the centre of the assembled glass plates, prepared for dispensing the gel solution. A volume of 50 µl of 10% APS was added to both the low and the high density solutions, and 10 µl of TEMED was added rapidly to the high density solution, mixed and poured into the gradient mixer chamber connected to the pump. A volume of TEMED (5 µl) was then rapidly added to the low-density solution, mixed and poured into the adjacent chamber. The gradient mixer pump was switched on and the valve between the two chambers opened to allow for the gel solution to be delivered. During this time the stacking gel was prepared with 5 ml 0% denaturant solution, 35 µl APS and 5 µl TEMED, which were poured directly into the high-density solution chamber of the gradient mixer after completion of delivery of

the gel. Finally, the comb was positioned between the two glass plates, and the cast gel allowed to polymerize for approximately 2 hours.

After gel polymerization, the comb was removed and the gel sandwich released from the casting stand. Non-polymerised material was rinsed off the gel sandwich with 1 X TAE running buffer using a syringe. The gel sandwich was then locked into the core apparatus. This system allows two DGGE gels to be run simultaneously, with two gel sandwich assemblies locking into the core apparatus adjacent from one another, to complete the upper chamber of the system. The system unit, or tank, was filled with approximately 7 l of 1 X TAE running buffer, which was pre-warmed in the tank by placing the power control module in place, and setting the temperature controller to 60ºC, and the ramp rate to 200ºC hr^{-1}. Once the desired temperature had been reached, the power control module was removed, and approximately 1 l of running buffer was temporarily decanted out of the tank. The core apparatus holding the two gel sandwich assemblies was then placed into the running buffer and the power control module replaced on top of the tank. The decanted running buffer was then poured carefully into the upper chamber, ensuring there were no leaks, up to the 'Run' mark, and the unit switched on again.

The samples were prepared by mixing 20 µl of sample with 4 µl DGGE 6 X loading dye (0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1 M EDTA (pH 8), 0.5% (w/v) SDS). The samples were loaded via the upper chamber using gel saving tips to ensure well integrity was maintained. The DGGE gels were run for 16 hours at a temperature of 60ºC at a 70 Volt constant current.

e. DNA visualization

DNA on the DGGE gel was visualized using SYBR® Gold Nucleic Acid Stain (Molecular Probes, Inc., Leiden, The Netherlands). The stain was diluted to a 1 X solution, using 1 X TAE buffer, in a plastic container covered with aluminium foil to prevent entry of light. The glass plates, once removed from the system, were carefully separated and the polyacrylamide gel was gently floated off into the staining solution. The gel was stained for approximately 40 min, with agitation (70 rpm), to ensure even staining, and finally

visualized by exposure to short wavelength UV light (320 nm) using a VersaDoc® Image Capturer (Bio-Rad Laboratories, München, Germany).

DGGE images were analyzed using Bio-Rad Image Analysis, Quantity One Software (Bio-Rad Laboratories, München, Germany) according to the manufacturers' instruction manual. The VersaDoc Image Capturer (Bio-Rad Laboratories, München, Germany) used to capture each image functions by expressing the signal intensity of each band in counts. The bands and corresponding count values for each image were detected using the Quantity One 1-D analysis software. Once the DGGE images were opened in this program the lanes were manually defined for each image. Lane-based background subtraction was performed using the 'rolling disk' method of subtraction setting, in order to remove background intensity from the lanes. The detection parameters for this were set to detect bands that had intensity counts greater than 100. At this point each band was identified and confirmed using the 'zoom box' function and the three-dimensional imaging function. The sensitivity parameter setting for band detection was changed from the default setting of 10 and increased to 20 for all images to ensure that all correctly ascribed bands were numbered.

CHAPTER 4

RESULTS

4.1 Diurnal temperature variation during a simulated HTCD

The first harvest (conducted on 5 June 2006) of stalks were subjected to a simulated 'winter harvest-to-crush delay' (HTCD) and, hence, were incubated at temperatures cycling between 8ºC and 20ºC. The second harvest (conducted on 3 July 2006) included those stalks representing a 'summer HTCD', which were incubated at temperatures cycling between 21ºC and 31ºC. The above cycling temperatures were chosen from the KwaZulu-Natal long-term mean (LTM) temperatures experienced over the past 10 years. The winter temperatures (8ºC to 20ºC) being the coldest temperatures recorded overall (experienced in Wartburg, KwaZulu-Natal, South Africa), and the summer temperatures (21ºC to 31ºC) being the warmest temperatures recorded overall (experienced in Mtubatuba, KwaZulu-Natal, South Africa). Figure 4.1 illustrates the temperature and humidity data output by the incubator software system. These outputs include the profile for the summer HTCD scenario (Days 0 and 4) and those for the winter HTCD scenario (Days 1 and 4).

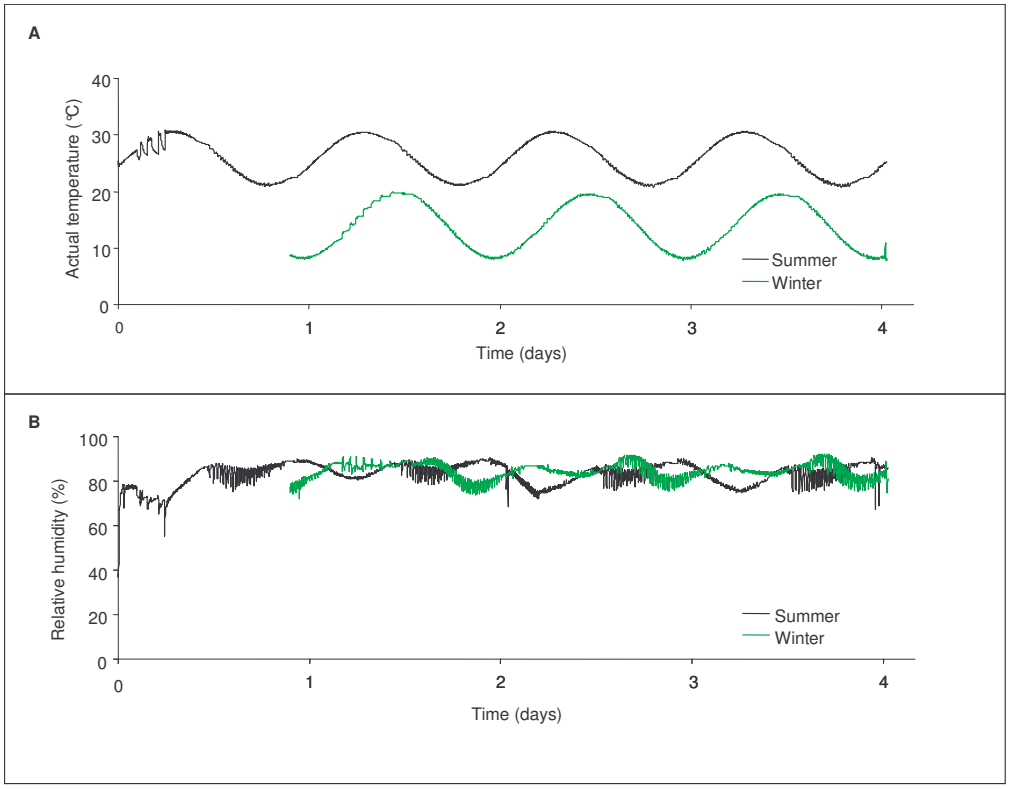


Figure 4.1: *Fluctuating temperature (A) and relative humidity (B) levels during the winter (—) and summer (—) deterioration experiments.* The summer profile reflects temperature and humidity between days 0 and 4, and the winter profile those between days 1 and 4. Data for the first day of the winter regime are not presented due to a data logger fault.

The incubator could regulate temperature but not relative humidity and the data output for the latter is merely a reflection of the humidity of the environment within the incubator chamber. Therefore, to avoid excessive condensation within the incubator the lid of the instrument was left slightly ajar during the two experiments to allow fresh air to circulate. Figure 4.2 shows the average relative humidity levels recorded for each day for winter and summer. These humidity profiles show a similar characteristic decrease in relative humidity with time, with the summer profile decreasing to a slightly lower level by day 4 than the winter profile.

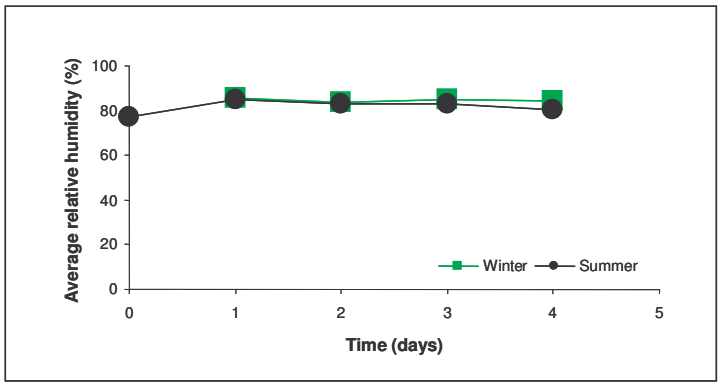


Figure 4.2: *Average relative humidity profiles recorded for winter* (■) *and summer* (●) *HTCD scenarios.* Summer averages correspond to levels recorded between days 0 and 4, and winter averages correspond to levels recorded between days 1 and 4. Data for the first day of the winter regime are not presented due to a data logger fault.

4.2 Evaluation of bacterial cell numbers during a simulated HTCD

It is widely accepted that microbial infection plays a major role in sugarcane deterioration after harvest, with *L. mesenteroides* being reported as the primary bacterial cause (Tilbury, 1968; Holzapfel and Schillinger, 1992; Eggleston, Legendre and Tew, 2004). However, limited microbiological data are available on the natural proliferation of bacteria within mature sugarcane stalks throughout the deterioration period, with bacterial numbers most commonly measured in juice extracted from deteriorated sugarcane. For this reason, this study focused on the enumeration of total bacterial cell numbers and *L. mesenteroides* cell numbers in sugarcane tissues, with a view to determining: (1) the effect of *L. mesenteroides* inoculation on the total microbial population numbers within sugarcane during a simulated HTCD; (2) the effect of ambient temperature on bacterial cell numbers; and (3) the effect of time between harvesting and crushing on bacterial cell numbers.

The detection of *L. mesenteroides* cells in all stalks at harvest (Day 0) (Figure 4.3 C and D) suggests the presence of this bacterium within the sugarcane plant. These results suggest that this bacterial species may possibly exist within the plant tissue as endophytes. However, the possibility cannot be excluded that the bacterium survived the surface-sterilisation procedures at sampling or that their ubiquitous nature within the

surrounding environment during sampling made infection unavoidable. Regardless of the source of contamination, the presence of these cells within tissue samples on the day of harvest indicates the aggressive nature of *L. mesenteroides* infection of freshly harvested sugarcane.

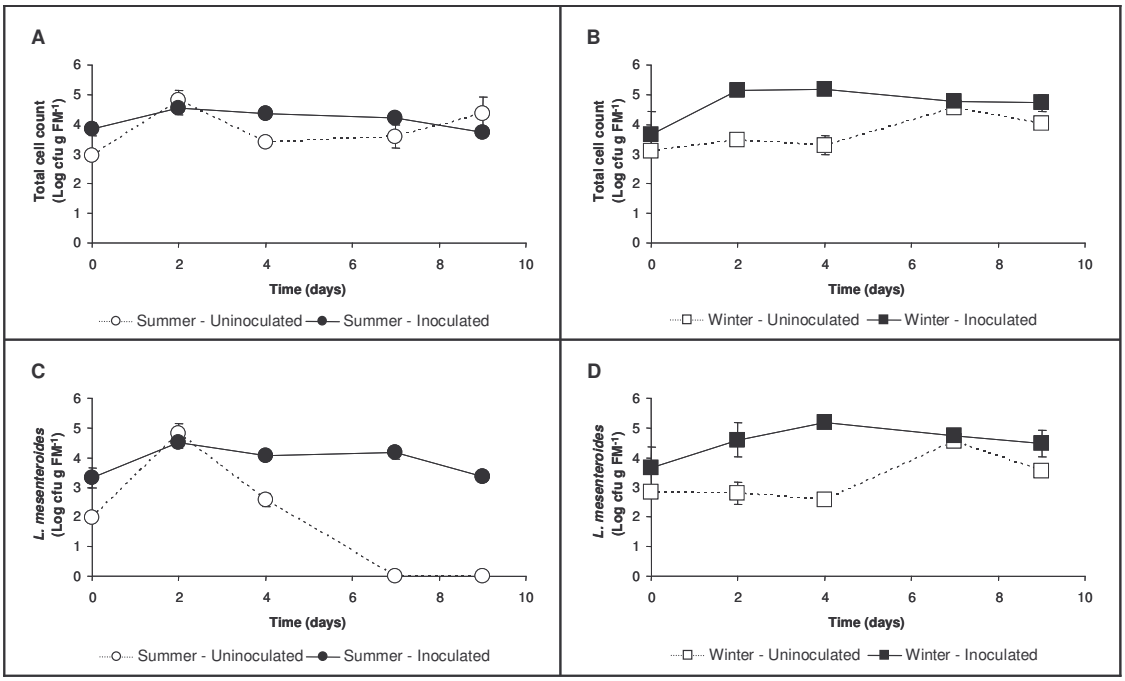


Figure 4.3: *Changes in bacterial cell numbers during stalk deterioration over 9 days.* Total cell numbers (A and B) and *L. mesenteroides* cell numbers (C and D) were monitored under winter (□,■), (B and D) and summer (○,●), (A and C) conditions. Stalks were either inoculated (——) or uninoculated (**.....**) with *L. mesenteroides*. Error bars denote standard error of the mean values (n = 3); absence indicates that the standard error bar was smaller than the data symbol.

For inoculated stalks incubated under winter conditions, the total cell number (Figure 4.3 B) and *L. mesenteroides* cell number (Figure 4.3 D) profiles were similar, indicating that this bacterium made up a very large proportion of the total culturable cell community. This trend was also apparent for inoculated stalks incubated under the simulated summer temperature regimen, where the total cell number profile (Figure 4.3 A) was similar to that of *L. mesenteroides* (Figure 4.3 C). Similarly, in uninoculated stalks incubated under the simulated winter temperature regimen, *L. mesenteroides* cell numbers comprised a large proportion of the total cell numbers throughout the

deterioration period. In all the above instances the percentage of *L. mesenteroides* cell numbers to total cell numbers ranged from 85 to 100% on the day of harvest (Figure 4.3 A, B and D). In contrast, the number of *L. mesenteroides* cells observed at Day 0 in the uninoculated stalks subject to high temperature (Figure 4.3 C) was lower; comprising 64% of the total cell numbers observed (Figure 4.3 A). Despite this initial difference, the *L. mesenteroides* cell numbers reached approximately 100% of the total cell count by Day 2 (Figure 4.3 A and C). After this initial increase between Days 0 and 2 under summer conditions, the *L. mesenteroides* numbers in uninoculated stalks declined, reaching undetectable numbers by day 7 (Figure 4.3 C). The total cell numbers in inoculated stalks subjected to the higher temperature displayed less dramatic variation than those of *L. mesenteroides*; with an initial increase between Days 0 and 2 being followed by a gradual decline over subsequent days (Figure 4.3 A and C).

4.3 Characterisation of bacterial populations during a simulated HTCD: Selective culturing technologies.

Despite *L. mesenteroides* being identified as the dominant (culturable) bacterium infecting deteriorating sugarcane (Figure 4.3), representatives of a number of other bacterial populations were isolated during the total cell count procedure. These bacterial populations were tentatively characterised by colony and cell morphology on TSA, and then further characterised by their ability to survive on and colonise the *Leuconostoc* selective medium (PES). The identity of the isolates was further assessed by means of PCR and *L. mesenteroides* specific primers with a view to confirming the results obtained from culture-dependent assays. Based on the capacity for growth on selective medium (PES) and colony morphology (TSA), one of the isolates (S7) was classified as *L. mesenteroides* (Table 4.1). This isolate served as the reference against which *L. mesenteroides* colonies were tentatively enumerated during the study using colony morphology and selective growth as classification criteria. It is this reference colony that was later confirmed as *L. mesenteroides* by PCR analysis. Typical *L. mesenteroides* colonies were identified on TSA growth medium as being grey-white in colour, producing an entire margin, displaying a smooth surface with a clear slime layer evident; and were notably slower growing compared to other bacterial colonies observed. The remaining bacterial isolates (W1 to S6) were those most frequently observed on the TSA plates

throughout the study (Table 4.1). These were taken as being representative of the bacteria comprising a substantial proportion of the remaining total cell count.

Five bacteria with distinctive colony morphology on TSA were isolated from stalks incubated under the simulated winter temperature regimen (Table 4.1). Of these five isolates (W1 to W5); only one was Gram negative (W3), the other four being Gram positive, or Gram variable. In terms of cell morphology, the Gram negative bacterium (W3) was rod-shaped and 2 µm in length, and the remaining four were cocci of variable diameter. Two of the isolates from the simulated winter temperature regimen (W1 and W2) showed noticeable growth on the selective medium (PES), whereas the remaining three isolates did not. The simulated summer temperature regimen rendered six morphologically distinct colonies. Notably, four of the six isolates were Gram negative, with only one isolate (S2) being Gram positive. Of the six isolates growing under the simulated summer temperature regimen four were coccoid in shape and the remaining two were rod-shaped.

Table 4.1: *Microbial populations isolated from sugarcane stalks incubated under simulated winter and summer conditions.* Microbial populations were isolated from the TSA plates used to enumerate total cell numbers. Isolates were selected on the basis of their frequency throughout the entire deterioration period for winter and summer conditions respectively. The use of selective medium (PES) and *L. mesenteroides* specific PCR served as a means to verify whether the microbial isolates were *L. mesenteroides*. The letters ''W'' and ''S'' within the reference codes refer to winter and summer plate sampling respectively, with each microbial colony isolated in numerical order.

Simulated Season	Reference code	Culture morphology on TSA plates:	Gram reaction	Cell morphology and approximate size	Growth on PES plates	Presence of *L. mesenteroides* specific PCR amplicon
Winter	W1	Light yellow colonies, slime layer evident, entire colonies.	Positive	cocci (0.5 µm)	+	—
	W2	Bright yellow colonies, slime layer evident, opaque, entire colonies.	Positive	rods (2 µm)	+	—
	W3	Orange colonies, no obvious slime layer.	Negative	cocci (0.5 µm)	—	—
	W4	White/peach colonies, smooth, no obvious slime layer.	Positive / variable	cocci, cell budding (4 µm)	—	—
	W5	Pure white colonies, opaque, smooth, entire margin.	Positive / variable	cocci, cell budding (4 µm)	—	—
Summer	S1	Light yellow colonies, slime layer evident, translucent colonies.	Negative	cocci (0.3 µm)	—	—
	S2	Bright yellow, slime layer evident, opaque, entire colonies.	Positive	rods (2 µm)	—	—
	S3	Mustard/yellow colonies, no obvious slime layer.	Negative	cocci (0.5 µm)	—	—
	S4	Red/pink, smooth colonies, no obvious slime layer.	Positive / variable	cocci (0.5 µm)	—	—
	S5	Pure white colonies, opaque, smooth, entire margin. No slime layer evident.	Positive / variable	cocci, cell budding (2 µm)	—	—
	S6	Cream/white colonies, opaque, smooth, entire margin. No slime layer evident.	Negative	rods (2.5 µm)	—	—
	S7	*L. mesenteroides* colony. Characteristic grey/white colonies, translucent, slime layer evident, entire margin, smooth surface.	Positive	short rods (1µm)	+	+

4.4 Characterisation of bacterial populations during a simulated HTCD: Molecular technologies.

Identification of the microorganisms isolated during the culture-dependent assays (Table 4.1) was conducted by means of direct sequencing and homology searches against the NCBI database. Extracted DNA from each isolate was used as the template for PCR using universal 16S rDNA primers, (section 3.5.1d), yielding a 230 base-pair product in all instances except for samples in lane 7 (W4), lane 8 (W5), and lane 13 (S5) (Figure 4.4). Multiple bands were apparent in lanes 7 (W4), 8 (W5), 9 (S1), 11 (S3) and 13 (S5) (Figure 4.4). The absence of a 230 base-pair product, combined with the presence of multiple bands in the above-mentioned lanes indicated that these samples may not be prokaryotes. As a result, samples W4, W5, S1, S3 and S5 were excluded from further analyses and the remaining samples' (namely W1, W2, W3, S2, S4, S6 and S7) PCR product was subjected to PCR purification (section 3.5.1d).

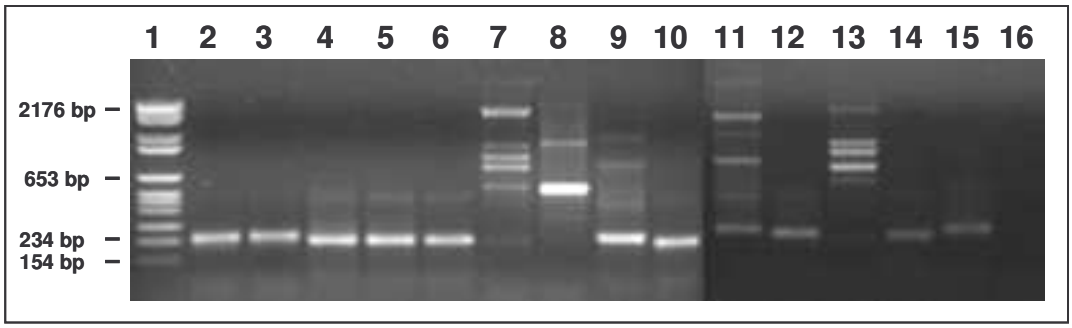


Figure 4.4: *Variable V3 region of 16S rDNA amplified from the microbial isolates.* Ethidium bromide stained 1.5% agarose gel showing the resulting 230 base-pair PCR product with each microbial isolates' genomic DNA as the template. Lane 1: Molecular weight ladder A; Lane 2: *L. mesenteroides* inoculum population (rep 1); Lane 3: *L. mesenteroides* inoculum population (rep 2); Lane 4: W1; Lane 5: W2; Lane 6: W3; Lane 7: W4; Lane 8: W5; Lane 9: S1; Lane 10: S2; Lane 11: S3; Lane 12: S4; Lane 13: S5, Lane 14: S6; Lane 15: S7; Lane 16: PCR negative control (water). See Table 4.1 for detailed descriptions of colony morphology of the microbial isolates.

Thereafter purified PCR product from the selected samples served as the template for sequencing reactions. The resulting nucleotide sequences were submitted to the BLAST (Basic Local Alignment Search Tool) program (Altschul *et al.*, 1990) for comparative

analysis against the NCBI database, making use of the 'megablast' algorithm. Results of the BLAST search are given in Table 4.2. The data in the table indicate the microorganisms with the most similar sequences in the database, accompanied with the corresponding accession number and relative identities. The Expect value (*E*) is a parameter that describes the number of hits occurring by chance within a database of that particular size (Altschul *et al*., 1990). An *E*-value of 0.0 indicates a highly significant similarity and alignment between sequences.

Table 4.2: *Identity of microbial isolates.* Colonies isolated on selective medium (PES) were PCR-amplified by means of universal 16S rDNA primers and then subjected to nucleotide sequence analysis. BLAST homology matches are presented for each culture isolate. (ND: 'not determined'; samples were excluded from further analyses due to the presence of multiple bands in the PCR product (Figure 4.4)).

Sample	Bacterial Isolate Identity	Accession Number	Identities	E-value
W1	*Curtobacterium*	EF 063717	96%	0.0
W2	*Clavibacter*	DQ 659089	93%	0.0
W3	*Microbacterium*	DQ 658940	98%	0.0
W4	ND	-	-	-
W5	ND	-	-	-
S1	ND	-	-	-
S2	*Microbacterium*	EU 181228	98%	0.0
S3	ND	-	-	-
S4	*Actinobacterium*	EU037282	96%	0.0
S5	ND	-	-	-
S6	*Brevundimonas*	EU 182890	98%	0.0
S7	*L. mesenteroides*	AB 362705	97%	0.0
L. mesenteroides inoculum (rep 1)	*L. mesenteroides*	DQ 061074	98%	0.0
L. mesenteroides inoculum (rep 2)	*L. mesenteroides*	DQ 061074	93%	0.0

All of the isolates (Table 4.2) have been reported as associated with plants (Holtsmark *et al*., 2007), the soil (Holt *et al*., 1994) and / or agricultural crop production (Egamberdiyeva *et al*., 2003). Figure 4.4 illustrates the instances whereby certain culture isolates, for example 'W4', 'W5' and 'S5', did not present with the expected 230 base pair fragment after PCR, this in combination with the corresponding samples'

morphological descriptions (Table 4.1) indicate that these isolates may possibly be eukaryotes.

Sequencing and identification of the two *L. mesenteroides* inoculum populations (rep 1 and 2) confirmed the identity of these two isolates (Table 4.2). Sample 'S7', a representative of the *L. mesenteroides* confirmation colonies isolated from the total plate count procedure was positively identified as *L. mesenteroides*, and was more closely aligned to an isolate with a different BLAST accession number to that of the inoculum population. This further supports the contention that this *L. mesenteroides* population, isolated using selective culturing, may be a natural population and not a result of the initial *L. mesenteroides* inoculation.

4.5 Variation in bacterial community composition during a simulated HTCD: PCR-DGGE

Due to the highly selective and inherently biased nature of culture-dependent microbiological analyses, culture-independent analyses were conducted to investigate the changes in bacterial community structure over time during post-harvest sugarcane deterioration. As for the PCR-mediated identification of bacterial isolates by sequence analysis (Section 4.4), this aspect of the investigation was based on genomic DNA extraction (Figure 4.5) and subsequent amplification of the variable V3 region of 16S rDNA of bacteria present in the sugarcane stalk during deterioration (Figure 4.6). However, in this instance, variations in bacterial communities over time and in response to temperature were monitored through the analysis of minor nucleotide variations between the amplified fragments. These variations were detected using DGGE and enabled the amplified fragments within the samples to form a profile of the microbial populations in both a qualitative and semi-quantitative manner (Muyzer *et al*., 1993), where each band is considered to represent an operational taxonomic unit (Diez *et al*., 2001).

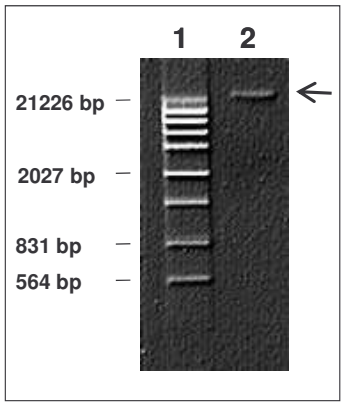


Figure 4.5: *Ethidium bromide stained 1.0% agarose gel showing a representative sample of genomic DNA extracted from frozen sugarcane tissue samples.* Lane 1: Molecular weight ladder B, Lane 2: representative sample of genomic DNA, indicated by arrow.

Using universal primers as described in section 3.5.4, fragments of approximately 230 base pairs were amplified during the PCR. Amplicon size was assessed by means of agarose gel electrophoresis against molecular weight ladder A (Figure 4.6).

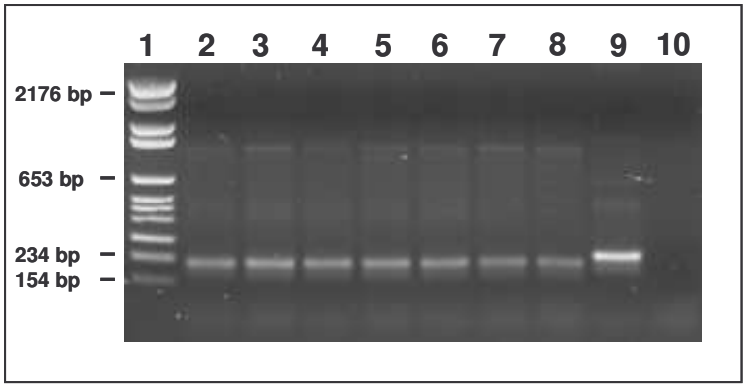


Figure 4.6: *Ethidium bromide stained 1.5% agarose gel showing the resulting 230 base-pair PCR product following amplification of the variable V3 region of 16S rDNA from genomic DNA isolated from sugarcane stalks.* Lane 1: Molecular weight ladder A, Lane 2 – 8: PCR product from tissue sample genomic DNA from winter, uninoculated stalks (lanes 2, 3 and 4: replicate samples from Day 0; lanes 5, 6 and 7: replicate samples from Day 4, lane 8: single sample from Day 9). Lane 9: PCR positive control (bacterial DNA), Lane 10: PCR negative control (water).

DNA extraction and subsequent PCR using the same 16S rDNA universal primers were also conducted on bacteria cultured from internodal tissue samples, as well as the *L. mesenteroides* population used as inoculum in HTCD experiments (Section 4.4, Figure 4.4). DNA amplified from these isolates was then mixed in an equimolar ratio, diluted, and further PCR amplified to create a 'culture-dependent' DGGE marker. Samples that did not produce the desired 230 base pair fragment (observed in lanes 7 (W4), 8 (W5) and 13 (S5) respectively) were excluded from further analyses.

4.5.1 DGGE profiling of stalk bacterial isolates

DNA amplified from the various microbial isolates (Table 4.1; Figure 4.4), *L. mesenteroides*, as well as the 'culture-dependent' DGGE marker, were then profiled by means of DGGE (Figure 4.7). Lane 1 represents the combined bacterial DNA that was used as a PCR template for the 'culture-dependent' DGGE marker. A second PCR was carried out to ensure a sufficient quantity of DGGE marker was available for subsequent DGGE gels. Lanes 8 and 14 represent this DGGE marker after PCR. The most prominent band (band 7) observed in the marker lane (lanes 1, 8 and 14) corresponded to the amplified DNA of the original *L. mesenteroides* cultures (lanes 2 and 3). Sample S7, the *L. mesenteroides* colony (Section 4.3) is profiled in lane 13. The pattern of fragment size fractionation observed for this *L. mesenteroides* (S7) was similar to those observed in lanes 2 and 3, although bands 5 and 6 were not present in lanes 2 and 3.

Of particular interest is the presence of more than one band per lane observed for single bacterial isolates. This phenomenon may result from the loading of an excess of DNA onto the gel prior to electrophoresis, and is often accompanied by band smudging. However, it has been reported in the literature that minor base changes may occur within the variable V3 region of 16S rRNA genes, resulting in the presence of multiple copies of ribosomal genes all belonging to the same bacterial species. This phenomenon is referred to as intraspecies heterogeneity and ultimately results in the presence of more than one band during DGGE (Dahllöff *et al.*, 2000; Yang and Crowley, 2000).

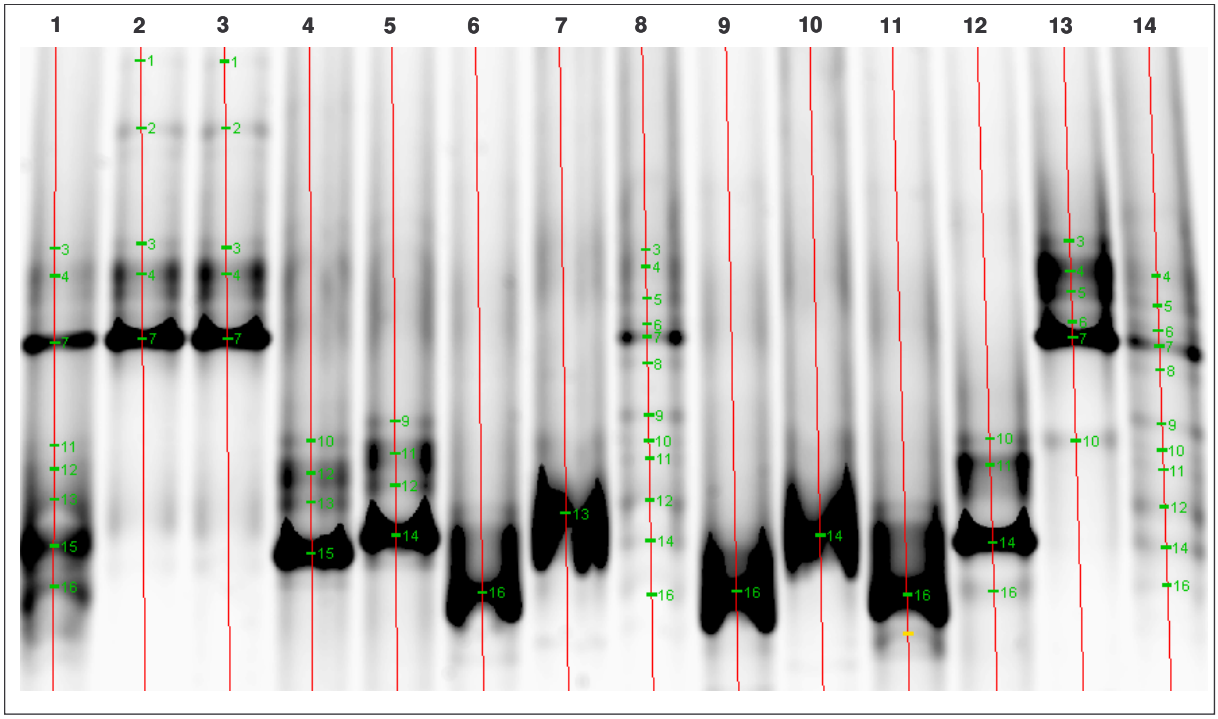


Figure 4.7: *DGGE profile of single bacterial isolates.* Lane 1: Combined bacterial DNA used as a PCR template for DGGE marker; Lane 2: *L. mesenteroides* inoculum population (rep 1); Lane 3: *L. mesenteroides* inoculum population (rep 2); Lane 4: bacterial isolate 'W1'; Lane 5: bacterial isolate 'W2'; Lane 6: bacterial isolate 'W3'; Lane 7: bacterial isolate 'S1'; Lane 8: DGGE marker; Lane 9: bacterial isolate 'S2', Lane 10: bacterial isolate 'S3'; Lane 11: bacterial isolate 'S4'; Lane 12: bacterial isolate 'S6'; Lane 13: bacterial isolate 'S7'; Lane 14: DGGE marker.

The limited number of samples that can be applied to a single DGGE gel, (16 wells per gel), in conjunction with the lack of appropriate DGGE size standards, also observed by Moeseneder *et al*. (1999) and Neufeld and Mohn (2005), necessitated the preparation of the 'culture-dependent' DGGE marker. This allowed for qualitative gel-to-gel comparisons to be made between the individual bacterial isolates' DNA profiled in Figure 4.7, and the amplified 230 base-pair fragments originating directly from the sugarcane tissue samples (profiled in Figures 4.8 to 4.12). The most intensely stained band in the DGGE marker lanes (band 7, lanes 1, 8, and 14, Figure 4.7) corresponded to the most prominent band produced by *L. mesenteroides* (lanes 2, 3 and 13, Figure 4.7). From this point it was inferred that providing the DGGE system was not altered in any way, the fifth band in the DGGE marker lane, observed as the predominantly stained band in the

DGGE marker profile, was always ascribed to the inoculated *L. mesenteroides* population.

4.5.2 Temperature, time and inoculation: Effects on bacterial communities

Due to the qualitative nature of the DGGE results presented, the 230 base-pair fragments originating from stalk tissues, namely the uninfected and infected winter samples (Figure 4.8 and 4.9) and the uninfected and infected summer samples (Figure 4.10 and 4.11) from days 0, 4 and 9 were each profiled on individual DGGE gels. This permitted the assessment of variability in banding patterns within the replicate samples (three replicates per sampling day) with the use of corresponding similarity matrices produced by Quantity One Software (Bio-Rad Laboratories, München, Germany) for each gel image. Low intensity bands were individually assessed and verified using the three dimensional imaging and magnification settings available with this software (as described in section 3.5.4e). Background stains and smears were subjectively examined in the same manner. Based on these analyses, numbers were only assigned to verifiable DGGE bands observed using the Quantity One image analysis software.

a. Bacterial community profiling: Uninoculated cane in winter

The DGGE profile in Figure 4.8 shows the uninfected samples incubated at low temperatures in triplicate for days 0, 4 and 9, together with the DGGE marker. Of particular interest was the high similarity observed within sample replicates, as well as the marked similarity observed across time. With the presence of a number of bands being consistent throughout the 9-day deterioration period, namely bands 7, 12, 16 and 18. Changes in banding patterns were limited to bands 9 and 8 appearing by day 4 and 9 respectively, as well as the appearance of band 14 by day 9 in lanes 11 and 12. Of further interest was the apparent absence of *L. mesenteroides* (represented by band 5 in the marker lanes 1, 5, 9 and 13) throughout the sample lanes despite the detection of this bacterium by selective culture techniques, albeit in relatively low numbers (Figure 4.3 D); possibly as a result of PCR bias or low template concentration compared to other bacterial populations. In contrast certain bands were observed throughout the sample lanes but did not appear evident within the DGGE marker lanes prepared with the bacterial isolates' DNA (Figure 4.7). This suggests that the microorganisms associated with these bands were not isolated, and thus possibly unculturable using the standard culture techniques used to make up the culture-dependent DGGE marker (3.5.4c).

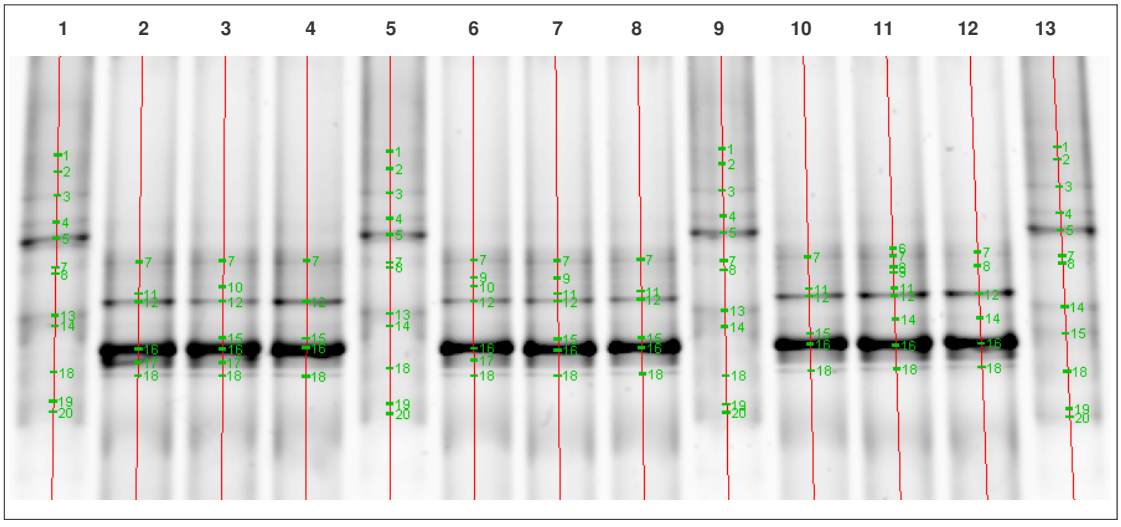


Figure 4.8: *DGGE profile for the uninoculated winter samples.* Lane 1: DGGE marker; Lanes 2, 3, 4: samples representing day 0; Lane 5: DGGE marker; Lanes 6, 7, 8: samples representing day 4; Lane 9: DGGE marker; Lanes 10, 11, 12: samples representing day 9; Lane 13: DGGE marker.

b. Bacterial community profiling: Inoculated cane in winter

The inoculated winter samples (Figure 4.9) differed from the uninoculated winter samples (Figure 4.8), in that the band indicative of the presence of *L. mesenteroides* was present in the samples by day 4, as indicated by the presence of band 5 in lanes 6, 7 and 8. This bacterium persisted in two of the three replicates (lanes 10 and 11) up to day 9, suggesting that the inoculation of the stalks was successful in creating a *L. mesenteroides* infection at a level sufficient for detection by means of PCR-DGGE by day 4. It was also observed that the average number of bands present on day 0, decreased by day 4 and 9, with the appearance of a unique band (band 4) on day 9 (lane 11), possibly associated with the endogenous *L. mesenteroides* population (S7) isolated in Section 4.3 and PCR-DGGE profiled (Figure 4.7). This association is based on the profile exhibited by the S7 isolate (Figure 4.7, lane 13), where a unique band (band 6) is discernable just above the *L. mesenteroides* specific band (band 7), which does not happen to appear in the *L. mesenteroides* inoculum population lanes (lanes 2 and 3), and thus could be attributed to the presence of a natural *L. mesenteroides* population.

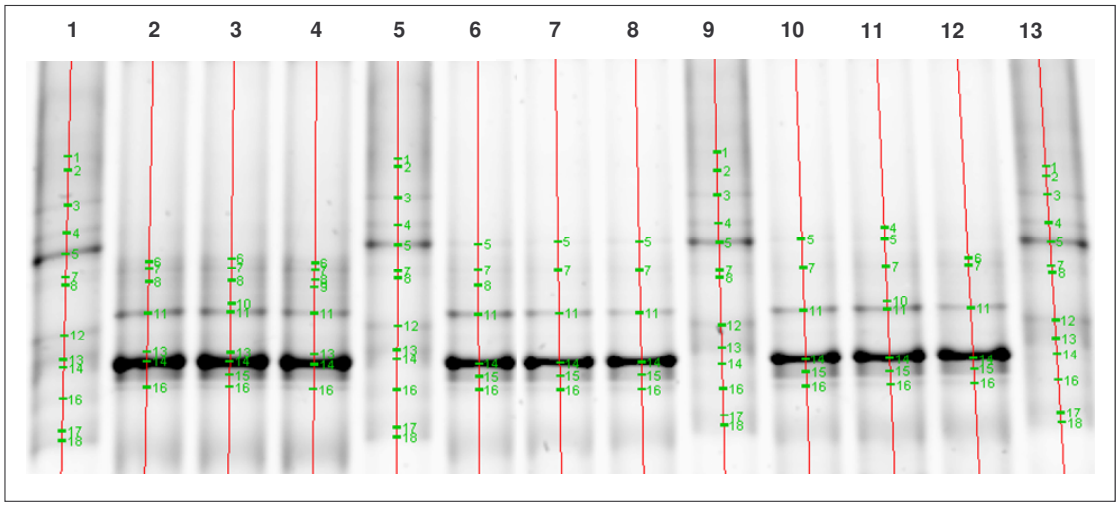


Figure 4.9: *DGGE profile of the inoculated winter samples.* Lane 1: DGGE marker; Lanes 2, 3, 4: samples representing day 0; Lane 5: DGGE marker; Lanes 6, 7, 8: samples representing day 4; Lane 9: DGGE marker; Lanes 10, 11, 12: samples representing day 9; Lane 13: DGGE marker.

c. Bacterial community profiling: Uninoculated cane in summer

In contrast to the inoculated cane subject to a simulated HTCD in winter, (Figure 4.9), *L. mesenteroides* was not detected in uninoculated cane during summer (Figure 4.10, band 5). However, the same band (band 4) that was observed in the inoculated samples in winter (Figure 4.9, lane 11) was observed in one lane on day 0, all three lanes on day 4 and finally in one lane on day 9. Once again relatively high similarity was observed within the sample replicates, with the more prominent bands remaining so throughout the 9-day deterioration period. Faint banding patterns did emerge however between days 0 and 4 (bands 8, 9 and 10). Of interest was the appearance of band 19 in lane 4 (day 0) which persisted throughout the deterioration period.

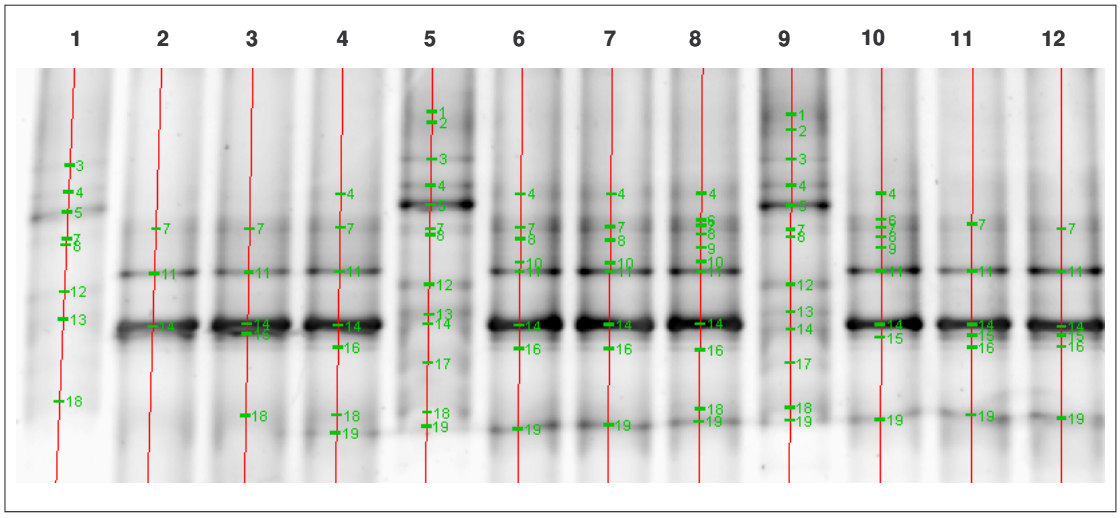


Figure 4.10: *DGGE profile for the uninoculated summer samples.* Lane 1: DGGE marker; Lanes 2, 3, 4: samples representing day 0; Lane 5: DGGE marker; Lanes 6, 7, 8: samples representing day 4; Lane 9: DGGE marker; Lanes 10, 11, 12: samples representing day 9.

d. Bacterial community profiling: Inoculated cane in summer

The inoculated summer samples displayed an increase in the number of bands between days 0 and 9, with bands 7 and 9 emerging on day 4 (lane 6), and bands 7, 8 and 9 appearing on day 9 (lane 11) (Figure 4.11). The *L. mesenteroides* inoculum population (band 5) did not appear in any of the sample lanes, however band 4 did appear on day 4 (lanes 7 and 8) and on day 9 (lanes 11 and 12), suggesting that a pre-existing, natural *L. mesenteroides* population infected the samples to the level observed in Figure 4.3 C. Of interest was the appearance of band 3 occurring on day 9 (lane 11), which did not appear in any of the previous DGGE profiles.

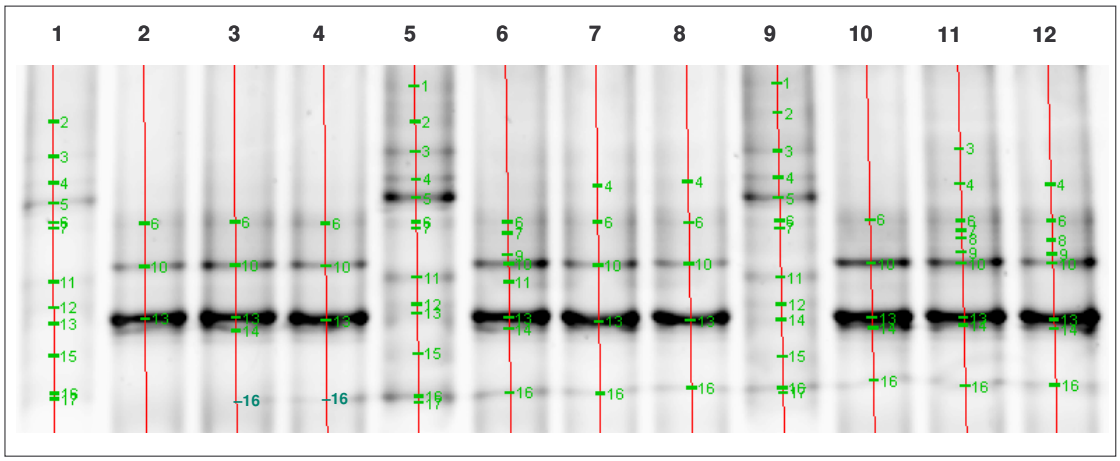


Figure 4.11: *DGGE profile for the inoculated summer samples.* Lane 1: DGGE marker; Lanes 2, 3, 4: samples representing day 0; Lane 5: DGGE marker; Lanes 6, 7, 8: samples representing day 4; Lane 9: DGGE marker; Lanes 10, 11, 12: samples representing day 9.

e. Comparison of inoculation, HTCD and temperature on bacterial communities

In order to compare banding patterns accurately across the four experimental treatments a single DGGE gel incorporating one representative sample from each triplicate set was prepared (Figure 4.12). Samples were chosen on the basis that their resulting profiles truly represented the three replicates of each treatment and time shown in Figures 4.8 to 4.11.

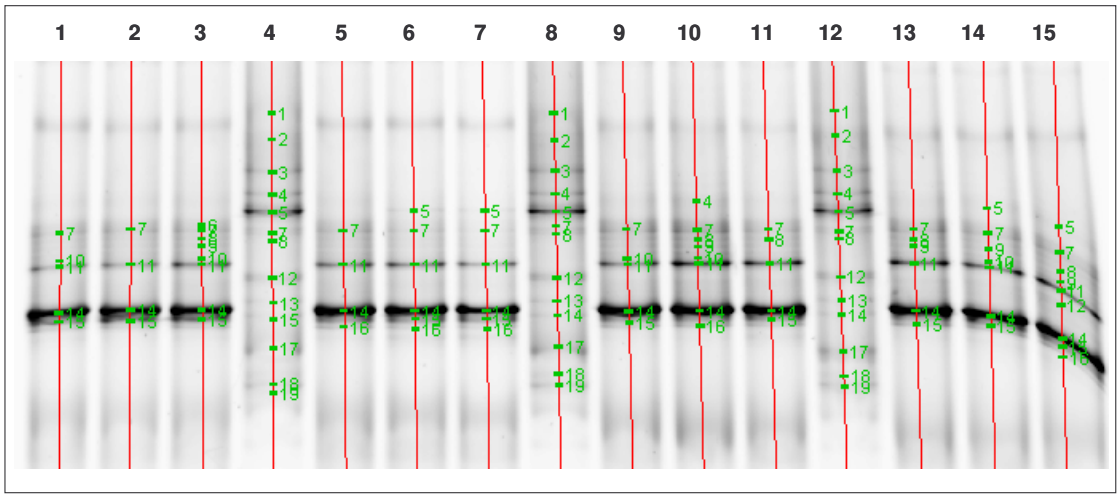


Figure 4.12: *DGGE profile comparing bacterial diversity across the four experimental treatments.* Lanes 1, 2, 3: uninoculated winter samples (day 0, 4, 9); Lane 4: DGGE marker; Lanes 5, 6, 7: inoculated winter samples (day 0, 4, 9); Lane 8: DGGE marker; Lanes 9, 10, 11: uninoculated summer samples (day 0, 4, 9); Lane 12: DGGE marker; Lanes 13, 14, 15: inoculated summer samples (day 0, 4, 9).

The resulting profiles (Figure 4.12) depict the high similarity in banding patterns observed across the different experimental treatments. Notwithstanding this, changes in banding patterns, and thus bacterial populations, were observed across time within each treatment. The first three lanes displaying the uninoculated winter samples (days 0, 4 and 9 respectively) did not contain the *L. mesenteroides* associated band (number 5). This band was observed however in two of the inoculated winter samples (lanes 6 and 7). Interestingly the uninoculated winter samples appeared to show an increase in microbial diversity with time with a number of additional bands emerging on day 9 (lane 3) compared to the inoculated winter samples.

The uninoculated summer samples showed an emergence of bacterial populations on day 4 (lane 10), together with the presence of band 4, a tentative pre-existing *L. mesenteroides* population. These bacterial populations however decreased to undetectable levels by day 9 (lane 11). The *L. mesenteroides* inoculum population was present in lanes 14 and 15 (the inoculated summer samples, day 4 and 9 respectively). A marked increase in bacterial diversity was also visible from day 0 through to day 9 in the inoculated summer samples, which was in contrast to the rest of the treatments.

Overall the samples incubated under warmer temperatures appeared to support a more diverse range of microbial populations with time compared to those incubated under the cooler temperatures.

Background stains, or artefacts, were observed near the top of the lanes in Figure 4.12. The presence of smears occurring as bands was disputed using the Quantity One image analysis software (3.5.4e), as the smears did not exhibit the same characteristics as the defined bands. The presence of smears or artefacts within the lanes of DGGE images has been observed and reported by other individuals using the same DGGE system (Govender, 2008). Background smears on DGGE gels, reported by McAuliffe and co-workers (2005), similarly dismissed the observed smears as artefacts.

Direct sequencing was performed on the DNA isolated from particular bands of interest within the comparative DGGE gel (Figure 4.12). This procedure was performed to compare identities of bacterial isolates obtained by means of culture-dependent assays to those obtained directly from the PCR amplification of DNA extracted from stalk material without an intermediate culturing step. However, sequences derived from the latter displayed multiple signals within the electrophoretogram, possibly arising from mixed template DNA co-migrating during DGGE size fractionation. In this instance, where multiple PCR products appeared to be present within a single DGGE gel, cloning of the gel extracted DNA would offer a means to distinguish sequence variation amongst amplicons of the same size.

4.6 Stalk protein concentrations during a simulated HTCD

Total protein analysis was conducted to ensure total protein levels were not affected by the applied deterioration conditions and artificial inoculation of *L. mesenteroides*, to enable the expression of sucrose and metabolite data on a total protein basis. The protein levels did not vary across the deterioration period for all treatments (Figure 4.13 A and B). Therefore average total protein values for each sample were determined and used as a basis for expression of metabolite concentrations.

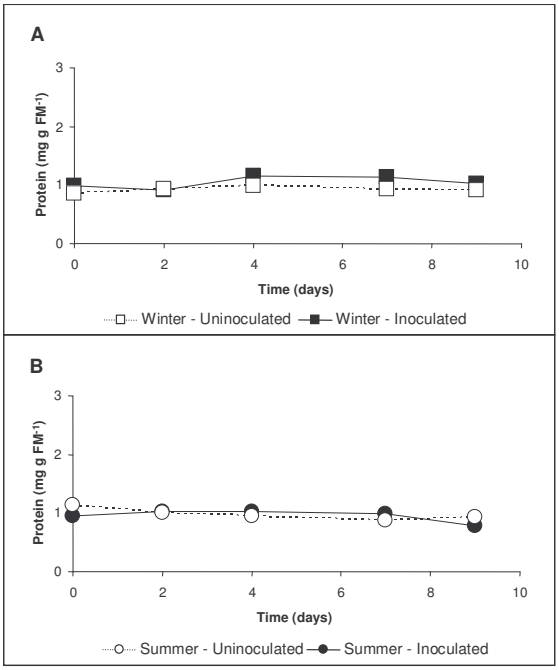


Figure 4.13: *Levels of total protein in Internode 11 under the summer and winter deterioration period of 9 days.* Levels of total protein were monitored under (A) winter (□,■) and (B) summer (○,●) conditions in stalks that were either inoculated (——) or uninoculated (**.....**) with *L. mesenteroides*. Error bars denote standard error of the mean values (n = 5); absence indicates that the standard error bar was smaller than the point symbol.

The expression of metabolites on the basis of average total protein concentration is an approach commonly used in biochemical and physiological sugarcane analysis. Total protein concentrations have been found to be consistent across mature sugarcane stalks (Whittaker, 1997, Bindon and Botha, 2001), as demonstrated by Figure 4.13. The use of fresh mass as the basis for data expression, a possible alternative to total protein concentration, was avoided as the sugarcane samples were likely to dry out during the deterioration period, particularly during the simulated summer temperatures. This dehydration of the sugarcane samples with time may have caused inconsistencies in the resulting data. The use of dry mass as the basis for data expression was also avoided as it was important to capture the precise metabolic status of the sugarcane stalk. In this study drying the cane prior to sample analysis was not practicable.

4.7 Stalk sugar concentrations during a simulated HTCD

One of the primary concerns relating to post-harvest sugarcane deterioration in the sugar industry is the loss of valuable sucrose as a result of biochemical, microbiological and chemical degradation and its impact on grower and miller profitability.

The majority of previous work on sucrose loss during cane deterioration has involved mill room-based determinations on extracted sugarcane juice or core juice samples (Smith, 1993; Yusof *et al*., 2000; Eggleston *et. al*, 2001; Saska, 2002). In this work, sucrose, glucose and fructose concentrations were determined by HPLC analysis of extracts derived from frozen internodal tissues. This approach for sugar analysis, together with the controlled temperature regimens under which deterioration was allowed to proceed, permitted the precise measurement of changes in the sugar content of sugarcane stalks with time during the deterioration process. In addition, inoculation of stalks with *L. mesenteroides* was undertaken to facilitate the measurement of changes in sugar concentrations associated with infection of harvested stalks by this primary agent of deterioration.

Average sucrose values on the day of harvest prior to the winter HTCD experiment (Figure 4.14 B) varied significantly ($p<0.05$) between 200 mg mg protein^{-1} in the uninoculated stalks and 300 mg mg protein^{-1} in the stalks that were inoculated with *L. mesenteroides*. A similar significant difference ($p<0.05$), albeit to a lesser extent, was observed in stalks subjected to the simulated summer HTCD (Figure 4.14 A). In the latter instance, the uninoculated stalks had an average sucrose concentration of 200 mg mg protein^{-1}, while 250 mg mg protein^{-1} was measured in the inoculated stalks. These differences result from the inherently high and variable concentrations of sucrose found in mature sugarcane stalks and the difficulty in precisely measuring small changes wrought by deterioration over time in such a high sucrose concentration environment. This phenomenon (Figure 4.14 A and B) is further illustrated by the lack of any overall trends in sucrose concentrations during the simulated HTCDs.

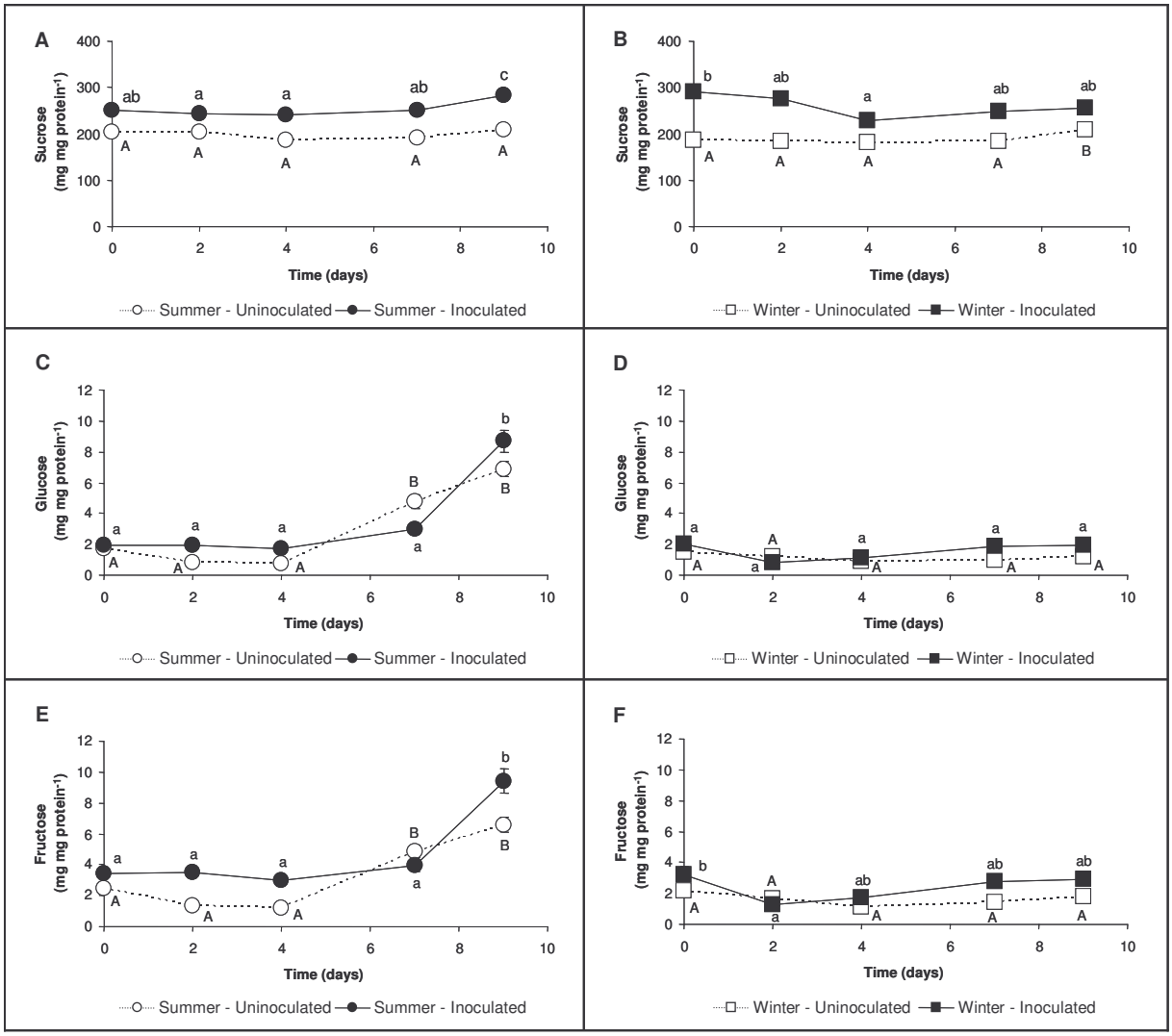


Figure 4.14: *Concentrations of sucrose, glucose and fructose during a simulated HTCD.* Sugar levels were monitored under (A, C and E) summer (○,●) and (B, D and F) winter (□,■) conditions in stalks that were either inoculated (——) or uninoculated (**.....**) with *L. mesenteroides*. Error bars denote standard error of the mean values (n = 15); absence indicates that the standard error bar was smaller than the data symbol. Symbols above or below data points denote significant difference ($p<0.05$) within data sets.

Under summer conditions, the levels of sucrose in both inoculated and uninoculated stalks did not change significantly ($p<0.05$) over the initial 7 days of the simulated HTCD (Figure 4.14 A). Between days 7 and 9, however, a slight increase in sucrose levels was observed in both uninoculated and inoculated stalks subjected to summer conditions, as

well as in the winter uninoculated treatment. This apparent increase in sucrose concentration may be due to inter-stalk variation and the difficulty of detecting minor changes in sucrose concentrations in the sucrose-rich environment of the stalk.

Significant differences ($p<0.05$) in glucose and fructose concentrations were observed in response to inoculation and temperature during the simulated HTCD (Figure 4.14 C, D, E and F). Glucose and fructose are liberated when sucrose is broken down in the stalk, either by stalk respiration or microbial activity. Hence, increases in their concentration indicate the rate of sucrose breakdown within the sugarcane stalks. Figure 4.14 C to F indicates that temperature has a pronounced effect on the concentration of these hexoses, with glucose and fructose levels increasing significantly ($p<0.05$) between days 4 and 9 in the uninoculated and inoculated summer treatments (Figure 4.14 C and E), compared to the slight changes observed in the uninoculated and inoculated winter treatments (Figure 4.14 D and F). The effect of *L. mesenteroides* on glucose and fructose levels was observed in the inoculated summer stalks, which, apart from day 7, exhibited higher glucose and fructose concentrations (Figure 4.14 C and E) when compared to the uninoculated stalks. However, these differences were insignificant ($p<0.05$). Similarly, no significant differences ($p<0.05$) in glucose and fructose concentrations between inoculated and uninoculated stalks occurred during the simulated winter HTCD (Figure 4.14 D and F).

4.8 Concentration of microbial by-products during a simulated HTCD

Quantification of the concentration of microbial metabolic by-products as a means to determine the extent of sugarcane deterioration has become a popular area of research over the past number of years. Metabolic by-products that are known to accumulate as a direct result of microbial proliferation include ethanol, lactic acid, acetic acid, dextran, mannitol, alternan and a number of other polysaccharides (Eggleston *et al.*, 2004). The accumulation of these by-products has previously been determined directly from the juice liberated from crushing deteriorated sugarcane stalks. However, for this study, the techniques were adapted to quantify the concentration of these microbial by-products in stalk tissue, with a view to assessing the precise changes occurring within the sugarcane stalk during HTCDs.

4.8.1 Lactic acid

Lactic acid is a microbial metabolic by-product produced by the Lactic Acid Bacteria during the process of heterolactic fermentation (Schlegel, 1993). Eggleston and co-workers (2001), among others (Yusof *et al.*, 2000, and Saska, 2002) have proposed that the degree of sugarcane stalk deterioration may be gauged by the accumulation of lactic acid, with titratable acidity being commonly measured as a function of lactic acid accumulation in the mill room environment.

Lactic acid (or L-Lactate) concentrations within stalks at harvest (day 0) were in the range 8 – 14 µg mg protein^{-1} across all treatments (Figure 4.15). Two days after harvest, a decrease in lactic acid levels was observed in both inoculated and uninoculated samples subjected to a simulated HTCD under both summer (Figure 4.15 A) and winter conditions (Figure 4.15 B). From days 2 to 7 lactic acid levels increased significantly ($p<0.05$) in both the summer treatments (Figure 4.15 A). During the summer HTCD, lactic acid in both inoculated and uninoculated samples reached maximum concentrations of 18 and 16 µg mg protein^{-1} by day 7, respectively, which was followed by a decrease to day 9 in the inoculated samples, while the concentration in the uninoculated samples continued to increase during this time.

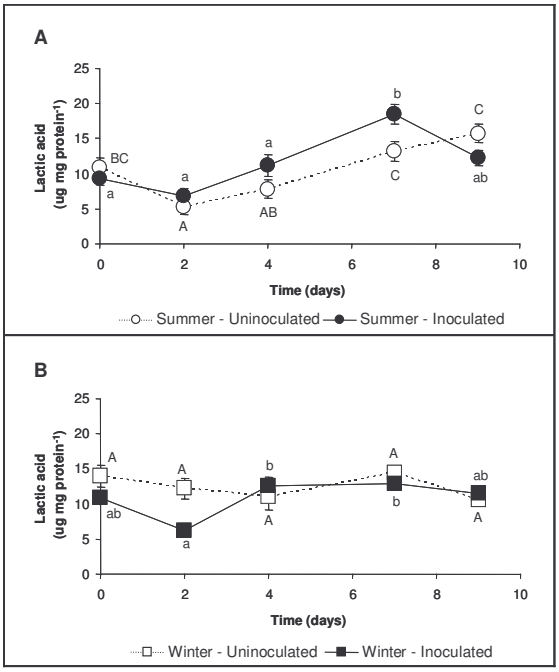


Figure 4.15: *Lactic acid concentrations during a simulated HTCD.* Lactic acid concentrations were monitored under (A) summer (○,●) and (B) winter (□,■) conditions in stalks that were either inoculated (——) or uninoculated (**.....**) with *L. mesenteroides*. Error bars denote standard error of the mean values (n = 15); absence indicates that the standard error bar was smaller than the data symbol. Symbols above or below data points denote significant difference ($p<0.05$) within data sets.

In contrast to the observation made during the summer HTCD, lactic acid concentrations in the uninoculated winter treatments did not vary significantly ($p<0.05$) over the nine day deterioration period. In the samples inoculated with *L. mesenteroides*, the initial decline in lactic acid concentrations was followed by a return to the concentration observed at harvest (Figure 4.15 B). Of interest is an apparent seasonal trend in lactic acid concentrations, with higher concentrations of lactic acid being found in the summer treatment samples overall compared to those of the winter samples (Figure 4.15).

4.8.2 Ethanol

Ethanol concentrations have been commonly used as a means to determine the extent of stalk deterioration (Amorim *et al*., 2000; Lionnet and Gooch, 2002; Saska, 2002). Like lactic acid, ethanol is produced as a by-product of microbial metabolism and is produced by a range of different microbes including bacteria, fungi and yeast (Prescott *et al*., 2002). The purpose of measuring ethanol was to examine the extent to which the appearance of ethanol correlated to bacterial proliferation, and in so doing determine whether ethanol concentrations could be used to predict potential dextran levels.

Ethanol concentrations on the day of harvest for all treatments were in the range 110 – 210 µg mg protein^{-1}. The effect of typical summer temperatures on ethanol accumulation is illustrated in Figure 4.16 A. Ethanol concentrations increased between days 0 and 2 in both the uninoculated and inoculated treatments, with a more marked increase occurring in the latter. After this initial increase, the concentration of ethanol declined, reaching similar concentrations between days 4 and 7 in both the inoculated and uninoculated stalks. At the end of the simulated summer HTCD, a significant ($p<0.05$) increase in ethanol concentration occurred in the uninoculated samples between days 7 and 9, reaching a concentration of approximately 410 µg mg protein^{-1}.

The ethanol concentrations detected in inoculated and uninoculated stalks subjected to simulated winter conditions (Figure 4.16 B) differed from those recorded for the summer treatments (Figure 4.16 A). In winter, ethanol levels increased gradually over time, although these increases were not statistically significant ($p<0.05$).

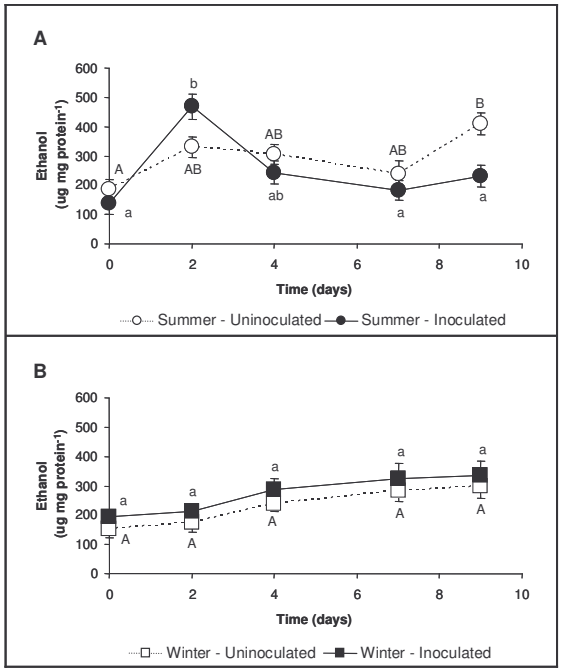


Figure 4.16: *Ethanol concentrations during a simulated HTCD.* Ethanol levels monitored under (A) summer (○,●) and (B) winter (□,■) conditions in stalks that were either inoculated (——) or uninoculated (**.....**) with *L. mesenteroides*. Error bars denote standard error of the mean values (n = 15); absence indicates that the standard error bar was smaller than the data symbol. Symbols above or below data points denote significant difference ($p<0.05$) within data sets.

Temperature appears to play a key role in the increase in ethanol observed between days 0 and 2 under the simulated summer temperature regimen (Figure 4.16 A), and this increase is pronounced in those samples that were inoculated with *L. mesenteroides* compared to those samples that remained uninoculated.

4.8.3 Dextran

The presence and accumulation of dextran in the mill, primarily ascribed to the receipt of consignments of heavily deteriorated sugarcane, is a common problem throughout the sugar milling regions of SA (Peacock and Schorn, 2002; Ravnö and Purchase, 2005). Knowledge of the causes and effects of dextran accumulation due to cane deterioration is essential to supply chain management in the industry and, hence, was an important objective of the current work. Accurate quantification of dextran and precise definition of

the conditions under which it accumulates is essential to the development of mathematical models to predict the effect of cane deterioration on industry profitability.

In this study, various means were employed to optimize glucose liberation from dextran through the enzymatic action of dextranase on dextran. In addition, a variety of technologies were employed to detect any glucose liberated by dextranase from dextran, namely enzyme-coupled assays analysis, HPLC and colourimetry. Despite extensive optimization of these methodologies dextran remained below the detection limits of the technologies in all tissue samples. Table 4.3 illustrates the inherent limitations of this method wherein the removal of starch and soluble glucose via alpha-amylase digestion is required, followed by alcohol precipitation. The step-wise loss in dextran through the process of alpha-amylase digestion, followed by alcohol precipitation was observed, with percentage recovery values as low as 21% being recorded from known dextran concentrations.

Table 4.3: *Influence of starch removal and alcohol precipitation on dextran recovery.*

ACTUAL	**RECOVERED**			
Concentration of dextran assayed (µg ml^{-1})	**After alpha-amylase digestion (µg ml^{-1})**	**Percent recovery (%)**	**After alpha-amylase digestion and alcohol precipitation (µg ml^{-1})**	**Percent recovery (%)**
2500	1449	58	604	24
2000	1219	61	417	21
1500	799	53	339	23
1000	547	55	264	26
500	329	66	259	52
0	0	0	0	0

To verify the selected technology, dextran analysis on selected replicate samples was conducted by the South African Sugar Milling Research Institute (SMRI). The data generated at SMRI confirmed that dextran levels in the stalks subjected to the simulated HTCD were below detection thresholds, thereby validating the methodology developed at SASRI.

To further assess the ability of the developed method to quantify dextran, the production of the polysaccharide was monitored in a pure liquid culture of *L. mesenteroides* grown in a sucrose medium (Atlas, 1997; section 3.5.1e). Substantial dextran levels could be

detected (section 3.5.1e), thereby confirming the identity of the bacterium as *L. mesenteroides*, as well as verifying the dextran quantification method. From this work, a minimum detection level of approximately 400 µg ml^{-1} of dextran was established. In this instance dextran production in sucrose broth occurred rapidly between days 2 and 3, where the highest concentration of glucose liberated following enzymatic action on the dextran was 125 µg ml^{-1}; corresponding to a dextran concentration of 1100 µg ml^{-1} (Figure 4.17 A and B). Of interest is the decline in dextran levels between days 3 and 4, finally forming a plateau between days 7 and 9.

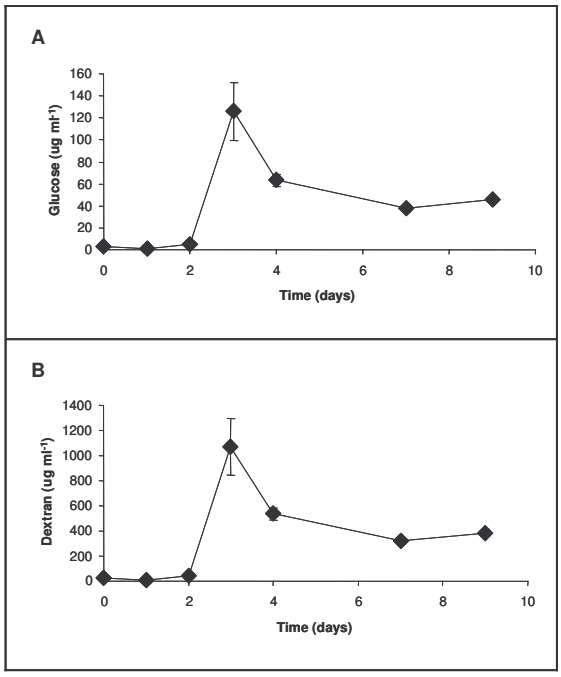


Figure 4.17: *Dextran production by L. mesenteroides.* Depicted are glucose (A) and consequent dextran (B) concentrations resulting from *L. mesenteroides* growth in a sucrose medium (Atlas, 1997; 3.5.1e) under the 'summer' temperature conditions. Error bars denote standard error of the mean values (n = 3); absence indicates that the standard error bar was smaller than the data symbol.

4.8.4 Mannitol

As dextran could not be detected in the sugarcane tissue samples an investigation into the accumulation of mannitol was conducted. The appearance of mannitol during deterioration has been suggested as a convenient alternative biochemical marker to

dextran, primarily due to the relative ease of quantification (Eggleston and Harper, 2006). Consequently, mannitol concentrations were assessed in inoculated and uninoculated stalks subjected to the simulated winter and summer HTCDs. The method employed was a modified technique of Eggleston and Harper (2006), an enzyme-coupled assay using the enzyme mannitol dehydrogenase. However, no mannitol was detected in the frozen tissue samples. Using current technologies no mannitol has reportedly been detected at SMRI either (PG Morel du Boil, 2007, Sugar Milling Research Institute (SMRI), University of KwaZulu-Natal, Durban, South Africa, pers. comm.). However ongoing investigations into the use of mannitol as a reliable post harvest sugarcane deterioration indicator is being carried out due to its relative ease of use and convenience (Eggleston and Harper, 2006).

4.9 Mill room scale assessment of sugarcane deterioration

Mill room analyses were conducted in parallel with the previously described microbiological and biochemical analyses of the effects of HTCDs on sugarcane stalks, with a view to investigating possible links between the resulting data. It is widely accepted that standard mill room analyses conducted on highly deteriorated sugarcane consignments in industry generally exhibit reduced Pol % cane, and thus lower sucrose levels, combined with a reduced level in purity (Pillay, 1994). However the results observed from mill room analyses of the simulated summer and winter HTCDs under investigation and illustrated in Table 4.4, show little evidence of loss in sucrose, or reduced Pol % cane across the four experimental regimens over time. In contrast the Pol % cane value was observed to increase slightly over the 9-day period in the uninoculated winter treatment and the inoculated summer treatment. Purity and ERC % cane values were also variable, lacking any obvious trend over time. The lack of clear trends in the data may be a result of the inherent variability amongst stalks within a single stool and between stools. The detection of experimental trends under such conditions may require extensive sampling and increase in the number of stalks constituting a single sample. However, such large-scale sampling was beyond the capacity of the current study, in that experimentation was constrained by the capacity of the incubator used to mimic summer and winter environmental conditions.

Table 4.4: *Mill room data set representing the simulated winter and summer HTCD samples for each sampling day.*

Temperature	Treatment	Duration (days)	Stalk mass (5/bundle, kg)	Dry Mass % cane	Fibre % cane	Brix % cane	Brix % dry mass	Purity	Pol % cane	ERC % cane	Cane value	Cane (g/stalk)	Suc (g/stalk)
Winter	**Uninoculated**	**0**	3.250	26.4	10.2	16.2	61.4	85.8	13.9	12.2	8.9	650.0	90.4
		2	3.050	27.5	11.8	15.7	57.1	87.9	13.8	12.3	8.9	610.0	84.2
		4	4.500	26.1	10.9	15.2	58.2	82.9	12.6	10.7	7.3	900.0	113.4
		7	3.000	26.5	11.1	15.4	58.1	87.0	13.4	11.8	8.5	600.0	80.4
		9	3.150	27.5	11.3	16.2	58.9	88.3	14.3	12.8	9.4	630.0	90.1
	Inoculated	**0**	4.050	24.8	9.7	15.1	60.9	88.1	13.3	11.9	8.7	810.0	107.7
		2	3.400	27.2	11.2	16.0	58.8	90.0	14.4	13.0	9.8	680.0	97.9
		4	3.300	26.1	10.9	15.2	58.2	86.8	13.2	11.7	8.3	660.0	87.1
		7	3.150	26.6	11.3	15.3	57.5	81.7	12.5	10.5	7.0	630.0	78.8
		9	3.100	26.4	11.1	15.3	58.0	86.3	13.2	11.6	8.2	620.0	81.8
Summer	**Uninoculated**	**0**	4.150	27.7	12.3	15.4	55.6	80.5	12.4	10.3	6.6	830.0	102.9
		2	4.050	27.0	10.0	17.0	63.0	88.2	15.0	13.4	10.2	810.0	121.5
		4	3.800	27.1	11.7	15.4	56.8	81.2	12.5	10.5	6.9	760.0	95.0
		7	4.100	28.6	12.6	16.0	55.9	91.9	14.7	13.5	10.1	820.0	120.5
		9	4.300	27.2	12.5	14.7	54.0	76.9	11.3	9.0	5.3	860.0	97.2
	Inoculated	**0**	4.450	29.1	12.6	16.5	56.7	78.8	13.0	10.6	6.8	890.0	115.7
		2	4.400	26.3	9.7	16.6	63.1	86.7	14.4	12.7	9.5	880.0	126.7
		4	4.050	27.9	12.2	15.7	56.3	87.3	13.7	12.1	8.7	810.0	111.0
		7	3.450	28.1	12.7	15.4	54.8	90.3	13.9	12.6	9.1	690.0	95.9
		9	4.400	28.4	11.8	16.6	58.5	86.7	14.4	12.7	9.3	880.0	126.7

CHAPTER 5

DISCUSSION

The major effects of post-harvest sugarcane deterioration have been investigated extensively over a number of years (e.g. Morel du Boil, 1995; Amorim *et al*., 2000; Yusof *et al*., 2000; Eggleston *et al*., 2001; Eggleston, 2002). The majority of these studies were conducted under field trial conditions and were useful in determining the general effects of deterioration, particularly due to the processing and analysis of relatively large sample sets under commercially-relevant mill conditions (Ravelo *et al*., 1991a; Smith, 1993; Solomon *et al*., 2001). However, due to the number of environmental and biological variables associated with field trials, the precise biological factors contributing to sugarcane deterioration are less well known.

The purpose of the present study was to determine the individual effects that duration of HTCD, ambient temperature and *L. mesenteroides* infection have on the biology of sugarcane stalks under controlled environmental conditions. To avoid the environmental unpredictability associated with field trial conditions, a custom-built, temperature controlled incubator was used to simulate diurnal temperature changes experienced under typical summer and winter harvesting conditions in the South African sugar industry. Simulation of the heavy microbial infection that frequently accompanies burning, harvesting and stock-piling cane prior to processing was achieved by inoculation of stalks with a known and consistent concentration of *L. mesenteroides* cells. The effect of duration of HTCD was investigated over a 9 day period, with sampling at days 0, 2, 4, 7 and 9. Various laboratory-based analyses were developed and used to examine the effect of each of these factors, namely ambient temperature, *L. mesenteroides* infection and HTCD, had on established post-harvest sugarcane deterioration indicators.

The use of controlled environmental conditions to simulate HTCDs and the subsequent laboratory based analyses to investigate and compare their precise effects on selected biochemical and microbiological markers of deterioration, represents the first of its kind at SASRI. The data generated from this type of investigation may in future be used for

the development of mathematical models to predict the commercial consequences of deterioration.

5.1 Post-harvest sugarcane deterioration in the South African sugarcane industry

Over the past few decades many researchers have investigated problems relating to post-harvest sugarcane deterioration (Egan and Rehbein, 1963; Irvine and Legendre, 1977; Lionnet, 1986a; Bacci and Guichard, 1994; Morel du Boil, 1995; Ravnö and Purchase, 2005; Corcodel and Mullet, 2007; Eggleston *et al*., 2008). However, despite this intense interest and study, quality-related problems resulting from post-harvest cane deterioration still occur frequently in the South African sugar industry and were the subject of dynamic discussion in 2007 at a Quality in the Sugar Industry Workshop, which was hosted by the South African Sugar Technologists' Association. Typical issues reported included the loss of valuable sucrose (or Pol) as sugarcane deteriorates, which results from biochemical inversion by plant and microbial respiratory activity. Such problems are further compounded by the accumulation of microbial metabolic by-products that negatively affect processing efficiency and productivity in extracting raw sugar from sugarcane. Metabolites of particular concern include ethanol, lactic acid, acetic acid, levan, alternan, mannitol and dextran (Eggleston *et al*., 2004). Issues associated with dextran accumulation are of the utmost concern not only in South Africa but across sugar industries worldwide, as this polysaccharide raises the viscosity of the extracted sugarcane juice and ultimately causes crystal deformation (Peacock and Schorn, 2002; Ravnö and Purchase, 2005). Dextran and other associated microbial metabolites become concentrated within the raw sugar crystals, negatively affecting the quality of sugar sent to the refinery (Morel du Boil, 1995). The loss in revenue for miller and grower alike escalates as more heavily deteriorated cane enters the mill; and as a result there is an urgent need to predict deterioration-associated problems in advance. At SASRI mathematical model development has been proposed to permit prediction of the causes and consequences of cane deterioration, however empirical data are required for model construction and subsequent validation. This work was initiated to generate empirical data for the purpose of model development to allow for the generation of best-practice advice for growers in the South African sugarcane industry.

5.1.1 Seasonal effect

Season, or climate, has long been known to have a pronounced effect on the rate of post-harvest sugarcane deterioration (Wood and Du Toit, 1972; Wood, 1976; Irvine and Legendre, 1977; Lionnet, 1986b; Morel du Boil, 1995). However, the precise effect that different ambient temperature alone has on sugarcane deterioration during HTCDs, and to what extent these effects differ, is less well known. In the current study, the controlled environment in which the cane samples were incubated allowed for the effects of temperature to be examined, although the additional effects of high humidity, heavy dew and rainfall were excluded.

The effect of temperature on the deterioration of sugarcane samples was evident in a number of different ways. One of the more substantial effects was the increase in glucose and fructose concentrations in the summer HTCD samples (Figure 4.14 C and E) compared to the consistently low glucose and fructose concentrations maintained in the winter HTCD samples (Figure 4.14 D and F). The presence of high invert concentrations, also expressed as low purity in the mill, has previously been reported during the deterioration of green, whole stalk harvested sugarcane (Wood and Du Toit, 1972; Wood, 1973; Smith, 1993; Eggleston *et al*., 2001). Lionnet (1986b) reported that the rate of decrease in purity and thus increase in concentration of invert sugars was higher in cane incubated at 34°C than at 19°C, which is in agreement with the above findings.

The significant increase in glucose and fructose concentrations however was not coupled to a decrease in sucrose concentration in the warmer HTCD (Figure 4.14 A) as may have been anticipated. On the contrary, sucrose concentrations appeared to increase in the infected sugarcane samples between day 7 and 9. The consistent protein concentrations found in different stalk samples (Figure 4.13 A and B), previously demonstrated by Bindon and Botha (2001), as well as Botha *et al*. (1996); confirm that the increase in sucrose observed is likely to be as a result of inherently high and variable concentrations of sucrose found in mature sugarcane stalks.

Although the effects of typical summer and winter temperatures on deteriorating sugarcane, as determined by mill room analyses (Table 4.4), generally reflected those

apparent from high-resolution HPLC analysis (Figure 4.14 A and B), the data were highly variable. This was particularly evident in Pol % cane values and the purity values across the two different temperature regimens (Table 4.4). High variability within and amongst data sets rendered impossible the detection of statistically-significant trends in sucrose concentrations over the HTCD or in response to temperature. An increase in the number of stalks constituting a single sample may have resulted in lower variability between sample replicates. However, increased sampling, treatment and incubation were beyond the capacity of the current study in that sample throughput was restricted by the capacity of the temperature-controlled incubator.

The effect of summer temperatures on the accumulation of lactic acid in deteriorating sugarcane stalks only became evident after day 2 (Figure 4.15 A). Thereafter, lactic acid concentrations increased significantly between days 2 and 7 in both the inoculated and uninoculated summer samples. In contrast, the lactic acid concentrations in the winter samples measured between days 4 and 9 did not change significantly from those measured at harvest (day 0) (Figure 4.15 B). These data suggest that lactic acid concentration could be explored as a potential biochemical marker of deterioration in green, whole-stalk sugarcane. However, the presence of lactic acid in cane at harvest (Figure 4.15 B), as previously noted by Lionnet (1986b) and Saska (2002), indicates that basal concentrations of this metabolite would have to be surveyed across environments, genotypes and agronomic management regimens before this parameter could be used diagnostically. Titratable acidity and juice pH have also been used as a measure of post-harvest sugarcane deterioration (Yusof *et al*., 2000; Eggleston, 2002; Mao *et al*., 2005), and it is assumed that lactic acid accumulation, resulting from microbial activity, is a factor contributing to the reduced pH typically found in deteriorated cane juice (Wood and Du Toit, 1972).

The effect of summer temperatures on ethanol accumulation was evident between days 0 and 2, with increases in the concentration of the alcohol occurring in both inoculated and uninoculated treatments (Figure 4.16 A). These elevated alcohol levels subsequently declined after day 2, although a sharp increase was observed between days 7 and 9. Under winter conditions, a gradual increase in ethanol concentration occurred in both inoculated and uninoculated stalks between days 0 and 9, although these changes were not significant (Figure 4.16 B). These data suggest that ethanol is

not an ideal indicator of the extent of post-harvest deterioration in green, whole-stalk sugarcane. The use of ethanol as a measure of post-harvest deterioration has received much attention in South Africa due to its rapid accumulation in juice liberated from burnt cane (Cox and Sahadeo, 1992), as opposed to green cane (Bacci and Guichard, 1994) used in this study.

Lionnet (1986b) showed that the factors that most influence deterioration of clean, whole stalk cane are temperature, burning or trashing and cane variety. Of these three variables, temperature was reported to be the most important, which appears to be corroborated by the findings of the current study. A recent collaboration between Eggleston and the South African SMRI to compare post-harvest sugarcane deterioration in Louisiana and South Africa revealed that the environment under which sugarcane is harvested plays a highly significant role (Eggleston *et al*., 2008).

5.1.2 Delays

The effects of HTCDs on the quality of sugarcane consignments delivered to mills for processing are well established (Cox and Sahadeo, 1992; Smith, 1993; Amorim *et al*., 2000). The average HTCD or burn-to-harvest-to-crush delay (BHTCD) in South Africa is estimated to be in the region of 60 to 70 hours; particularly in areas making use of benchmarking systems such as the SLIP (Sugar Logistics Improvement Plan) (Giles, 2007). However, delays have been known to exceed 200 hours, predominantly in the small-scale sector, where BHTCD of several weeks are relatively commonplace (Davis and Archery, 2007). Reports that cane delays are kept to below 24 hours in Australia (Lionnet and Moodley, 1994; Planting, 2005) indicate that the average delays in South Africa are extraordinarily high. Possible reasons for this may include difficulties in co-ordinating harvesting and logistics from small-scale growers to the mill, thus negatively affecting supply chain management (Mahlangu and Lewis, 2008). Recent increase in environmental awareness is also resulting in larger burns occurring less frequently (Ravnö and Purchase, 2005; Kent, 2008). Extenuating circumstances such as runaway fires through sugarcane fields and heavy frosts, particularly in the Midlands area, can also cause longer than average HTCDs. The use of innovative benchmarking techniques (e.g. SLIP System) used in certain areas in SA has proven extremely beneficial in reducing HTCD and BHTCD and highlighting significantly long delay areas.

The extent of post-harvest sugarcane deterioration is primarily determined by the length of the HTCD (Brokensha *et al*., 1975; Lionnet and Pillay, 1987; Yusof *et al*., 2000). The effect of HTCD on deterioration is compounded by other factors such as environment (e.g. temperature, humidity, rainfall) and extent of microbial infection, which must be taken into account when investigating the accumulation of deterioration-associated metabolites. The appearance of these metabolites (e.g. glucose, fructose, lactic acid and ethanol) was more evident under a simulated summer temperature regimen than a winter one, confirming that temperature plays an important role in determining the extent of deterioration. However, the pattern of appearance of each of these metabolites under high temperature was quite different throughout the duration of the delay. For instance glucose and fructose levels became elevated at the end of the delay (days 7 to 9), lactic acid during the latter stages (days 4 to 7) of the HTCD, and ethanol both at the beginning (day 2) and at the end (day 9). These different trends indicate the diversity of the physiological and microbiological processes that constitute deterioration. This complexity makes the selection and use of a single biochemical marker of deterioration very difficult, even under the highly regulated conditions used in this study. This also emphasizes the need for a comprehensive study of the biology of sugarcane post-harvest deterioration.

5.1.3 Infection

A number of factors contribute to the level of microbial infection in sugarcane after harvest; two of the main factors include the method of harvest, whether it is burnt or green cane harvested, and the extent to which the cane stalks are damaged either before or during harvest. In South Africa 89% of sugarcane is burnt prior to harvest (Davis and Archery, 2008), which is predominantly manually harvested as whole stalks. Burning cane prior to harvest removes the bulk of the trash and allows the cane to be harvested with relative ease compared to green cane harvesting. However, the burning process also negatively affects the plants defenses by destroying the protective wax layer on the surface of the stalk, rendering it more vulnerable to microbial infection (Bevan and Bond, 1971). In the few areas where mechanisation is being used in South Africa the sugarcane is chopped into billets, which exposes a greater surface area for potential infection (Vickers, 1968; Ivin, 1972; Wood, 1976; Ravnö and Purchase, 2005).

The level and extent of microbial infection is also highly dependent on the environment in which the cane is harvested, stored, and transported (Eggleston *et al*., 2008). As a result sugarcane is prone to varying degrees of bacterial, fungal and / or yeast infection (Bevan and Bond, 1971; Cerutti de Guglielmone *et al*., 2000).

Members of the lactic acid group of bacteria have received much attention regarding post-harvest sugarcane deterioration, in particular *L. mesenteroides* (Egan and Rehbein, 1963; Bevan and Bond, 1971; Fulcher and Inkerman, 1974; Ravelo *et al*., 1991b; Brown and Inkerman, 1992; Eggleston *et al*., 2008). This microorganism is reported to be the predominant cause of dextran formation in deteriorating sugarcane and in the milling process. For this reason, sugarcane samples in this study were inoculated with *L. mesenteroides* at harvest to mimic infection levels observed in burnt cane. The overall effect of inoculation with *L. mesenteroides* was relatively small. In both summer and winter the glucose and fructose concentrations in the inoculated samples increased to slightly higher levels compared to the uninoculated samples by day 9, however these changes were not significant (Figure 4.14 C, D, E and F). In summer the lactic acid and ethanol concentrations in the inoculated samples reached higher levels compared to the uninoculated samples, indicating that *L. mesenteroides* infection was more pronounced under summer conditions (Figure 4.15 A and 4.16 A). The most pronounced effect of infection was evident for ethanol, where a significant increase occurred between days 0 and 2 under summer conditions (Figure 4.16 A).

5.1.4 Microbial by-products negatively affecting mill room processing

Apart from the loss of valuable sucrose as a result of post-harvest sugarcane deterioration, the accumulation of microbial by-products that negatively affect mill and factory processing are of major concern to millers and growers alike (Ravelo *et al*., 1992; Morel du Boil and Wienese, 2002; Kulkarni and Warne, 2004; Eggleston and Harper 2006; Foster and Bryan, 2007). These by-products are made up of a number of oligosaccharides and polysaccharides, with dextran being of primary concern (Hidi and Staker, 1975; Morel du Boil, 2000; Rauh *et al*., 2003; Ravnö and Purchase, 2005). Surprisingly, however, no dextran was detected in the sugarcane samples throughout the HTCD in this study suggesting that the sugarcane samples did not reach deterioration levels commonly experienced in industry. Dextran is a polysaccharide

produced by the bacterium *L. mesenteroides*; therefore it may be assumed that the conditions within the HTCD experiment were not ideal for dextran production by the inoculated bacterium. Ravnö and Purchase (2005) found that burnt cane was more prone to dextran formation than unburnt, and billeted cane more vulnerable than wholestick cane. In a similar study Irvine and Legendre (1977) showed that dextran accumulated naturally on the cut ends of deteriorated green cane stalks, but that it could not be detected in the inner segments. They reported that the amount of dextran increased with time in both the terminal and inner segments, but that the rate of increase was slowest in the centre. Ultimately it was concluded that a higher production of dextran occurred near cut and dying cells, with dextran concentration being proportional to the amount of cells damaged by burning, bruising and freezing (Irvine and Legendre, 1977).

The minimally invasive technique used for inoculation of *L. mesenteroides* into internode 11 of each experimental stalk in this study may have limited the extent to which this bacterium could infect the surrounding inner stalk tissue. *L. mesenteroides* cells were quantified throughout the HTCD and despite a slight increase between the day of harvest and day 2 in the inoculated stalks; the *L. mesenteroides* cell numbers remained fairly constant (reaching approximately 1 x 10^5 colony forming units per gram of fresh tissue, Figure 4.3 C and D). Therefore it is likely that prolific *L. mesenteroides* infection primarily requires a large majority of the stalk cells to be damaged or dead, as may occur in burnt cane or frost-damaged cane. Under such conditions, it is likely that bacteria may more easily gain entry into the damaged cells (Morel du Boil, 2001; Eggleston and Legendre, 2003). Studies conducted by Ivin and Bevan (1973) showed that approximately 1 x 10^6 viable bacterial cells were required per millilitre of disintegrated extract before a detectable dextran concentration could be achieved. Although that study is not directly comparable, it is of note that the bacterial cell numbers within inoculated tissues in this study only reached concentrations of up to 1 x 10^5 colony forming units per gram fresh tissue. In addition, it would appear that plant defenses to pathogen invasion are sufficiently intact in unburnt stalks to suppress *L. mesenteroides* invasion and subsequent dextran accumulation.

In the South African sugar industry, data show that dextran concentrations tend to become more problematic towards the end of the harvesting season, correlating strongly

with high rainfall periods (Ravnö and Purchase, 2005). Although this is mainly attributed to rain-induced delays, it is likely that increased moisture conditions support an increase in microbial proliferation, particularly *L. mesenteroides* as observed by Eggleston and Legendre (2003), and allow an increase in microbial transmission between cane stalks. As humidity and moisture in the surrounding environment were not considered in the current study, it is impossible to indicate whether these factors may have contributed to the apparent lack of *L. mesenteroides* proliferation after inoculation.

5.1.5 Varietal effect: Unburnt versus burnt sugarcane

Although not examined in the current study, reports in the literature indicate that variety may contribute to the extent of deterioration (Ivin and Bevan, 1973; Wood, 1973; Wood, 1976; Irvine and Legendre, 1977). Research undertaken at SASRI by Wood (1973a) found differences in the rates of deterioration in unburnt cane of different varieties within four to seven days after harvest, with these differences becoming progressively greater with time. However, in burnt cane this effect was not as distinct, with the different varieties showing similar deterioration rates throughout the deterioration period (Wood, 1973). The differences observed between burnt and unburnt cane of the same varieties could be as a result of a number of different factors. Cane that is harvested unburnt, or green for instance, continues to respire for a minimum of 200 hours post-harvest (Watt and Cramer, 2008), with respiration rates determined primarily by temperature. Sucrose inversion rates and purity levels may differ according to variety, as well as the extent to which the variety is resistant to post-harvest infection in terms of overall stalk rind strength and impermeability, pith: fibre ratios and perhaps more complex plant-host defense mechanisms. In contrast when sugarcane is burnt prior to harvest the integrity of the cane stalk is compromised, respiration in the stalk ceases, and the waxy stalk surface is destroyed, leaving the entire stalk vulnerable to a higher degree of infection compared to unburnt cane (Irvine and Legendre, 1977). Burning sugarcane prior to harvest may therefore limit the effect that variety has on post-harvest sugarcane deterioration rates.

5.2 Harvesting techniques and their effect on deterioration indicators

There is a rapid international trend towards the adoption of green-cane harvesting techniques for a number of reasons, particularly increased environmental awareness, pressure to reduce smoke and fall-out (or ash), the potential for increased sucrose recovery, and the desire to prevent soil moisture-loss, degradation and erosion (Richard *et al*., 2001; Viator *et al*., 2006; Corcodel and Mullet, 2007; Kent, 2008). The value of trash has also received attention recently as an alternative energy source, for example in the applications of boiler fuel (Kent, 2008). Implementation of mechanical harvesting, although requiring high initial capital expense, allows farmers to harvest their cane without burning and thus potentially benefiting from the value of their trash. However, a large majority of sugarcane grown in South Africa is still harvested burnt. One of the main reasons for this, especially with respect to the larger commercial farms that can afford to implement mechanical harvesting, is that the South African sugar industry is largely located in areas with very hilly topography, rendering the use of mechanical harvesting almost impossible. Burning cane prior to harvest acts as an effective means of avoiding the negative factors associated with the alternative of manual green cane harvesting. Negative factors include the time consuming and laborious task of manually removing trash from green cane stalks; as well as the inherent increase in tops and trash delivered to the mill (Bernhardt, 1994; Kent, 2007). Higher trash content in cane has substantial effects on the operation, recovery and overall performance of a sugar mill, which can largely be avoided with burnt cane harvesting. A number of studies however, have proven that deterioration rates in green cane are far reduced compared to burnt cane (Foster, 1969; Bacci and Guichard, 1994; Lionnet and Moodley, 1994; Eggleston *et al*., 2008). Reasons behind reduced deterioration rates in green cane have been mentioned, and include the intact stalk rind acting as a protective barrier against desiccation and microbial infection, and potentially possessing bacteriostatic properties, as observed by Bevan and Bond (1971). However, if green cane is billeted then it deteriorates more rapidly than whole-stalk harvested green cane (Vickers, 1968; Ivin, 1972; Irvine and Legendre, 1977; Eggleston *et al*., 2001; Solomon *et al*., 2007). Ultimately if a biochemical marker is to be effective in determining deterioration then it must be tailored to the specific harvesting system used. For example the use of mannitol as a deterioration indicator has been developed and is suitable for Louisiana (green,

billeted cane), however, this same marker does not seem readily transferable to South Africa (burnt, whole-stalk).

5.3 Microbiology of post-harvest sugarcane

Microbiological degradation is known to be the largest contributing factor to post-harvest deterioration associated losses in sugarcane (Lionnet, 1986b; Eggleston, 2002). Microbial populations of fungi, yeasts and bacteria have all been isolated from deteriorating cane (Tilbury, 1968; Bevan and Bond, 1971; Cerutti de Guglielmone *et al*., 2000). The type and extent of microbial infection is determined by a range of different factors, namely environmental pressures such as temperature, humidity, moisture availability and presence or absence of oxygen. Time between harvest and crushing and the method of sugarcane harvest also play an important role. However, little is known about the exact effect and influence of these factors on the different microbial communities, and their infection levels, within deteriorating cane stalks. The presence and accumulation of *L. mesenteroides* in the extracted juice from deteriorated sugarcane is well documented (Tilbury, 1968; Bevan and Bond, 1971; Ivin and Bevan, 1973; Ramos *et al*., 1992; Purchase, 2001). The high prevalence of *L. mesenteroides* in deteriorated cane juice, coupled with the capacity of the organism to produce dextran and other by-products has resulted in this bacterium receiving a great deal of attention with regards to post-harvest sugarcane deterioration (Cerutti de Guglielmone *et al*., 2000; Eggleston *et al*., 2008).

5.3.1 Effect of L. mesenteroides infection on the total bacterial population

Leuconostoc mesenteroides is naturally found in relatively low numbers on the surface of living, undamaged plant tissue, along with far less fastidious microorganisms, such as Gram–negative bacteria and aerobic spore formers (Holzapfel and Schillinger, 1992). However, the release of nutrients from surface cracks and, or mechanical damage, render the sugarcane plant highly vulnerable to opportunistic *L. mesenteroides* infection (Bevan and Bond, 1971). The inoculation of *L. mesenteroides* in this study was intended to mimic infection resulting from the burning of sugarcane prior to harvest, a process widely known to exacerbate *L. mesenteroides* infection. The effect of *L. mesenteroides* infection was then assessed according to total bacterial cell numbers within sugarcane

stalks incubated under different temperature conditions. Under typical winter conditions, *L. mesenteroides* was found to be the predominant bacterium throughout the HTCD in both inoculated and uninoculated samples (Figure 4.3 B and D). Inoculation caused total bacterial numbers to increase from 1 x 10^3 cells per gram fresh tissue to 1 x 10^5 cells per gram fresh tissue between days 0 and 2, an increase typically observed over 7 days in the uninoculated samples at the same temperature (Figure 4.3 B); despite the apparent presence of *L. mesenteroides* in the uninoculated samples on day 0. This result illustrates the resistance of undamaged, green cane stalks to initial post-harvest microbiological infection under cool climatic conditions.

Leuconostoc mesenteroides inoculation in summer contributed no effect to the observed increase in total bacterial concentration between days 0 and 2 (Figure 4.3 A). The initial *L. mesenteroides* inoculation on day 0 in summer gave rise to the predominance of this bacterium in these inoculated samples throughout the HTCD (Figure 4.3 C). The *L. mesenteroides* population existing in the uninoculated summer samples however, fell to undetectable levels by day 7 (Figure 4.3 C). This suggests that although the optimum temperature range for *L. mesenteroides* growth is between 20 and 30°C (Garvie, 1984), other environmental, physiological or microbiological factors may play an important role in determining infection levels within harvested cane stalks. For example, other infecting microorganisms present in higher concentrations in the sugarcane stalk may effectively out-compete and thus inhibit lower concentrating *L. mesenteroides* populations. Other factors such as a larger degree of stalk damage, coupled with increased humidity, are however known to favour an increase in *L. mesenteroides* infection (Ivin *et al*., 1973; Morel du Boil, 1995). Nevertheless, the overall number of bacterial cells in the uninoculated summer samples remained at approximately 1 x 10^3 colony forming units per gram of tissue between days 4 and 7, with an increase in bacterial cell numbers observed between day 7 and 9. This highlights the presence of other bacterial populations within sugarcane stalks and their proliferation under warm, relatively dry conditions. However, the extent of deterioration-associated losses contributed by these other microbial populations' remains undefined.

5.3.2 Bacterial species isolated from green post-harvest sugarcane

Investigation into the bacterial species existing within the harvested sugarcane during the HTCDs was carried out using culture-based techniques initially (Table 4.1), followed by preliminary identification of the microorganisms using molecular technologies. The incorporation of the antifungal agent, cyclohexamide, into the growth medium limited this study to bacterial species. Other than the work done by Egan and co-workers in 1963, few recently performed studies have focused on the microbial flora existing within harvested green cane stalks, with more emphasis previously placed on the microorganisms existing within first expressed juice in the mill (Purchase, 2001; Ravnö, 2001).

A range of different bacterial species were isolated and successfully identified from the sugarcane stalks at various stages throughout the two HTCDs (Table 4.2), all of which have previously been found to be associated with the agricultural environment in some form or another. For example, *Curtobacterium flaccumfaciens* is known to be pathogenic towards soyabeans (Huang *et al*., 2007), and has a number of other hosts. *Clavibacter xyli* subsp. *xyli* is a pathogenic endophyte and causes ratoon stunting disease (RSD) in sugarcane (James and Olivares, 1997). *Clavibacter michiganensis* infects tomatoes, as well as other solanaceous plants (Holtsmark *et al*., 2007). Particular strains of *Microbacterium* are known to exhibit plant-growth-promoting effects on crops such as cotton, wheat and maize (Egamberdiyeva *et al*., 2003), and have also been associated with nitrogen fixation and certain sugarcane varieties. Certain species belonging to the Genus *Brevundimonas* are endophytes (Zheng *et al*., 2008) and some species are known to be soil dwelling microorganisms (Holt *et al*., 1994). This level of microbial diversity is typical of that previously found within the sugarcane stalk (Bevan and Bond, 1971; James and Olivares, 1997; Insuellas de Azeredo *et al*., 1998).

5.3.3 Molecular profiling of cultured bacterial isolates using PCR-DGGE

In order to compare the identified bacterial isolates described in 5.3.2 with the populations PCR-amplified directly from the stalk tissue, the amplified DNA of the bacterial isolates (Figure 4.4) were first DGGE profiled individually alongside the combined amplified fragment mixture, or 'culture-dependent' DGGE marker (Figure 4.7). The *L. mesenteroides* inoculum profile (lanes 2 and 3, Figure 4.7), although highly similar to the S7 isolate profile identified as *L. mesenteroides* (lane 13, Figure 4.7), did not contain bands 5 and 6 (corresponding to bands 3 and 4 in Figures 4.8 to 4.12). This result, together with differing accession numbers obtained for the inoculum population and the S7 isolate, respectively (Table 4.2), confirms that the S7 isolate was a natural population of *L. mesenteroides* and not contributed by the initial inoculation. Ultimately however, and despite the differences observed between the natural *L. mesenteroides* profile and the inoculum profile, the appearance of bands numbered 1 to 5 in the DGGE marker lanes all reflect the presence of *L. mesenteroides*. Each of the other bacterial bands migrated further down the denaturing gradient gel compared to the *L. mesenteroides* associated bands (Figure 4.7), presumably as a result of their higher GC ratios (Muyzer *et al.*, 1993) within the 230 base-pair fragment under investigation.

The bacterial isolates displayed highly similar fragment migration profiles, with the dominant bands occurring in close proximity to each other (ranging from bands 13 to 16). Isolates W3 and S2 were both identified as *Microbacterium* species (Table 4.2); and the fragment migration distances for both of these isolates were equal (band 16, Figure 4.7). Although the rest of the isolates were identified under different genera, the larger group, and or family, in which each isolate is classified, is suspected to play an important role in the distance of band migration within a denaturing gradient. Isolates W1, W2, W3, S2 and S4 all belong to the group Actinobacteria, of these W1, W2, W3 and S2 are of the family Microbacteriaceae, and S4 is of the family Micrococcaceae. Whilst the bacterial isolates from the same family, or group, may have similar GC contents, and show significant sequence similarity within their amplified fragments, the link between each bacterial group in terms of similar fragment migration distances (e.g. S4 and S6 both at band 14) remains unclear. This phenomenon is not unique and has been demonstrated previously by Muyzer and co-workers (1993).

Ultimately, similar fragment migration distances between isolates, in conjunction with the phenomenon of intraspecies heterogeneity occurring in a number of the isolates, caused the 'culture-dependent' DGGE marker to become relatively indistinct. Therefore, precise classification of each band within the DGGE marker was difficult to interpret and accurately identify. One of the major pitfalls associated with the DGGE technique is the lack of consensus regarding standards for normalization (Neufeld and Mohn, 2005), where a number of other fingerprinting methods have the advantage of using commercially available size standards. The 'culture-dependent' DGGE marker was produced in order to overcome this drawback and allow for qualitative gel-to-gel comparisons to be made. However, the use of fluorophore-labelled primers, recently demonstrated by Neufeld and Mohn (2005), may offer an alternative means to allow for a more definitive internal standard to be produced. Optimisation of the concentrations of denaturants used in the polyacrylamide gel gradient to deliver more narrow gradients may permit increased migration distances and higher resolution between fragments of similar GC contents (Muyzer *et al*., 1993). The introduction of a second gradient, otherwise known as Double Gradient-DGGE has also been shown to further assist in improving the resolution of DGGE profiles, as assessed by Haruta and co-workers (2002), for bacterial community analysis. These technological modifications could be implemented in further studies to characterize and catalogue the endophytic communities existing within harvested sugarcane.

5.3.4 Bacterial community structures in post-harvest green sugarcane

The use of modern molecular technologies to investigate the diversity of microbial communities within complex environments has become a popular area of research over the past decade (Muyzer *et al*., 1993; Haruta *et al*., 2004; Fontana *et al*., 2006). Whilst culturing techniques remain an important component of microbial isolation and enumeration studies, their use has proven to be limited with respect to community structure analysis, primarily due to their inherent bias selectivity. Culture-based investigations in this project focused primarily on the determination of bacterial infection levels and detection of the predominant bacterial species residing within the sugarcane stalks throughout the delay period. With the culture-independent analyses, emphasis was placed on identifying bacterial community diversity within the sugarcane stalks over the HTCD, using the molecular profiling technique PCR-DGGE. This allowed for the

detection of changes to bacterial populations over the 9-day HTCD period, as well as across the different temperature and inoculation regimens.

The results of the molecular analyses indicated that temperature and HTCD duration had minimal effect on overall bacterial diversity, with similar fragments, and thus bacterial species, dominating across samples under both simulated temperature regimens. However, minor changes in banding patterns and thus bacterial species were evident, particularly with respect to bands of lower signal intensity. In the uninoculated winter samples, the number of bands increased from day 0 through to day 9 indicating an increase in bacterial diversity with time (Figure 4.8), which also occurred in the uninoculated summer samples between days 0 and 4. This is in contrast to the inoculated winter samples, in which a decrease in the number of bands was observed over the HTCD (Figure 4.9). The effect of *L. mesenteroides* inoculation was observed by the presence of the corresponding band (band 5) by day 4 and 9 in the inoculated winter samples (Figure 4.9); with no evidence of *L. mesenteroides* in the uninoculated, winter samples throughout the delay period (Figure 4.8). Inoculation of this bacterium therefore appeared to have an overall inhibitory effect on the number of different bacterial populations developing during the HTCD under cool conditions. DNA fragments were present in the stalk sample lanes that may correspond to those derived from the cultured bacterial isolates in the DGGE marker lanes, but confirmation of their exact bacterial identities was not possible (Figure 4.8 and 4.9).

The presence of band 4, associated with the pre-existing *L. mesenteroides* population in the inoculated summer samples (Figure 4.11) is in contrast to the same samples profiled in Figure 4.12, where band 5 (or the inoculum population) appears to be the resident population. It is assumed, however, that under higher temperatures, the inoculum population became dominant over the HTCD, as was evident in the bacterial enumeration analysis (Figure 4.3 D). The DGGE profile comparing bacterial diversity between each experimental treatment (Figure 4.12) reflects the small change in overall bacterial diversity across temperature and over the HTCD period, with the most discernable increase in bacterial diversity over time occurring within the inoculated summer samples. This may be as a result of the inoculum population creating an environment more conducive to bacterial colonization under simulated summer

conditions, compared to the uninoculated summer conditions and both inoculated and uninoculated winter regimens.

To avoid future problems related to DGGE band visibility and weak signal intensity, particularly in the images required for publishing, it may be worthwhile to follow the work done by Moeseneder and co-workers (1999) who captured the image of one DGGE gel in three different exposure times to obtain one optimally illuminated image, one oversaturated image, and one undersaturated image. Schematic drawings of the band patterns could then be produced by combining the information from all three images acquired from one gel, thereby increasing the information retrievable from one gel (Moeseneder *et al*., 1999). Further modifications to the PCR-DGGE technique would include additional testing to determine the optimum concentration of PCR product to be applied to the denaturing gradient gel to increase low band signal intensity, a common problem observed in Figures 4.8 to 4.12. The use of fluorophore-labelled primers could be incorporated into the system to inexpensively improve the sensitivity and versatility of this technique (Neufel and Mohn, 2005), potentially increasing low band signal intensities further. Optimisation of the concentrations of denaturants used in the polyacrylamide gel gradient, or as mentioned, the introduction and optimisation of a second gradient (Double Gradient-DGGE) may also be worthwhile to improve the resolution of the DGGE profile (Haruta *et al*., 2002).

5.3.5 Culturing versus non-culturing techniques

The limitations associated with traditional microbiological methods, which rely strongly on culture enrichment techniques for the analyses of community diversity, are well documented (Reysenbach *et al*., 1992; Yang *et al*., 2001; Haruta *et al*., 2006). With the isolation of only a fraction of the total microbial biomass being possible, the capacity of those techniques to characterise microbial diversity within complex environments is very restricted (Bruce *et al*., 1992; Hugenholtz *et al*., 1998). However, recent advances in molecular phylogenetic approaches, which are based on the amplification and comparative analysis of 16S rRNA genes from natural microbial communities, have alleviated a number of the problems associated with culture-based techniques (Hugenholtz *et al*., 1998; Case *et al*., 2007).

The array of microbiological analyses carried out in this study has permitted the comparison of the culture-dependent and culture-independent approaches used. The cultivation and enumeration analyses showed that *L. mesenteroides* was the predominant bacterial isolate prevailing within the harvested cane samples throughout the delay period, except during the latter stages of the summer HTCD in uninoculated stalks. In contrast however, the *L. mesenteroides* associated bands only appeared in the DGGE profiles (Figure 4.12) at concentrations above approximately 1 x 10^4 colony forming units per gram of fresh tissue (Figure 4.3 C and D), and with relatively low signal intensity. Hence, in contrast to the culture-dependent methods, PCR-DGGE indicated that *L. mesenteroides* was not the dominant bacterial population within the post-harvest cane samples.

Further to this, the PCR-DGGE technique highlighted a common problem experienced when using cultivation based analyses whereby a number of unculturable microbial populations are typically overlooked (Nakatsu, 2007). For example the presence of band 11 (Figure 4.12), a particularly dominant population throughout the sample lanes, among others (e.g. band 9 and 10), did not correspond to any of the bands within the marker lanes, and thus were undetected using the culture-based analyses. This shows the biased nature of traditional culture-based techniques; with a limited spectrum of microbial populations being isolated. Coupled to this was the distorted community profile created by the culture-plating analyses, where *L. mesenteroides* appeared to be the dominant population, whilst the molecular-based analyses reflected a completely different scenario in terms of the overall number of bacterial populations and their relative proportions within the total community. The overall effect of these other bacterial populations on the deteriorative process within the post-harvest cane samples however remains unknown.

The consistent banding patterns observed throughout the DGGE profiles highlight the specific environmental niche created by the green cane harvested stalk. The high similarity between DGGE profiles shows the high reproducibility of this molecular technique, an important requirement for any microbial diversity study (Nakatsu, 2007). However, culture-independent analyses, including PCR-DGGE are not completely infallible, and a number of discrepancies may arise when using this technique. For example, PCR-amplified products may not accurately reflect the full microbial population

due to different copy numbers of small-subunit rRNA genes existing, and, or possible bias occurring during the amplification process (Suzuki and Giovannoni, 1996; Haruta *et al*., 2002). Problems may also be attributed to the PCR components and the specific parameters used (Lopez *et al*., 2003), with primer specificity and correct annealing temperatures being crucial. There also appears to be a threshold concentration of DNA template required within the environmental sample to allow for PCR amplification (Muyzer *et al*., 1993; Suzuki and Giovannoni, 1996). This was apparent in a number of instances where the presence of *L. mesenteroides* was observed on the culture plates, but with no corresponding appearance in the DGGE profile. The phenomenon of intraspecies heterogeneity caused by microvariations within the targeted region of the 16S rRNA genes causing more than one band to appear on a DGGE profile further complicates the interpretation of the resulting band profile in analysing samples from mixed microbial communities (Dahllöff *et al*., 2000; Case *et al*., 2007).

Although the cultures isolated directly from the harvested cane samples were not directly evident within the DGGE profiles, the overall results obtained showed merit in the use of both culture-dependent and culture-independent analyses for future microbial diversity studies looking at specific environmental niches. It can be concluded that the microbial community structure within post-harvest sugarcane is highly complex, and comprehensive microbial characterisation of this environment is required for a better understanding of the processes occurring therein.

5.4 Recommendations and conclusion

The primary aim of this study was to determine the influence of temperature, time and *Leuconostoc* infection on the biochemistry and microbiology of harvested sugarcane stalks. The concentration of specific metabolites was measured, encompassing those of commercial value (sucrose) and those which are potential indicators of deterioration (glucose, fructose, lactic acid, ethanol and dextran). The overall effect of season, or temperature was primarily reflected by increasing glucose and fructose levels in summer compared to the constant levels measured under the winter conditions. Also known as purity in the mill, a more precise evaluation of invert concentrations in cane consignments could potentially indicate the extent of cane deterioration. Despite basal concentrations of lactic acid found on the day of harvest (day 0), the possibility of this

metabolite serving as a marker for post-harvest deterioration in green cane exists, with increasing concentrations of lactic acid occurring in summer. In contrast the use of ethanol as a potential indicator of post-harvest deterioration in green cane was ruled out. The inability to detect dextran and mannitol in the samples combined with consistent sucrose levels and scattered mill room data across the delay period may have shown that the cane samples did not reach deterioration levels commonly experienced in industry. The additional effect that burning cane prior to harvest may have on increasing deterioration rates; as well as the negative effects associated with large scale stockpiling, mechanical loading and delivery of cane consignments from the field to the mill was also not considered. Ultimately however, for a biochemical marker to be effective as a post-harvest deterioration indicator it should become apparent even at low levels of deterioration, it should also be adapted and tested for the type of harvesting system used (e.g. for green cane, or burnt cane), and the particular type of environmental conditions to which the cane is exposed (e.g. Louisiana: green cane harvesting system under humid conditions).

Culture-based analyses found that *L. mesenteroides* was the dominant bacterial species within the samples, despite the limited detection of deterioration associated indicators. A number of other bacterial species were also isolated and identified along with *L. mesenteroides*; however the level to which these microorganisms proliferated was limited to a maximum of 1×10^5 colony forming units per gram of fresh tissue. PCR-DGGE analyses revealed that *L. mesenteroides* was not the dominant population, and showed that the level of bacterial diversity within the samples was fairly consistent across the differing treatments and with time. Future work should initially focus on broad-based microbial investigations under different harvesting and environmental conditions, including a combination of both culture-based and molecular-based analyses, to determine the identities of the microbial populations contributing to the post-harvest deterioration process. Another approach that may prove useful in determining total microbial activity in sugarcane is the use of Fluorescein Diacetate (FDA). FDA is hydrolyzed by a number of different microbial enzymes, and the product of this enzymatic conversion is fluorescein, which can be visualized and quantified with spectrophotometry or fluorometry (Schnürer and Rosswall, 1982). In terms of the scope for PCR-DGGE analyses, future work should focus on the choice of PCR primer, optimisation of the denaturant gradient for the PCR fragment in question, and finally

cloning of selected bands of interest from the DGGE gel to assist in the identification of unculturable populations. In the interest of formulating industry-relevant recommendations, the major microbial cause of deterioration should be established, based on the specific harvest method used and the environmental conditions prevalent for the specific milling area under question, as this will assist in finding a biochemical marker associated with that particular type of infection.

Recommendations to reduce the impact of post-harvest sugarcane deterioration should primarily include reducing the harvest to crush delay period, particularly if the sugarcane is burnt. Alternatively, if delays cannot be reduced, the method of green cane harvesting should be seriously considered, as indications from this study and the literature indicates that green cane deteriorates at a rate lower than that commonly reported for burnt cane. However, as this is an extremely labour intensive task when performed manually, growers should also consider the economic impact and related cost implication that this method of harvesting may have on their overall sugarcane production costs.

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