Investigation of exopolysaccharide producing bacteria isolated from milled sugarcane

by

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Thesis presented in partial fulfilment of the
requirements for the degree of
Master of Science in
Plant Biotechnology
at the
University of Stellenbosch

UNIVERSITEIT • STELLENBOSCH • UNIVERSITY

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December 2012

Declaration

I the undersigned, hereby declare that the work contained in this thesis is my own work (Unless to the extent explicitly otherwise stated) and that I have not previously, in its entirety or in part submitted it at any university for a degree.

Signed:……..

Date:…04/08/2012……

Abstract

The deterioration of harvested sugarcane as a result of bacterial growth causes major losses of sucrose and a build-up of exopolysaccharides (EPS). Polysaccharides present during production increase the massecuite viscosity, which negatively influences evaporation and crystallisation. In this study 38 culturable EPS-producing bacteria were isolated from milled sugarcane. Analysis of the EPS showed the ubiquitous presence of glucose, however, 14 polysaccharides also contained mannose, fructose or galactose. *In vitro* treatment using *Chaetomium erraticum* dextranase to evaluate is effectiveness indicated that 37 of the EPS were hydrolysed to some extent. There were 21 polysaccharides that were only partially digested. The capacity of the isolates to produce EPS on different sugars indicated a correlation between sucrose and polysaccharide formation in 37 isolates. The results indicate there are more species involved in EPS production than previously thought as well as the presence of non-dextran polysaccharides.

Opsomming

Bakteriële groei veroorsaak ‘n afname in gehalte, sukrose en ‘n verhoging in die hoeveelheid van eksternepolisakkeriede (EPS). Die verhoogde konsentrasie van polysakkariede gedurende die verwerkingsprosses veroorsaak ‘n verhoging in “massecuite” viskositeit. Hierdie verskynsel het ‘n nadelige uitwerking op die verdamping en kristalvorming van die produk. In gemaalde skuikerriet was 38 groeibare EPS-produserende bakterieë geisoleer. Die geanaliseerde EPS van hierdie bogenoemde bakterieë was daar in almal glukose teenwoordig. In 14 van hulle was mannose, fruktose en galaktose ook gevind. Die *in vitro* effektiwieteit van *Chaetomium erraticum* dekstranase op die EPS het gewys dat 37 het tot ‘n mate gehidroliseer maar 21 was net gedeeltelik verteer. As gevolg van die bo-genoemde resultate was daar gevind dat sukrose was ‘n noodsaaklike subtraat vir EPS produksie in die geisoleerde bakterieë. In hierdie studie was bevestig ‘n groter verskiedenheid EPS-produserende bakterieë gevind was en dat hulle assosiasie aan sukierriet prossering meer kompleks is as wat vooreen gedink was.

Acknowledgements

I would like to thank my family for their unwavering support and understanding during this degree. You have been springboard that has kept me going even through the turbulence.

I want to thank my friends who have been there to share manic highs, confusion, laughs, mood swings, inappropriate sarcasm and mind numbing lows. Without you I would have gone insane a long time ago.

Looking forward to forging my future
In my own way
Never tied to ordinary standards
Dawn of my realization
I've drawn the line of who I am
Everything as it is

The people with whom I have worked the late nights, written with in silence, worked shoulder to shoulder with over the past 5 years, you have my deepest gratitude and respect. I would also like to thank Dr Paul Hills for the steady source of sensible advice.

I would like to thank the IPB for hosting me during my studies. I would also like to extend my thanks to two supervisors Dr Gavin M George and Prof Jens Kossmann who have guided me through the most trying academic endeavour thus far.

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List of Abbreviations

^{o}C	Degrees Celsius
EPS	Extracellular polysaccharide
CAZy	Carbohydrate active enzymes
CPS	Capsular polysaccharides
eV	Electron Volt
FAOSTAT	Food and agriculture organisation statistical database
Fru	Fructose
g	Gravitational force
Gal	Galactose
GC-FID	Gas chromatography-Flame ionization detector
GC-MS	Gas chromatography-Mass spectrometry detector
GDP	Guanosine diphosphate
Glc	Glucose
GT	Glycosyltransferases
HePS	Heteropolysaccharides
ICUMSA	International Commission for Uniform Methods of Sugar Analysis
LAB	Lactic acid bacteria
LB	Luria broth
Man	Mannose

mg/l	Milligrams per litre
MRS	de Man, Rogosa and Sharpe
MSTFA	Methyl-N-(trimethylsilyl) trifluoroacetamide
SASRI	South African Sugarcane Research Institute
SDM	Semi-defined media
SL	SASRI LB
SM	SASRI MRS
Suc	Sucrose
TDP	Thymidine diphosphate
TFA	Trichloroacetic acid
TLC	Thin layer chromatography
UDP	Uridine diphosphate
v/v	volume per volume
w/v	weight per volume

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Chapter 1

Literature Review

1.1. Industry

The production value of sugar derived from sugarcane was estimated at US$ 53 billion in 2010 (FAOSTAT). Sugarcane is an important crop in many tropical and sub-tropical regions of the world (Promraksa *et al.*, 2009). It is traditionally harvested by hand by cutting it whole or chopped, burnt or trashed, and more recently by mechanical billeting (Lionnet, 1986; Singh and Solomon, 2003). The chopped cane is then transported to the sugar mill where it can be stored in the open for up to 5 days before processing (Yusof *et al.*, 2000; Solomon, 2009). The minimization of this storage period is critical to reduce the break down products produced by microbial deterioration and subsequent losses of extracted sugar.

1.2. Production of sugar

After harvesting the cut cane is processed in the sugar mill to extract the accumulated sucrose. The general scheme of sugar production can be seen in Figure 1. The harvested sugarcane is milled and pressed to release the sugarcane juice (Promraksa *et al.*, 2009; Chauhan *et al.*, 2011). To facilitate extraction of the juice through diffusion, the cane is imbibed with water with sprayers in the milling process. The juice is then clarified to remove impurities. Clarification uses various techniques such as defecation, sulphitation, carbonation or chromatography to remove polyphenols, solids and other impurities (Kulkarni, 1996; Cheesman, 2005). After clarification the juice contains between 83-85% water.

To facilitate crystallization, the water content of the juice is reduced to 35-45% by evaporation (Kulkarni, 1996; Cheesman, 2005). The evaporation is done under

vacuum to reduce the energy input required to boil the juice and speed up the evaporation due to a lowered boiling temperature (Kulkarni, 1996). The resulting syrup after the first evaporation step is sometimes clarified to remove the concentrated impurities (Kulkarni, 1996; Cheesman, 2005). The clarified syrup is then processed to precipitate the sucrose to ~99.4% (Kulkarni, 1996)..After evaporation, contains ~15% non-sucrose matter (Kulkarni, 1996).

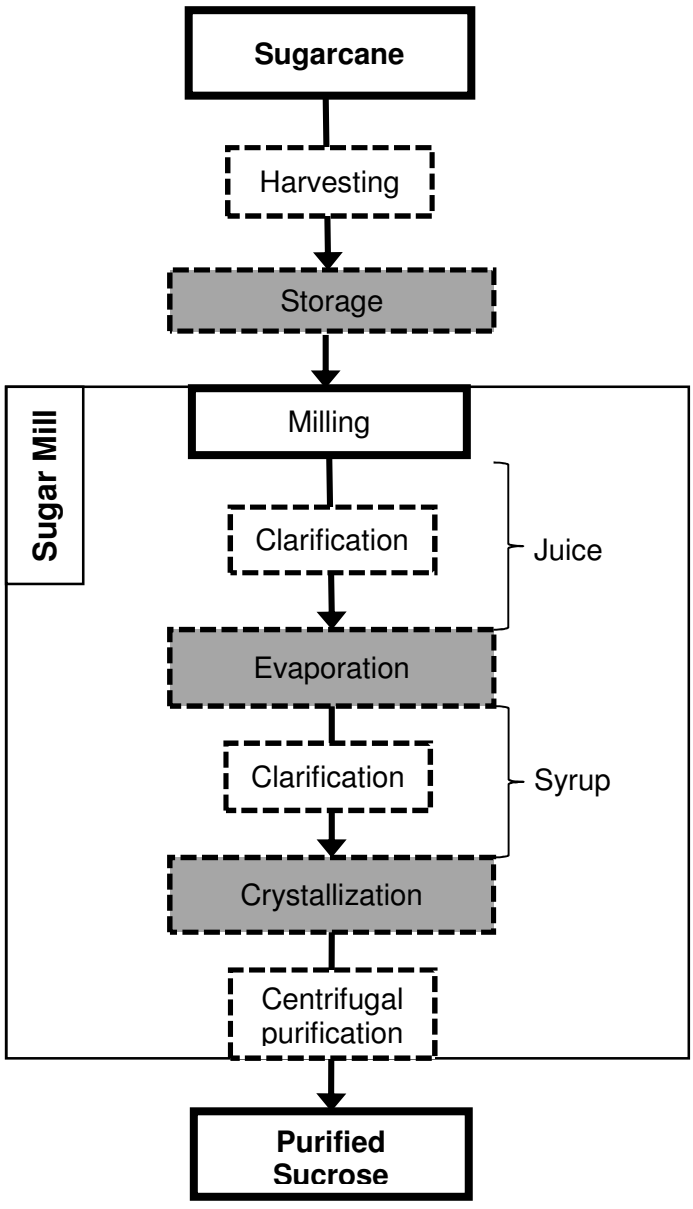

Figure 1. Simplified diagram of sugarcane processing to sugar (adapted from Kulkarni, 1996; Chauhan *et al.*, 2011)

The crystallization stage of sugar production can account for between 50-60% of total sucrose loss (Kulkarni, 1996). The syrup is evaporated and is used to grow seed nuclei in the crystallization process, once the sucrose in the molasses or massecuite is exhausted the heating stops and more syrup is added (Kulkarni, 1996; Cheesman, 2005). This crystallization process is repeated three times to recover as much sucrose as possible (Cheesman, 2005). The massecuite with precipitated sucrose is then centrifuged to collect all the sucrose crystals of sufficient size and remove the molasses (Kulkarni, 1996; Promraksa *et al.*, 2009; Solomon, 2009; Chauhan *et al.*, 2011). The high viscosity of the molasses requires two centrifugation steps to separate out the sugar, finally the purified sugar is centrifuged with water to remove the brown coloration (Cheesman, 2005). The sugar is dried and separated into the different grades of sugar through filter screens (Kulkarni, 1996).

1.3 Deterioration of harvested sugarcane

Deterioration of the cane commences when the cane is cut, and continues until it is processed in the sugar mill (Promraksa *et al.*, 2009). Cane juice contains up to 18% sucrose, 0.5% reducing sugars as well as adequate protein and mineral salts for microbial growth, the pH range of 5-5.5 makes it selective for the growth of acidophilic microorganisms such as yeast and lactic acid bacteria (LAB) (Solomon, 2009). Sugarcane is stored at ambient temperature before being processed in the mill and this can increase deterioration at higher ambient temperatures (Yusof *et al.*, 2000). The rate of deterioration of sugarcane is influenced primarily by temperature and rainfall, and other factors such as humidity, cane variety and state of the stalk (whole/chopped, burnt/trashed) can exacerbate its effects (Lionnet, 1986; Singh and Solomon, 2003; Solomon, 2009).

Cane deterioration is a result of two processes; the first is natural inversion of sucrose by endogenous enzymes, the second involves the infection of cane by microorganisms which secrete sucrose metabolizing enzymes and enter through the cut ends or damaged sites of the stalk (Lionnet, 1986; Solomon, 2009). The

contaminating bacteria in harvested sugarcane are introduced through soil, cutting blades and epiphytic flora of the plant (Solomon, 2009). Soil bacteria are only inoculated during cutting, *Leuconostoc* species, however, are known to enter physically damaged cane before harvesting (Solomon, 2009; Saxena *et al.*, 2010). The spread of bacterial infection is rapid, up to 15 cm from the cut sites after 1 hour and 30 minutes (Solomon, 2009). The losses as a result of an extended 'cut-to-crush' delay can be responsible for a loss of up to 30% of total extractable sucrose and increased concentrations of deterioration products (Morel du Boil, 1995; Eggleston, 2002; Solomon. 2009; Saxena *et al.*, 2010). Up to 90% of the deterioration during storage is through the agency of spoilage bacteria, the remainder is the result of chemical inversion (Eggleston, 2002; Promraksa *et al.*, 2009; Solomon, 2009).

The milling of poor quality (deteriorated) cane has a negative effect on profitability, to the extent that it results in processing problems up to and including factory shut down (Eggleston, 2002; Solomon, 2009). The process of sugarcane deterioration is separated into two distinct types that occur simultaneously after harvesting; sour cane and stale cane (Solomon, 2009). The flow rate of syrup during sugar processing varies and in the regions of stagnation and low speed, bacterial growth is accelerated (Solomon, 2009). Cane souring is of particular concern due to its association with *Leuconostoc mesenteroides* (Solomon, 2009), which was the first causative organism identified in sugarcane deterioration (Egan, 1965). Sugarcane contains an endophytic microbial flora, including *Acetobacter*, *Enterobacter*, *Pseudomonas*, *Aeromonas*, *Vibrio*, *Bacillus* species, and LAB which increase rapidly during staling and reduce juice quality (Solomon, 2009). Genera such as *Leuconostoc*, *Xanthomonas*, *Aerobacter* and yeast are usually present in the cut ends or damaged sites after harvesting, and are known to produce mucoid material (Solomon, 2009). The ability to produce exopolysaccharides is widespread amongst LAB (Ruas-Madiedo and de los Reyes-Gavilán, 2005). Facultative anaerobic bacteria, such as *Leuconostoc* species, grow rapidly in mud coated cane, as well as cane stacked in large piles with poor ventilation (Solomon, 2009). Amongst all the bacteria involved in sugarcane deterioration, *Leuconostoc* infection is still considered as one of the main causes of factory processing difficulties (Egan, 1965; Soetaert *et*

al., 1995; Eggleston, 2002; Solomon, 2009). *Leuconostoc mesenteroides* is an ubiquitous soil bacterium and known to produce lactic acid as well as dextran (Solomon, 2009). There is a significant increase of dextran and reducing sugars, with a concomitant decrease in pH and sucrose percentage, over the course of cane left to stale over several days (Morel du Boil, 1995; Singh and Solomon, 2003).

Dextran or dextran-like polysaccharides are the primary polysaccharide associated with sugarcane spoilage (Eggleston, 2002). Dextran is produced by the extracellular enzyme dextransucrase, which is secreted by *Leuconostoc* species (Eggleston and Monge, 2005; Saxena *et al.*, 2010). Dextransucrase hydrolyses sucrose and polymerises the glucose portion to form dextran and releases fructose (Robyt, 1996; Robyt *et al.*, 2008).

$$\text{Sucrose} + \alpha\text{-(1,6)-D-glucosyl}_n \rightarrow \alpha\text{-(1,6)-D-glucosyl}_{n+1} + \text{D-fructose}$$

Dextransucrase is problematic for sugarcane processing because it does not require ATP or cofactors (Soetaert *et al.*, 1995; Leathers *et al.*, 1997). *Leuconostoc* species are also known to be able to secrete more than one type of dextransucrase (Côté and Robyt, 1982; Zahnley and Smith, 1995; Robyt, 1996; Remaud-Simeon *et al.*, 2000). Dextran, an extracellular glucose homopolysaccharide, has been shown to interfere with downstream processing in sugar production and can result in significant losses in the recovery of sucrose (Lionnet, 1986; Monsan *et al.*, 2001; Eggleston, 2002; Eggleston *et al.*, 2004; Eggleston and Monge, 2005; Ravnö and Purchase, 2005; Eggleston and Harper, 2005; Eggleston *et al.*, 2009; Jiménez, 2009; Solomon, 2009; Vettori *et al.*, 2012).

1.4 Processing complications during sucrose production

There are three processes in the sugar refinery that are crucial for processing; the first is the evaporation to concentrate the massecuite, the second is the transport of the syrup through the different processes and the third is the precipitation of sucrose from the supersaturated syrup (Kulkarni, 1996). The processing of sugar relies

heavily on evaporation to concentrate the massecuite and precipitate the sucrose. During normal processing the extracted juice is concentrated to syrup and then further concentrated for crystallization, this increases the viscosity significantly and makes movement from the crystallizers to the centrifuges a challenge (Kulkarni, 1996; Eggleston *et al.*, 2004). The decreasing water content has the compounding effect of increasing the concentration of contaminating secondary products accumulated during harvesting and storage before milling.

The contamination of the juice with polysaccharides that increase the viscosity of the massecuite are of concern during refining (Morel du Boil, 1995; Eggleston, 2002; Ravnö and Purchase, 2005; Eggleston and Monge, 2005; Khaddour *et al.*, 2012). The milling of severely deteriorated cane can result in the shutdown of factory operations due to inability to move the massecuite (Eggleston and Harper, 2005). Any increase in viscosity has a number of detrimental effects including the reduction of evaporation rates and slowing the rate of crystallization (Eggleston, 2002; Eggleston and Monge, 2005; Eggleston *et al.*, 2004). Polysaccharides in the syrup can significantly retard crystal growth to the point where crystallization is inhibited (Abdel-Rahman *et al.*, 2008; Solomon, 2009).

During crystallization sucrose precipitates onto the surface of growing crystals, during this process impurities can become incorporated into the crystal lattice (Solomon, 2009; Kulkarni, 1996; Khaddour *et al.*, 2012). The impurities in the supersaturated solution effect crystallization in two ways; any increase in viscosity retards the mass transfer of sucrose onto the crystals and the second is obstructing sucrose incorporation into the crystal (Kulkarni, 1996). This obstruction is primarily the effect of oligo- and polysaccharides present in the massecuite (Solomon, 2009; Abdel-Rahman *et al.*, 2008; Eggleston and Monge, 2005). The presence of oligosaccharides during crystal growth has been shown to elongate the crystals by preferential absorption of dextran on the growing sugar crystal (Morel du Boil, 1991). The concentration of oligosaccharides required to negatively influence crystal formation is as low as < 4 mg/l (Morel du Boil, 1995). The crystals formed include platelets as the result of growth along only one axis and needle-like crystals

(Kulkarni, 1996). The detrimental impact of these viscosity-altering deterioration products requires management to minimize their influence.

1.5 Value of the Dextran

Commercial dextran can be produced either chemically or from bacteria grown on sucrose (Mehvar, 2000). The production of glucansucrase products is approximately 10 g per liter of culture (Leemhuis *et al.*, 2012). Expression of glucansucrases in heterologous organisms such as *Escherichia coli* or *Bacillus* species enables the high level production that can be used for enzyme immobilization (Swistowska *et al.*, 2008; Biedendieck *et al.*, 2007; Gómez de Segura *et al.*, 2004). The cost of sucrose is approximately ZAR 20 per kg which makes it an economical substrate for immobilized enzyme reactor systems for dextran production.

Dextran is used widely in the medical and pharmaceutical industries due to its non-toxicity and biocompatibility with humans (Kaewprapan *et al.*, 2012). Clinical grade dextran is used as a blood flow enhancer or plasma volume expander (Mehvar, 2000). The value of dextran has been expanded through the production of derivatives which have been shown to have a range of functions. These functions include; suitable nanoparticles for hydrophobic drug delivery (Kaewprapan *et al.*, 2012), anticancer drugs (Mehvar, 2000), sulfated dextrans have been shown to have anti-HIV properties (Nakashima *et al.*, 1989; Neyts *et al.*, 1995), as well as therapeutic protein conjugation (Mehvar, 2000). In addition to derivatization of dextran the enzyme itself has been used to glycosylate unnatural acceptors and enhance their physicochemical properties (Woo *et al.*, 2012). These characteristics make these enzymes scientifically, medically, commercially and industrially useful.

The α-glucans produced by dextransucrases has the added advantage of being indigestible by human digestive enzymes and are classified as fiber (Leemhuis *et al.*, 2012). This has been exploited using *Weissella* strains which are commonly used in

sourdough fermentations and produced dextran without major pH decreases (Katina *et al.*, 2009). It has been shown that native sugarcane dextrans have similar physiochemical properties to commercial dextran used in controlled release tablets (Gil *et al.*, 2008).

The value of dextran depends on the average molecular weight range. The dextrans with a molecular weight of 1.5 – 2.8 MDa are commercially sold for ca. ZAR 19,000 per kg. The value increase makes production of dextran from sucrose a valuable industry. The average molecular weight of dextrans produced by the dextransucrase from *L. mesenteroides* can be modified by alteration of substrate concentration, pH and temperature (Kim *et al.*, 2003; Falconer *et al.*, 2011). The sucrose to acceptor ratios can be used to control oligosaccharide molecular weights (Leemhuis *et al.,* 2012). The identification of novel dextransucrases which had more determined product sizes in terms of chain length of dextran would be highly commercially applicable.

1.6 Enzymatic production of polysaccharides by bacteria

Polysaccharides are widely distributed in nature and provide a means to store energy, provide protection, adhesion to surfaces, structural support and allow flexibility (Di Cango *et al.*, 2006; Badel *et al.* 2011). The production of energy storage molecules, usually polysaccharides, when environmental conditions are favourable, is a typical survival mechanism for organisms (Fettke *et al.*, 2006). Polysaccharides are divided into two major classes, homopolymers and heteropolymers (Sutherland, 1979). Homopolymers (HoPS) are composed of a single monosaccharide, whereas heteropolymers (HePS) consist of different sugars (Leemhuis *et al.*, 2012). The polysaccharides that are most relevant during sugarcane deterioration are EPS. The production of EPS is a characteristic found commonly in LAB and many other bacterial species (Ruas-Madiedo and de los Reyes-Gavilán, 2005). Microbial EPS

are divided into two main types; the capsular polysaccharides are anchored onto the cell wall itself, and the EPS which diffuse into the medium (Broadbent *et al.*, 2001; Van Hijum *et al.*, 2006; Ruas-Madiedo and de los Reyes-Gavilán, 2005; Tayuan *et al.*, 2011). EPS produced by bacteria are not metabolised as a storage or energy source by the bacteria that produce them (Ruas-Madiedo and de los Reyes-Gavilán, 2005). The production of EPS by microorganisms alters the physicochemical properties of its immediate environment, such as, suspension stabilization and viscosity (De Vuyst *et al.*, 2001; Freitas *et al.*, 2011; Donot *et al.*, 2012). The alteration of the physicochemical properties of the extracted juice has detrimental effects on processing. The EPS produced by spoilage bacteria, such as dextran, are of particular concern to sugar processing.

1.7 Enzymatic synthesis of polysaccharides

Enzymes known as glycosyltransferases (GT) are able to transfer sugar moieties from a donor to an acceptor, and linking them via a glycosidic bond (Vogt and Jones, 2000). Glycosyltransferases are a diverse enzyme family that are responsible for the biosynthesis of oligo- and polysaccharides (Taniguchi *et al.*, 2002). The current number of GTs both known and putative is 12 000, which include proteins from both prokaryotes and eukaryotes (Taniguchi *et al.*, 2002). GTs classification is based on the sugar which they transfer (Breton and Imberty, 1999). The most up to date online resource is CAZy (Carbohydrate Active Enzymes database; www.cazy.org) which currently contains 113 glycoside hydrolases, 91 glycosyltransferases, 19 polysaccharide lyases, 15 carbohydrate esterases and 52 carbohydrate-binding module families (Cantarel *et al.*, 2009). There is evidence that some GTs can contain glycosyl hydrolase-like folds (Hidaka *et al.,* 2004; Lovering *et al.,* 2007). Glycosyltransferases can be split into two main groups; Leloir (nucleotide sugar dependent) and non-Leloir (di- or oligosaccharide-dependent) GTs (Figure 2).

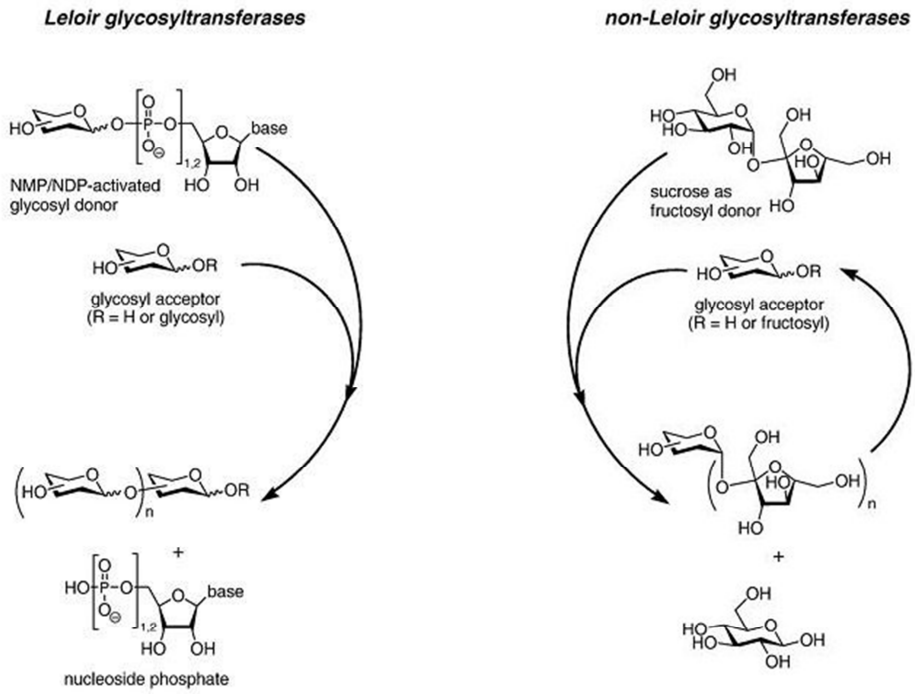

Figure 2. The two classes of glycosyltransferases (Weijers *et al.*, 2008)

1.8 Leloir Glycosyltransferases

The Leloir GTs utilizes nucleotide sugars to build both complex oligo- and polysaccharides (Figure 2) (Weijers *et al.*, 2008). Reactions derive their energy to drive the reactions from the bond between nucleotide and sugar. Leloir GTs has no sequence homology to one another, other than the sialyltransferases (Breton and Imberty, 1999). Leloir GTs are responsible for biosynthesis of glycoconjugates on the cell membranes in mammalian systems and plants, fungi and bacteria cell wall polysaccharides (Lim, 2005; Bowles *et al.*, 2006; Weijers *et al.*, 2008). Heteropolysaccharides and some homopolysaccharides are produced by Leloir GTs (Sutherland, 1979; De Vuyst *et al.*, 2001; Freitas *et al.*, 2011). Bacterial polysaccharides such as acetan, curdlan and xanthan are produced through the Leloir pathway (Griffin *et al.*, 1994; Sutherland, 2001; Letisse *et al.*, 2002; Jin *et al.*, 2008). The synthesis of HePS involves several biosynthetic steps and is linked to central carbon metabolism (Sutherland, 1979; Ramos *et al.*, 2001; Freitas *et al.*, 2011). HePS are built in oligosaccharide subunits that are then polymerised and

exported. The general scheme of synthesis was described by Sutherland (1979) and Freitas *et al.* (2011) (Figure 3). The general synthesis of these polysaccharides uses activated sugars as donors, the monosaccharides are sequentially transferred to a lipid carrier, once the repeating oligosaccharide unit is complete it is cleaved and polymerised during secretion into the media (Sutherland, 1979; Freitas *et al.*, 2011).

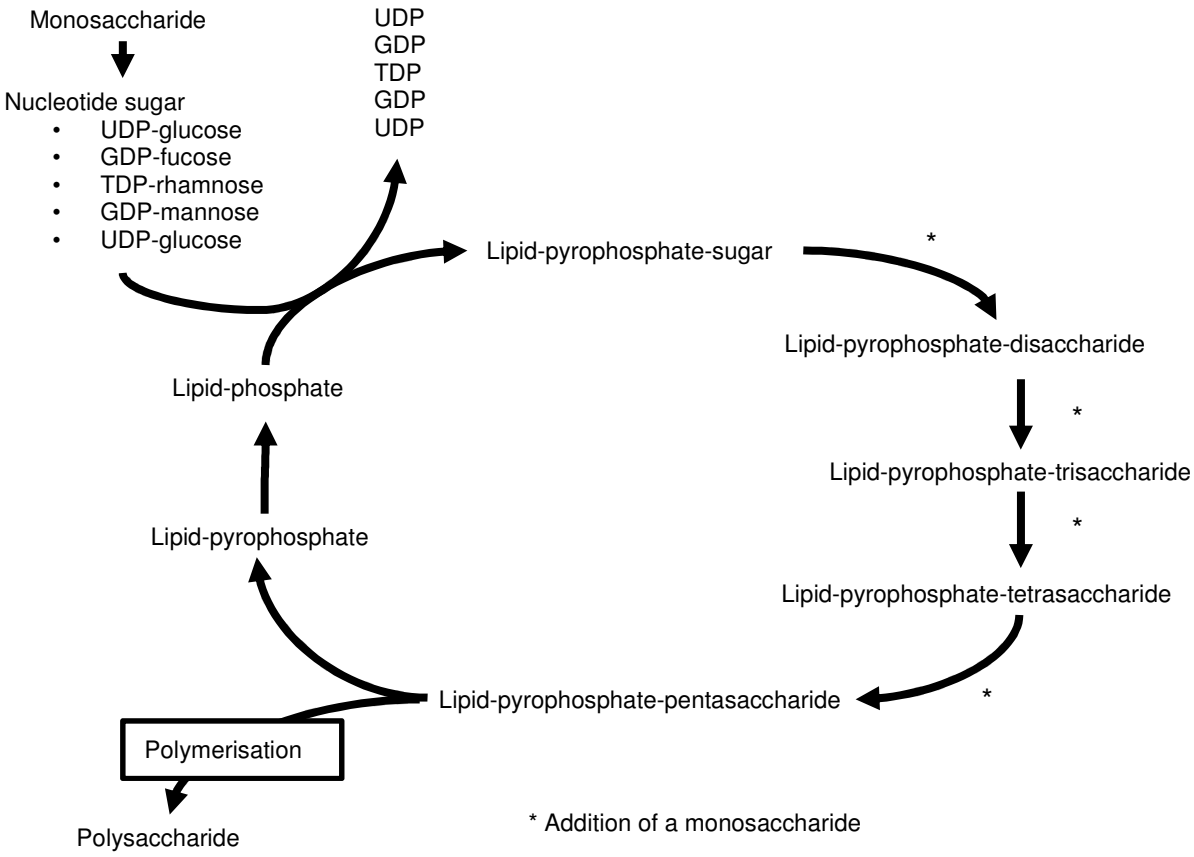

Figure 3. Assembly of Leloir polysaccharides (adapted from Sutherland, 1979; Freitas *et al.*, 2011)

The major Leloir-type GTs have two different catalytic mechanisms of action, the retaining and inverting (Lairson and Withers, 2004). The mechanism of inverting GTs has been shown to be a single displacement with a base inactivation of the acceptor

leading to the formation of an α→β glycosidic linkage (Weijers *et al.*, 2008; Lairson and Withers, 2004). The mechanism of retaining GTs is not well understood, but the proposed mechanism of action involves a double displacement reaction that allows formation of an α→α or β→β glycosidic bond (Davies, 2001; Persson *et al.*, 2001; Tvaroška *et al.*, 2003; Lairson and Withers, 2004; Faijes and Planas, 2007). The α→α bond is common amongst the LAB homo- and heteropolysaccharides (Badel, 2011).

1.9 Non-Leloir Glycosyltransferases

The transglycosidases are non-Leloir GTs that are capable of using non-activated di-, oligo- and polysaccharides for glycosyl donation (Monsan *et al.*, 2001; Lloyd *et al.*, 2004; Bresolin *et al.*, 2006; Weijers *et al.*, 2008). These transglucosidases do not require high energy substrates such as nucleotide or phosphorylated sugars or complex cofactors for activity (Soetaert *et al.*, 1995; Monsan *et al.*, 2010; Leathers *et al.*, 1997). A special class of transglucosidases found amongst microorganisms and plants are the sucrases (Monsan *et al.*, 2001; Seibel *et al.*, 2006; Velázquez-Hernández *et al.*, 2008; Weijers *et al.*, 2008).

The sucrases use sucrose as a high energy donor for the synthesis of high molecular weight polysaccharides (Weijers *et al.*, 2008). The β-(1-2) glycosidic bond of sucrose has been shown to contain -24.5 $kJ.mol^{-1}$ (Tewari and Goldberg, 1989). Weijers *et al.* (2008) postulated that the hydrolysis of sucrose releases energy which can then be used for oligo- or polysaccharide formation. This enzymatic mechanism of dextran synthesis is important because it does not require organic cofactors (Soetaert *et al.*, 1995; Leathers *et al.*, 1997). The polysaccharides formed by sucrases are limited to glucans and fructans, and the enzymes synthesising them are known as glucansucrases and fructansucrases respectively (Monsan *et al.*, 2001; Van Hijum *et al.*, 2006; Seibel *et al.*, 2006; Weijers *et al.*, 2008). Sucrases that produce α-linked glucans are restricted to LAB, whereas those producing fructan are spread amongst Gram-negative and Gram-positive bacteria (Van Hijum *et al.*, 2006).

Glucan and fructan sucrases are classified as glycosyl hydrolase family 70 and 68 respectively, according to the CAZy classification system (http://afmb.cnrs-mrs.fr/CAZY/; Cantarel *et al.*, 2008). In contrast to the Leloir GTs, glucansucrases display high levels of sequence identity which extends to sequence-function similarities, such as requiring a primer, activation by exogenous dextran, structure and size of products produced as well as affinity towards different acceptors (Remaud-Simeon *et al.*, 2000; Monsan *et al.*, 2010; Malik *et al.*, 2009). There are approximately 150 glucan sucrases that are currently recorded on the CAZy database (Cantarel *et al.*, 2009). Sugarcane deterioration is thought to be largely the result of dextransucrase which is a sucrase secreted by *Leuconostoc* species (Soetaert *et al.*, 1995).

1.9.1. Glucansucrases

Glucansucrases can produce a number of different glucans such as dextran, amylose, reuteran, mutan as well as alternan (Remaud-Simeon *et al.*, 2000; Notararigo *et al.*, 2012; Monsan *et al.*, 2010). The classification system is based on the carbon linkage that exists between the sugars (Morales *et al.*, 2001; Van Hijum *et al.*, 2006). Glucansucrases are extracellular enzymes that are typically between 120 and 200 kDa in size (Van Hijum *et al.*, 2006; Leemhuis *et al.*, 2012). The glycosidic bond of sucrose is used for catalysis of an α-1/2/3/4/6 glucosidic bond with retention of the anomeric carbons configuration (Remaud-Simeon *et al.*, 2000; Monsan *et al.*, 2001; Van Hijum *et al.*, 2006; Suwannarangsee *et al.*, 2007; Vettori *et al.*, 2011). The glucansucrases cannot use sucrose as an acceptor for transglycosylation reactions, however, they can use sucrose hydrolysis products (Van Hijum *et al.*, 2006).

Dextransucrases (EC 2.4.1.5) has been shown to have a processive reaction (Monsan *et al.*, 2001). Dextran has a predominance of α-(1,6) linkages (>50%) with random branches at the 2,3 and 4 position (Monchois *et al.*, 1996; Robyt *et al.*, 2008; Purama *et al.*, 2009; Falconer *et al.*, 2011). Dextrans can be divided into three major

classes: class one is an α-(1,6) backbone with branches at positions 2, 3 or 4; class two contains a non-consecutive α-(1,6) and α-(1,3) backbone with α-(1,3) branch linkages; and class three has an α-(1,3) backbone with α-(1,6) branch points (Naessens *et al*, 2005). Amylosucrase (EC 2.4.1.4) produces a glucan which is comprised of mainly α-(1,4) linkages, much like amylose found in starch (Büttcher *et al.*, 1997; Rolland-Sabaté *et al.*, 2004). Amylosucrases are restricted to *Neisseria* spp. and *Deinococcus radiodurans* (Büttcher *et al.*, 1997; Rolland-Sabaté *et al.*, 2004; Pizzut-Serin *et al.*, 2005). Alternansucrase (EC 2.4.1.140) has alternating α-(1,6/3) linkages with α-1,3 branches (Monsan *et al.*, 2001). Mutan (EC 2.4.1.5) is a glucan comprised of more than 50% α-(1,3) linkages, the remaining linkages are α-(1,6) (Monsan *et al.*, 2001; Shiroza *et al.*, 1987). Reuteran (EC 2.4.1.5) has a predominance of α-(1,4) linkages in its structure (Kralj *et al.*, 2004; Leemhuis *et al.*, 2012). Some strains contain more than one glucan-sucrase gene (Remaud-Simeon *et al.*, 2000; Zahnley and Smith, 1995; Van Hijum *et al.*, 2006; Shimamura *et al.*, 1994). *Leuconostoc mesenteroides* has been known to produce both dextran (with variable degrees of branching) and alternan (Kang *et al.*, 2005). The general mechanism of glucan synthesis by glucansucrases can be seen below in Figure 4.

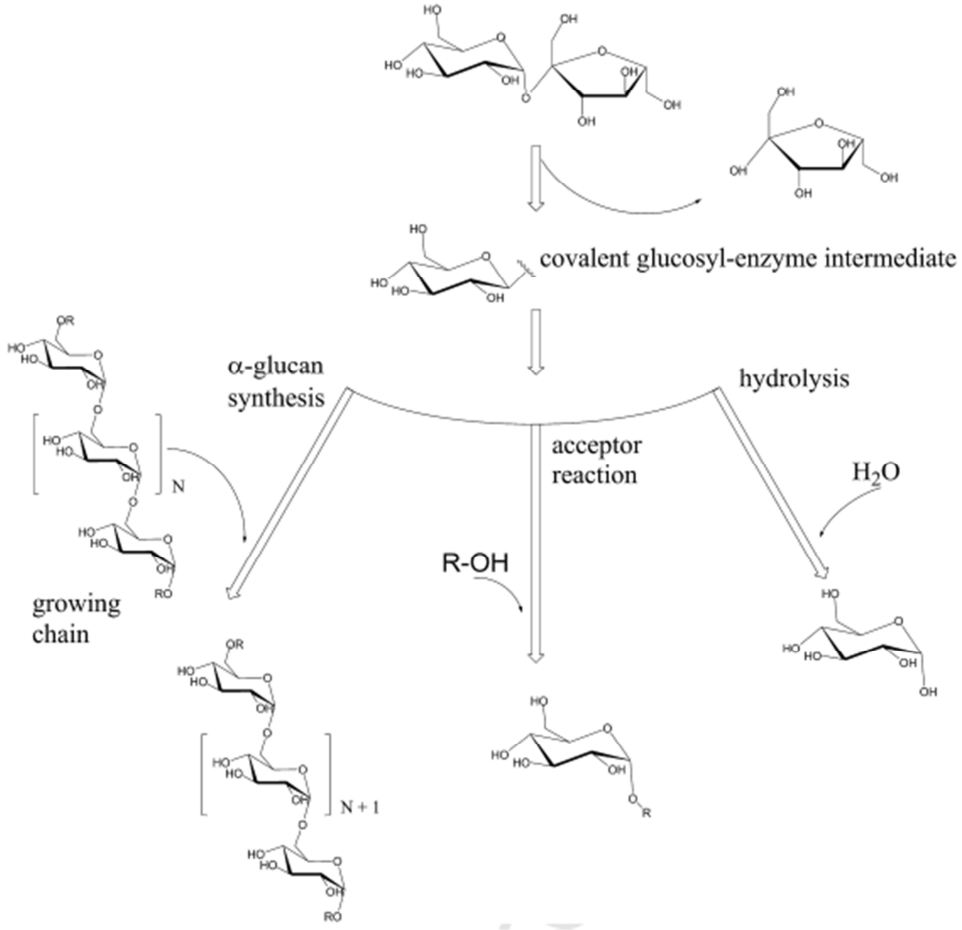

Figure 4. Three major reaction components of dextransucrase (Leemhuis *et al.*, 2012)

In addition to the production of glucans, glucansucrases can produce low molecular weight oligosaccharides in the presence of acceptors like maltose in addition to sucrose (Koepsell *et al.*, 1952). This acceptor molecule has been shown to vary significantly from just glycosyl moieties and can include phenol and aromatic alcohols (Mena-Arizmendi *et al.*, 2011; Monsan *et al.*, 2001). There are glucansucrases that are unable to utilize sucrose as an acceptor, these are then able to use the free glucose generated during hydrolysis of sucrose as an acceptor (Leemhuis *et al.*, 2012; Van Hijum *et al.*, 2006).

1.9.2 Fructansucrases

The fructansucrases (E.C. 2.4.1.10) are able to produce levan and inulin from sucrose (Monsan *et al.*, 2001; Van Hijum *et al.*, 2006). Inulosucrase is found exclusively in LAB, whilst levan sucrase is distributed in both Gram-negative and positive bacteria (Van Hijum *et al.*, 2006). Levan is comprised of β-(2-6) linked fructose containing β-(2-1) linked branches, while inulin has mainly β-(2-1) with β- (2-6) branches (Van Hijum *et al.*, 2006).

Levan sucrases have been shown to catalyse three different reactions; the hydrolysis of sucrose, polymerisation of fructose and the hydrolysis of levan (Kang *et al.*, 2005). The three reaction components of glucan- and fructansucrases are similar, but they share no sequence homology and have divergent products (Figure 4) (Van Hijum *et al.*, 2006). *Leuconostoc* and *Streptococcus* strains are known to produce β-fructans to some extent (Malik *et al.*, 2009). Numerous Gram-positive bacteria such as *Bacillus* spp. produce levansucrases (Donot *et al.*, 2012). Fructosyltransferases catalyse both sucrose hydrolysis and fructooligosaccharide synthesis (Ghazi *et al.*, 2007). The ratio of these two processes influences the production efficiency of fructooligosaccharides: the sucrose concentration and the enzymes ability to bind the acceptor and the exclusion of water (Ballesteros *et al.*, 2006). These enzymes and polysaccharides are not described in current literature regarding the deterioration of sugarcane and/or sugar production processing difficulties.

1.10 The aims and objectives of this study

There is an association of *Leuconostoc* bacteria and dextran with the deterioration of sugarcane in sugarcane mills (Solomon, 2009). In conjunction there is a lack of knowledge concerning the bacterial species involved in cane deterioration, in addition to the polysaccharides produced during their growth. The products formed

during bacterial growth in the harvested cane are not fully elucidated, evidence of this is seen in the hard to boil massecuite phenomenon which is still not understood (Eggleston *et al.*, 2011). The massecuite will not boil due to the action of an unknown component which is assumed to be produced during deterioration.

This study aims to investigate the cultivatable EPS-producing bacteria isolated from milled sugarcane. The EPS produced by these bacteria are of primary importance and the monosaccharide composition was analyzed to determine if all the EPS are dextran-like or there is a component of non-dextran based polysaccharide present. The EPS produced by these bacteria were analyzed for its sensitivity to *Chaetomium erraticum* dextranase and to evaluate the potential efficacy of the treatment *in vitro*. The production of EPS on different sugars was also evaluated to determine the dependence on the presence of sucrose. The dependence of EPS production on sucrose and a glucose-based polysaccharide is anecdotal evidence for the presence of a dextransucrase enzyme being secreted.

Chapter 2

Paper for submission to Food Microbiology

2.1. Title

Diverse exopolysaccharide producing bacteria isolated from milled sugarcane: Implications for cane spoilage and sucrose yield

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2.2. Abstract

Bacterial deterioration of sugarcane during harvesting and processing is correlated with significant loss of sucrose yield and the accumulation of bacterial polysaccharides. Dextran, a homoglucan produced by *Leuconostoc mesenteroides*, has been cited as the primary polysaccharide associated with sugarcane deterioration. Polysaccharides raise the viscosity of the massecuite, inhibiting evaporation and crystallisation. A culture-based approach was used to isolate extracellular polysaccharide (EPS) producing bacterial strains from milled sugarcane stalks. 16S rRNA sequencing analysis grouped 38 isolates into 12 genera. This study implicates several bacterial genera not previously associated with EPS production in sugarcane deterioration. Sucrose dependent polysaccharide formation was demonstrated for 33 of the isolates. Gas chromatography (GC) based monosaccharide analysis of purified polymers revealed 24 EPS consisting solely of glucose (homoglucans), while the remainder also contained galactose, mannose or fructose. The polysaccharides were treated *in vitro* with dextranase, full digestion was was achieved for only 15 extracts. Dextranase treatment does not fully address EPS build-up in deteriorated cane and, in addition, produces oligosaccharides which interfere with crystal formation.

2.3. Introduction

The estimated production of sucrose from sugarcane was valued at 53 billion dollars (FAOSTAT, 2010). During production, sucrose is precipitated from juice released from crushed sugarcane stalks (reviewed by Solomon, 2009). Cut sugarcane is stored at ambient temperature for an average of 3 - 5 days before processing (Solomon, 2009; Yusof *et al.*, 2000). This 'cut-to-crush delay' allows for losses as high as 20-30 % of extractable sucrose and a concomitant accumulation of bacterial EPS (Saxena *et al.*, 2010; Solomon. 2009; Eggleston, 2002; Morel du Boil, 1995). Cut sugarcane deterioration is influenced by several abiotic and biotic factors and is exacerbated by high ambient temperatures (Eggleston, 2002; Yusof *et al.*, 2000; Lionette, 1986). Sucrose degradation is mainly due to bacterial metabolism and chemical inversion (Solomon, 2009). Indeed, Eggleston (2002) showed that 95% of the sucrose loss can be attributed to spoilage bacteria.

Microorganisms utilise sucrose as a carbon source and for the synthesis of oligo- and polysaccharides. The impact of EPS on the production of sugar is an industrial concern due to raised viscosity of the massecuite, which inhibits evaporation and crystal formation (Lionnet, 1986; Ravnö and Purchase, 2005; Eggleston *et al.*, 2004; Eggleston and Monge, 2005; Eggleston and Harper, 2005; Jiménez, 2009; Abdel-Rahman *et al.*, 2008; Promraksa *et al.*, 2009). The impact of the polysaccharides on the production of sugar is, therefore, an industrial concern.

Dextran, produced by *Leuconostoc mesenteroides,* has been cited as the primary EPS produced during sugarcane deterioration (Eggleston, 2002; Eggleston and Monge, 2005; Eggleston *et al.*, 2009; Aquino and Franco, 2009; Solomon, 2009). Dextran is synthesized by an extracellular dextransucrase enzyme, using sucrose as the sole substrate. Bacterial dextran consists of α (1→6)-linked glucose polymers with α (1→3) or occasionally α (1→4)- or α (1→2)- branched linkages (Purama *et al.*,

2009). Other EPS producing microorganisms such as *Penicillium* spp., *Streptococcus* spp., *Lactobacillus* spp. (Kulkarni and Kulkarni, 1987), *Xanthomonas albilineans* (Blanch *et al*., 2006) and *Acetobacter diazotrophicus* (Arrieta *et al*., 1996) were shown to be present at cut ends and damaged sites of the cane after harvesting.

The growth of ubiquitous microorganisms during sugarcane processing is of primary concern to sugarcane mills. Several management and remediation strategies have been reviewed by Solomon (2009), where he stressed the importance of optimal cutting practises and the minimization of time between cutting and processing to reduce both bacterial spoilage as well as intrinsic invertase activity. Dextran has been shown to be the most problematic and abundant EPS produced during sugarcane deterioration (Solomon, 2009, Eggleston *et al.*, 2008; Aquino and Franco, 2009). Accumulation of the polysaccharide in sugarcane juice during processing can be controlled through good management practises and the use of the enzyme dextranase (Solomon, 2009; Eggleston and Monge, 2005). This enzyme hydrolyses α (1→6)-glucans to oligomers of between 2-10 glucose units which reduce the viscosity of massecuite (Eggleston *et al*., 2009). The presence of bacterial species producing EPS other than dextran is not addressed in current strategies for treatment of deteriorated sugarcane.

This study investigates the culturable EPS-producing bacterial diversity associated with sugarcane after processing, the monosaccharide composition of the polysaccharides, the relative production of EPS on different sugars, as well as sensitivity to dextranase treatment are reported.

2.4. Materials and Methods

Preparation of milled sugarcane. Rain irrigated Sugarcane stalks were cut below ground level, the tops removed, and stalks stacked outside in bundles for 3 days at the South African Sugarcane Research Institute (SASRI) laboratory in Durban, South

Africa. Average temperature and humidity values (day/night) during storage were 26°C/19°C and 94%/59%, respectively. Stalks were weighed, blended with double the volume of water and filtered through a mesh funnel. The milled filtrate was cooled to 20°C and passed through filter paper containing 3 g of celite.

Selection of EPS producing isolates. A dilution series of the milled filtrate was was plated onto De Man, Rogosa and Sharpe (MRS) (Merck, Darmstadt, Germany) and Luria Bertani (LB) (Merck, Darmstadt, Germany) both supplemented with 2% sucrose, and incubated at 30°C for 48 hrs to allow for sufficient polysaccharide production. EPS production was confirmed with the string test (Fang *et al*., 2004) by touching a sterile inoculation loop to individual colonies. The formation of a string (>5 mm) upon lifting of the loop was considered positive.

16S rRNA gene sequence analysis. Genomic DNA was extracted according to Babalola *et al*. (2009) and used as a template for 16S rRNA amplification. Universal 16s rRNA primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') and U529 (5'-ACCGCGGCKGCTGGC-3') (McInnerty *et al*., 1995; Watanabe *et al*., 2001) were used to generate amplicons with the following protocol: 94°C for 5 min; followed by 25 cycles consisting of 98°C for 20 s, 55°C for 20 s and 72°C for 1 min; and finally 72°C for 10 min. Amplicons were cloned by ligation into pJET 1.2™ (Fermentas, Burlington, Ontario, Canada) and sequenced using BigDye terminator V3.1. Post sequencing clean-up was done using Centri-sep columns prior to analysis on a Life Technologies 3730xl sequencer. Contigs were submitted to Genbank using BLAST (http://blast.ncbi.nlm.nih.gov) to identify the isolates. A sequence database was set up using sequences published on GenBank for type strains of the closest BLAST hit. Alignments were done in ClustalX and manually adjusted in Se-Al (Rambaut, 2007). Nexus files were analysed in PAUP* v4.0b10 (Swofford, 2001) using the BioNJ option, with confidence levels in nodes determined using a bootstrap analysis of 1000 replicates.

Purification of the EPS. EPS was isolated from cultures grown in semi-defined medium (SDM) supplemented with 5% sucrose (Bauer *et al*., 2009) at 22°C. EPS purification was performed according to Bauer *et al.* (2009) with slight modification. The cultures were placed in a boiling water bath for 10 min to facilitate EPS release and protein denaturation. Cultures were cooled and treated with pronase® (Roche, Basel, Switzerland) (0.5 mg/ml) at 37°C for 1 h. Proteins were precipitated by the addition of 5 ml 80% (m/v) trichloroacetic acid, followed by incubation on ice (30 min) and centrifugation (10000 *g* for 30 min at 4°C). EPS was precipitated from the supernatant by the addition of 3 volumes absolute ethanol (Frengova *et al*., 2000) and pelleted by centrifugation (10000 g for 30 min at 4°C). The pellet was resuspended in 5 ml MilliQ (MQ) water (Millipore, Bilerica, MA, USA) and dialysed overnight in SnakeSkin® dialysis tubing (MWCO 3500 kDa) (ThermoScientific, Rockford, IL, USA) against 20 l of MQ water. EPS samples were freeze-dried on a BenchtopK (VirTis, Warminster, PA, USA) for 24h and stored at -20°C.

Hydrolysis and derivatisation of the EP*S.* Purified polysaccharide (2 mg) was hydrolysed at 120°C for 2 h in the presence of 300 µl of 2M Trifluoroacetic acid , the hydrolysate was washed twice with 500 µl of methanol. Derivatization was performed by adding 140 µl methyloxyamine in pyridine (20 mg/ml) and incubation at 37°C for 30 min, followed by the addition of 70 µl of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and an incubation step of 2 h at 37°C.

Gas chromatography-based analysis of the EPS monosaccharide composition. Gas chromatography (GC) was used to determine monosaccharides present in the EPS hydrolysate. Glucose, galactose, mannose and fructose were used as standards. A combination of GC-flame ionization detector (GC-FID) and GC-mass spectrometry (GC-MS) was used. A Hewlett Packard 4550 GC-FID system fitted with an auto sampler and Rtx®-5MS (30 m by 0.25 mm by 0.25 µm film thickness) column was used. The GC operating conditions were as follows: injection port temperature, 280°C; detector temperature, 250°C; initial oven temperature, 120°C; hold for 0 min; first ramp 10°C/min to 160°C; hold for 0 min; second ramp 1.5°C/min to 220°C; hold for 0 min; third ramp 20°C/min to 280°C; hold for 3 min; flow rate, helium column, ca.

1 ml/min; injection mode split less. GC-FID results were confirmed by selecting an EPS from each genera of isolates and analysing the sample using GC-MS.

GC-MS. Samples were analysed with an Aligent Technologies (Agilent Technologies, Santa Clara, CA) 6890N Network GC system coupled to a 5975 inert Mass Selective Detector. Electron impact ionization was performed at 70 eV. GC-MS conditions were as follows: column, Rtx®-5MS (30 m by 0.25 mm by 0.25 µm film thickness); carrier gas, Helium; flow rate 1 ml/min; Split, 1:5; injector temperature, 280°C; initial temperature, 70°C; hold for 0min; first ramp, 10°C/min to 76°C; hold for 0 min; second ramp, 8°C/min to 310°C, hold for 4 min; and MS transfer 280°C. Mass-to-charge ratios (*m*/*z* values) were scanned from 40 to 550.

Dextranase treatment. Purified polysaccharide (10 mg) was resuspended in 1 ml of 50 mM sodium phosphate buffer (pH 6) containing 5 % Dextranase (*C. erraticum*) and incubated at 55°C for 16 h. The dextranase-treated polysaccharide was concentrated using a GeneVac EZ2 bench top evaporator to a final concentration of 0.1 mg/µl. The effect of the dextranase treatment was visualised using thin layer chromatography (TLC). The assay was optimized on Dextran T500 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

A total of 10 µg enzymatically-hydrolysed polysaccharide samples adjacent to non-hydrolysed polysaccharide with glucose, maltose, maltotriose and dextrimaltose as standards spotted onto a silica gel 60 (F_{254}) TLC plate (Merck, Darmstadt, Germany). The mobile phase was 2:5:1.5 (by volume) acetic acid:1-propanol:water modified from Kang *et al*. (2009). Plates were sprayed with sulphuric acid (5%) in ethanol and developed at 100°C for 10 min.

Relative EPS production. Single colonies from each isolate were struck onto SDM (Bauer *et al*., 2009) supplemented with 2% (m/v) sucrose, glucose and fructose respectively. The isolates were grouped according to their phylogenetic groups which were identified as part of a separate study (Figure 5). EPS production was assessed after incubation for 16 h at 22°C (Figures 7-9).

2.5. Results

2.5.1 Identification and characterisation of exopolysaccharide-producing bacteria

In this study, 38 isolates were selected for EPS production when grown on sucrose. Each isolate was genotyped by sequencing of a 512 bp section of the 16S rRNA gene. Alignments of the ribosomal sequences to those published in GenBank revealed a diverse population of bacteria encompassing 12 genera (Figure 5).

Isolates were grouped into five clades (Figure 5): Clade 1 includes *Acinetobacter* spp., *Psychrobacter* sp., *Enhydrobacter aerosaccus*; Clade 2 is comprised of *Enterobacteriaceae* spp. and *Poryphorymonas* sp.; Clade 3 includes *Weissella* sp., *Leuconostoc* spp. and *Streptococcus* spp.; Clade 4 is comprised of *Bacillus* spp.; and Clade 5 comprises of *Microbacterium ginsengisoli*, *Micrococcus luteus* and *Propionibacterium acnes*.

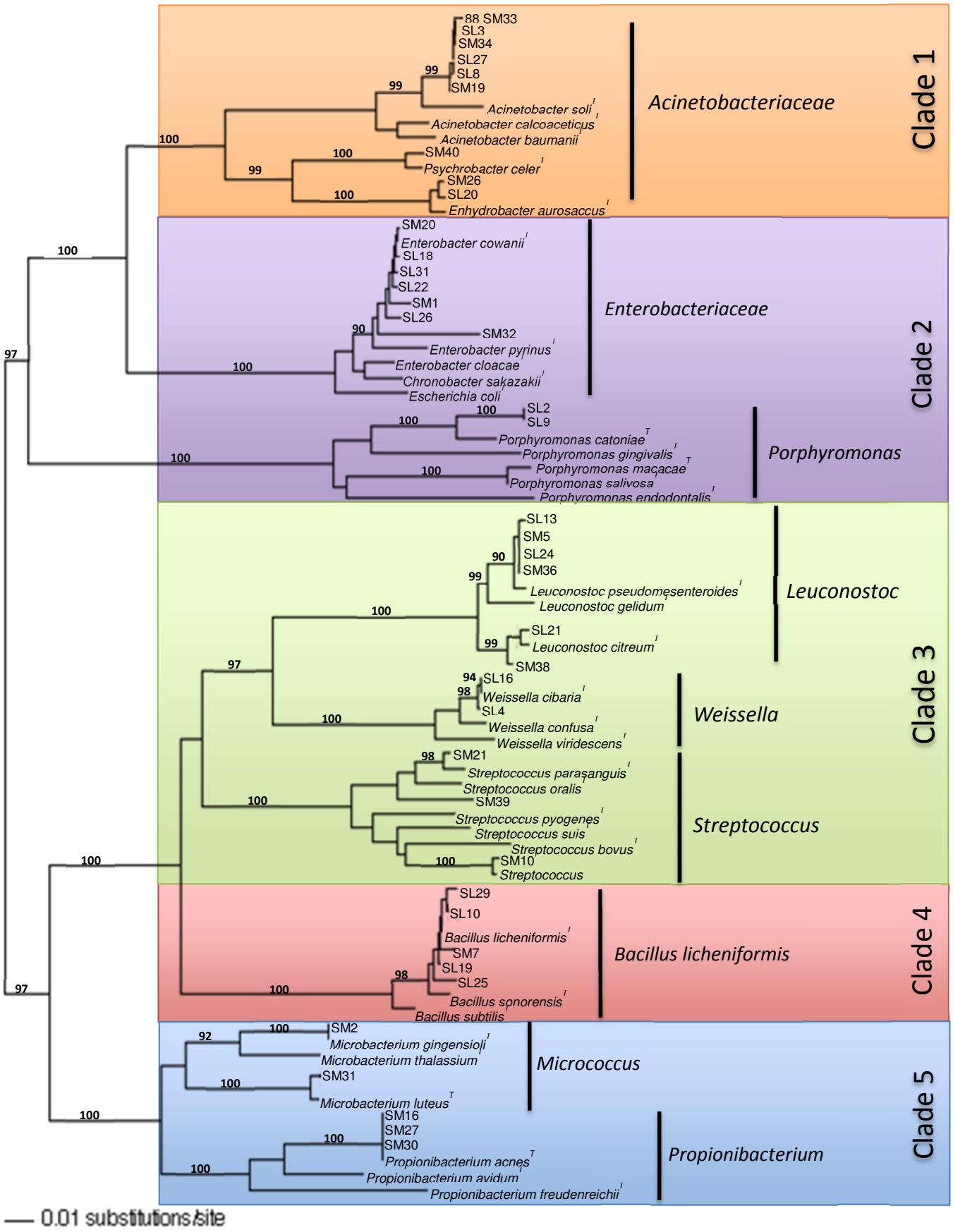


Figure 5. Condensed neighbour-joining phylogenetic tree of the isolates identified in milled sugarcane

Literature was scrutinized for close relatives with the ability to produce Capsular polysaccharide (CPS) or EPS (Table 1) the majority of which have been associated with the human epidermis, oral cavity or soil.

Table 1. Polysaccharide production by species related to isolates implicated in sugarcane deterioration

Isolate	Nearest relative	CPS	EPS	Isolation source	References
SL19, SM7, SL25, SL10, SL29	*Bacillus licheniformis*	+	Levan, Dextran, Mannan, HePS	Soil, marine and fresh water, compost, rumen of cattle and bread	Larpin *et al*., 2002; Singh *et al*.,2011; Yakimov *et al*., 1997; Ghaly *et al*., 2007; Bergey and Boone, 2009; MacLean *et al*., 1990; Priest, 1989; Sikorski and Nevo, 2005; Schembri *et al*., 2004; Priest, 1989
SM32, SL31, SM1, SL26, SL18, SL22, SM20	*Enterobacteriaceae* sp.	+	Colanic acid, HePS	Soil, water, milk powder, vegetation and alimentary canal	Stevenson *et al*., 1996; Grant *et al*., 1969; Blood and Curtis, 1995; Muytjens *et al*., 1988; Schembri *et al*., 2004; Alves *et al*., 2010; Sutherland, 2001 and 1994
SM38, SL21	*Leuconostoc citreum*	-	Dextran, Alternan	Human sources	Solomon, 2009; Egan, 1965; Soetaert *et al*., 1995; Van der Meulan *et al*., 2007; Maina *et al*., 2008; Bounaix *et al*., 2010; Bounaix et al., 2009; Holland and Liu, 2011; Eggleston, 2002; Eggleston and Monge, 2005; Eggleston *et al*., 2008; Aquino and Franco, 2009
SM5, SL13, SM36	*Leuconostoc pseudomesenteroides*	+	Dextran, Mutan, Alternan, Levan	Plants, raw milk, cheeses and meat	Egan, 1965; Soetaert *et al*., 1995; Solomon *et al*., 2009; Van der Meulen *et al*., 2007; Maina *et al*., 2008; Bounaix *et al*., 2010; Bounaix *et al*., 2009; Holland and Liu, 2011; Leathers *et al*., 1997; Cote and Robyt, 1982; Eggleston, 2002; Eggleston and Monge, 2005; Eggleston *et al*., 2008; Aquino and Franco, 2009; Bevan and Bond, 1971
SL4	*Weisella confusa*	+	Dextran, Alternan, Levan	Sugarcane, human faeces, fermented food (chilli bo and Tapai), canine ear and human gall	Katina *et al*., 2009; Wang *et al*., 2010; Ganzle and Schwab, 2009; Van der Meulen *et al*., 2007; Bjorkroth *et al*., 2002; Tieking *et al*., 2003
SL16	*Weisella cibaria*	+			
SM2	*Microbacterium ginsengisoli*	No literature	No literature	Ginseng field soil	Park *et al*., 2008
SM31	*Micrococcus luteus*	+	None	Mammalian skin, amber	Deng *et al*., 2010; Hase *et al*., 1972; Young *et al*, 2010; Greenblatt *et al*., 2004
SL27, SL3, SL8, SM34, SM33, SM19	*Acinetobacter* sp.	+	HePS	Human skin flora, sewage, soil and water	MacLean *et al*., 2009; Haseley *et al*., 1994; Pirog *et al*, 2003; Pantophet, 2008; Peleg *et al*., 2008
SM26, SL20	*Enhydrobacter aerosaccus*	No literature	No literature	Human skin	Gao *et al*., 2007
SM40	*Psychrobacter* sp.	+	No literature	Brown seaweed, seawater, human skin, fish, guniea pigs, contaminated air samples	Lee *et al*., 2006; Yoon *et al*., 2005; Hudson *et al*., 1987; Kondakova *et al*., 2012; Juni and Heym, 1986
SL2, SL9	*Porphyromonas* sp. Oral taxon	+	None	Oral cavity of animals	Brunner *et al*., 2010; Dewhirst *et al*., 2010; Paramonov *et al*., 2001; Fournier *et al*., 2001
SM16, SM27, SM30	*Propionibacterium acnes*	+	None	Human skin, oral cavity	Brüggemann *et al*., 2004; Holland *et al*., 2010; Dewhirst *et al*., 2010; Bek-Thomsen *et al*., 2008
SM39	*Streptococcus spp.*	+	Glucan	Oral cavity	Gibbons and Banghart, 1968; Rukke *et al*., 2011; Dewhirst *et al*., 2010
SM10	*Streptococcus equinus*	+	Glucan	Alimentary canal	Coutinho and Henrissat, 1999; Takagi *et al*., 1994; Bergey and Boone, 2009; Aquino and Franco, 2009
SM21	*Streptococcus parasanguinis*	+	None	Oral cavity	Bergey and Boone, 2009; Garnett *et al*., 2012; Dewhirst *et al*., 2010

Monomer composition of exopolysaccharides produced on sucrose

Purified EPS isolated from cultures grown in the presence of sucrose were hydrolysed and monosaccharide composition determined using GC-MS and GC-FID (Table 2). The bacterial isolates EPS monomer composition indicates that glucose-based EPS were produced by 24 of the 38 isolates, the rest were comprised of glucose and galactose, mannose or fructose. Mannose was present in 10 of the EPSs purified.

Table 2. EPS monosaccharide composition, dextranase susceptibility and relative polysaccharide production of bacteria isolated from milled sugarcane.

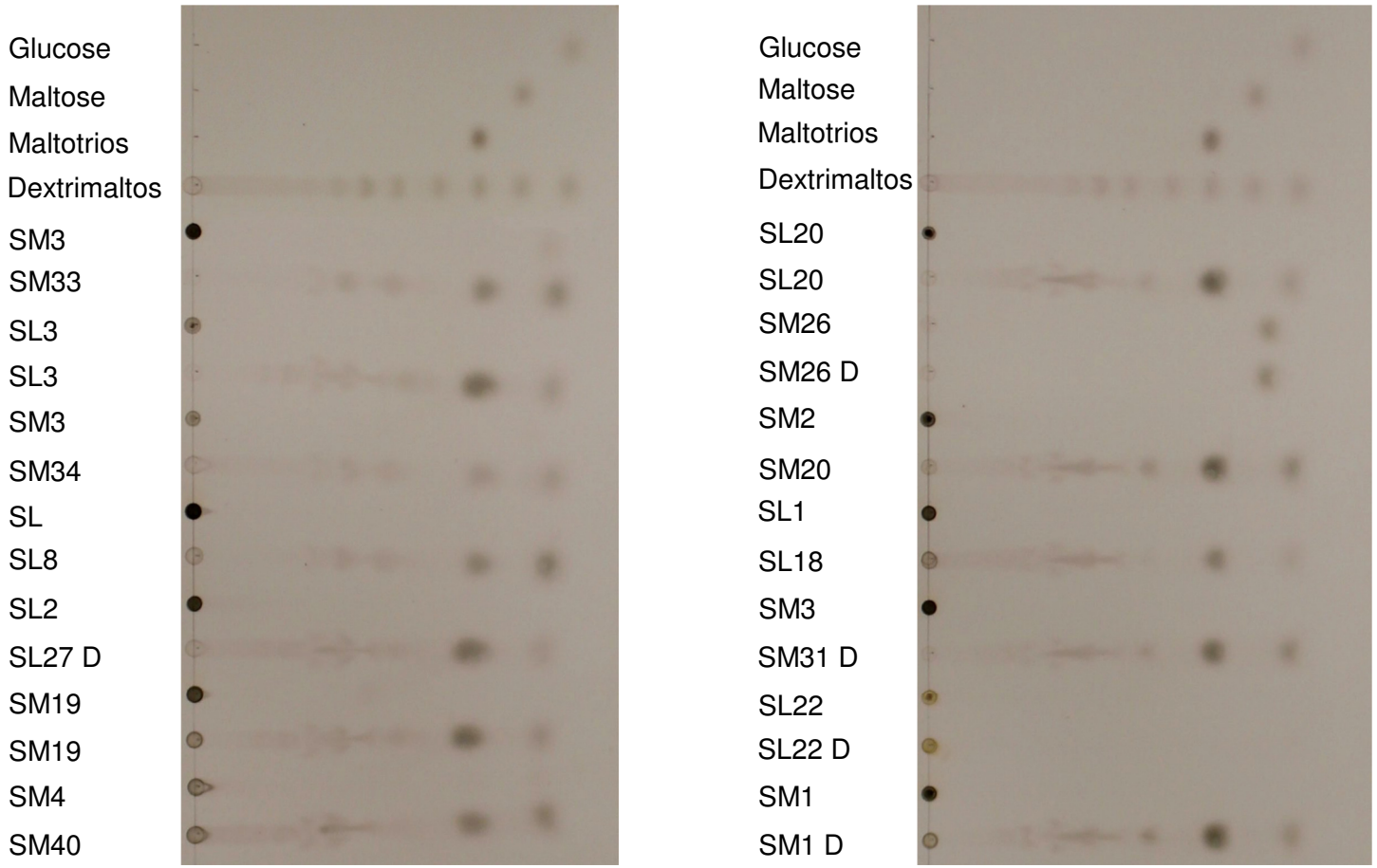
Phylogenetic group	Nearest type strain	Isolate	Selection[a]	Digested by Dextranase (*Chaetomium erraticum*)[b]	Sucrose-based EPS monosaccharide composition				Relative EPS production		
					Glc	Gal	Fru	Man	Suc	Glc	Fru
Moraxellaceae	*Acinetobacter* spp.	SM33	E	++	■				■		
		SL3	E	++	■				■		
		SM34	E	+	■				■		
		SL8	E	+	■			■	■		
		SL27	E	+	■				■		
		SM19	E	+	■			■	■		
	Psychrobacter sp.	SM40	E	+	■			■	■		
	Enhydrobacter aerosaccus	SL20	E	++	■				■		
		SM26	E	++	■		■				
Enterobacteriaceae	*Enterobacteriaceae* sp.	SM20	E	+	■				■		
		SL18	S	+	■			■	■	■	■
		SM31	E	++	■			■	■		
		SL22	S	-	■	■			■	■	■
		SM1	E	+	■				■		
		SL26	E	++	■				■		
		SM32	E	++	■				■		
Porphryromonadaceae	*Porphyromonas* sp. Oral taxon	SL2	E	++	■				■		
		SL9	S	++	■				■	■	■
Leuconostocaceae	*Leuconostoc pseudomesenteroides*	SL13	E	++	■				■		
		SM5	E	+	■				■		
		SM36	E	++	■				■		
	Leuconostoc citreum	SL21	E	+	■				■		
		SM38	E	+	■				■	■	
	Weisella cibaria	SL16	E	+	■		■		■		
	Weisella confusa	SL4	E	++	■				■		
Streptoccocaceae	*Streptococcus equinus*	SM10	E	++	■			■	■		
	Streptococcus sp.	SM39	E	+	■				■		
	Streptococcus parasanguinis	SM21	E	+	■			■	■		
Bacilliaceae	*Bacillus licheniformis*	SL29	E	+	■				■		
		SL10	E	++	■				■		
		SM7	E	+	■			■	■		
		SL19	E	+	■		■		■		
		SL25	E	+	■			■	■		
Microbacteriaceae	*Micrococcus luteus*	SM31	E	++	■				■		
Propionibacteriaceae	*Propionibacterium acnes*	SM16	E	+	■			■	■		
		SM30	E	+	■				■		
		SM27	E	++	■				■		
Microbacteriaceae	*Microbacterium ginsengisoli*	SM2	E	+	■				■		

[a] Isolates selected for string test (S) or EPS production (E)

[b] Purified EPS digested overnight to evaluate the sensitivity to dextranase (- indicates no digestion; + indicates some digestion; ++ indicates full digestion)

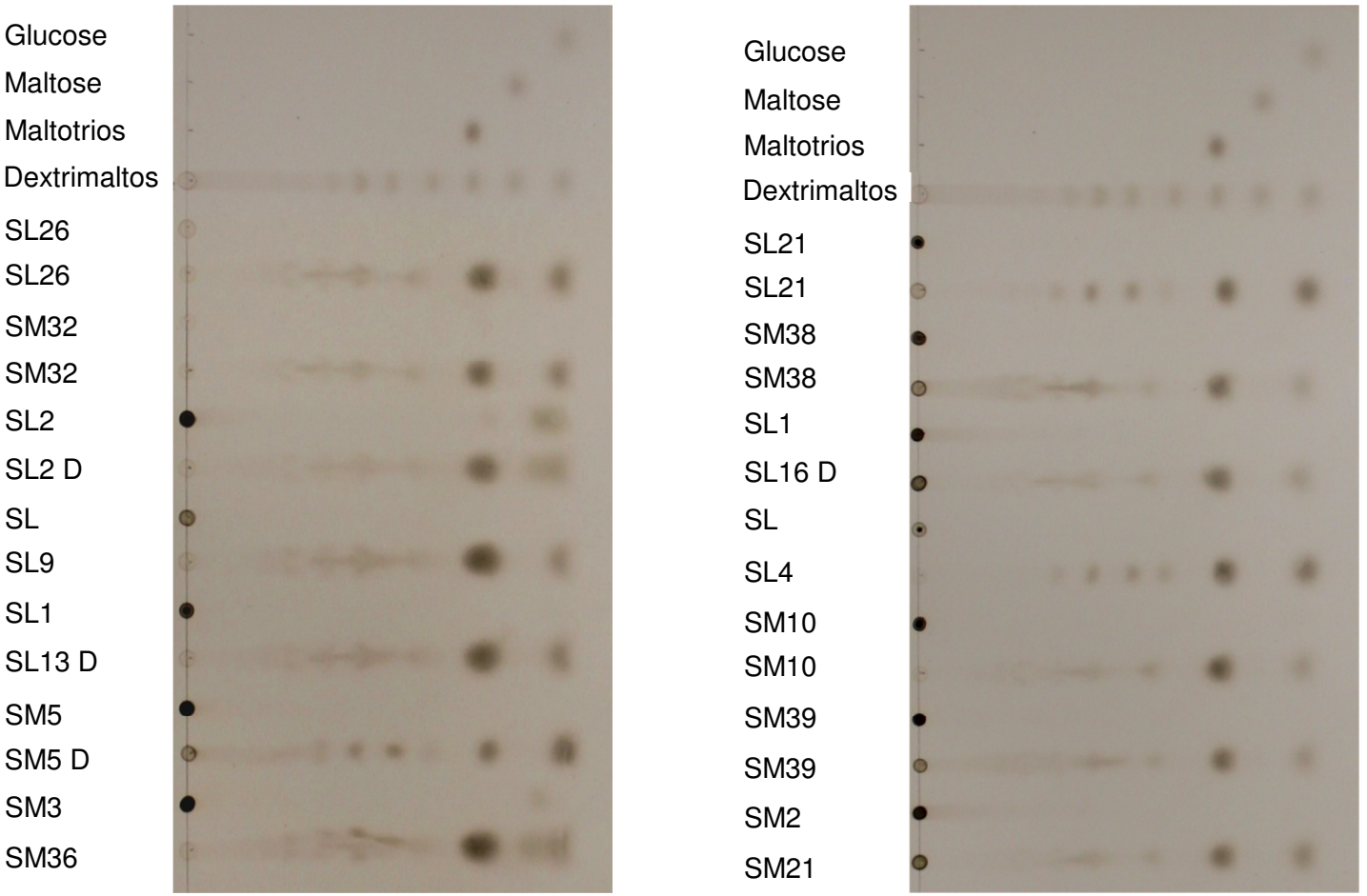
2.5.4. Dextranase susceptibility.

Considering that all purified EPS fractions contained glucose, samples were treated with dextranase to evaluate susceptibility to degradation by this enzyme (Table 2 and Figure 6-8). Full digestion profiles were obtained for 14 of the 24 homoglucan EPS and two of the mannose containing fractions. All of the EPS shown to contain fructose were partially digested by dextranase. The EPS from isolate SL22, containing glucose and galactose was not digested by dextranase treatment (Figure 4).



D – Dextranase treated

Figure 6. Thin layer chromatography of dextranase treated EPS from the Acinetobacter, Psychrobacter, Enhydrobacter and Enterobacteriaceae species with undigested control and glucose, maltose, maltotriose as well as dextrimaltose as standards



D – Dextranase treated

Figure 7. Thin layer chromatography of dextranase treated EPS from the Enterobacteriaceae, Porphyromonas, Leuconostoc, Weisella and streptococcus species with undigested control and glucose, maltose, Maltotriose and Dextrimaltose as standards

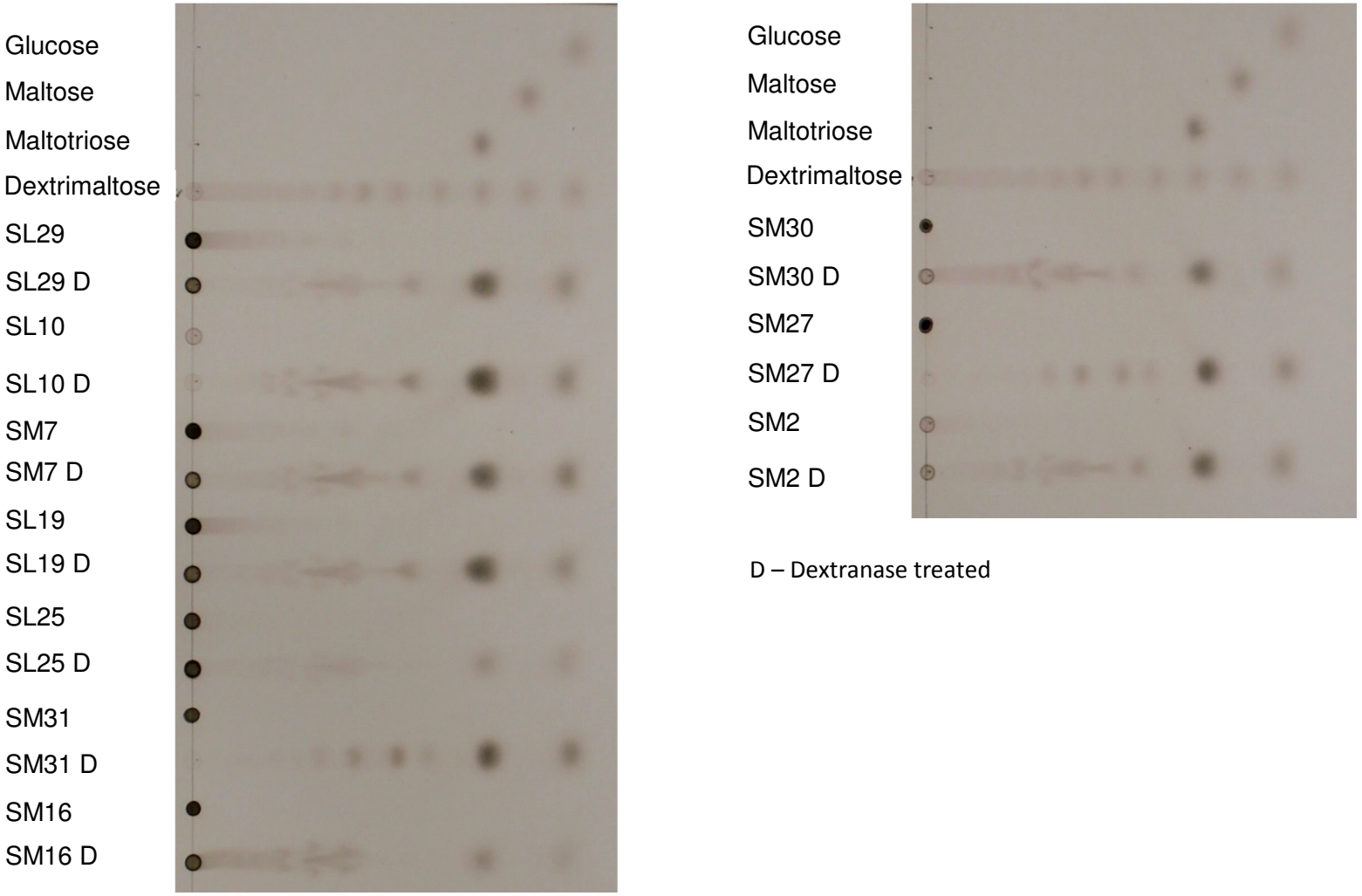


Figure 8. Thin layer chromatography of dextranase treated EPS from the Streptococcus, Bacillus, Micrococcus, Propionibacterium and Microbacterium species with undigested control and glucose, maltose, Maltotriose and Dextrimaltose as standards

2.5.5. Relative production of EPS on different sugars

The isolates cultured in this study were grown on SDM plates containing sucrose, glucose and fructose for 16 h. There is a bias towards production of EPS on sucrose in comparison to glucose and fructose. The isolates that produce EPS on sucrose and not glucose or fructose indicate an EPS operon that is induced by the presence of sucrose or the secretion of a sucrase type enzyme.

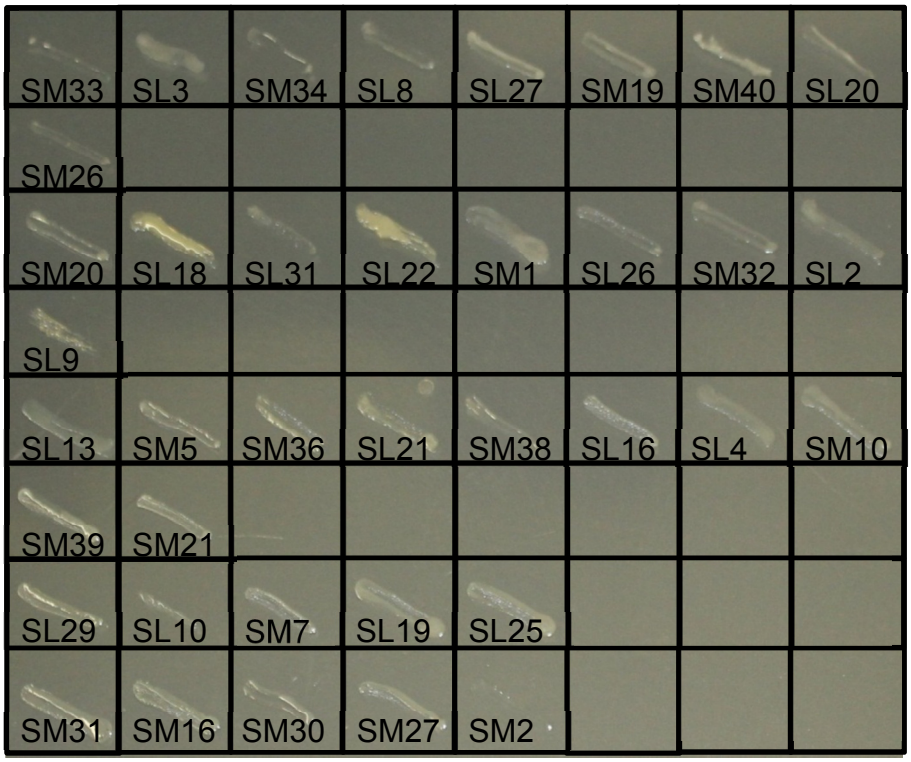


Figure 9. Relative exopolysaccharide production of isolates on semi-defined media supplemented with glucose (2%)

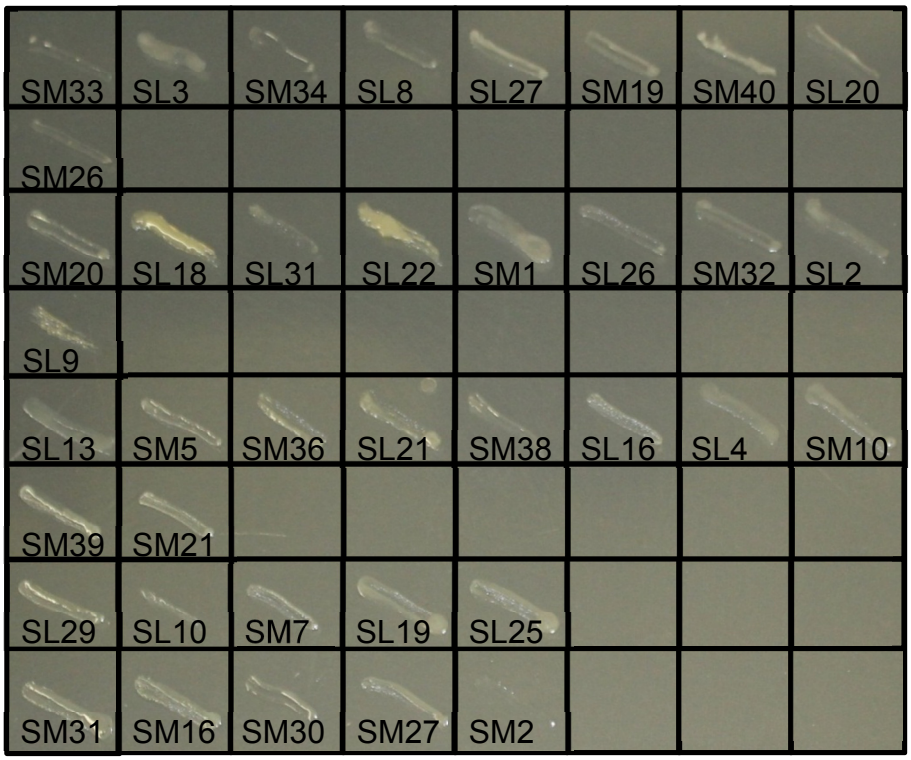


Figure 10. Relative exopolysaccharide production of isolates on semi-defined media supplemented with fructose (2%)

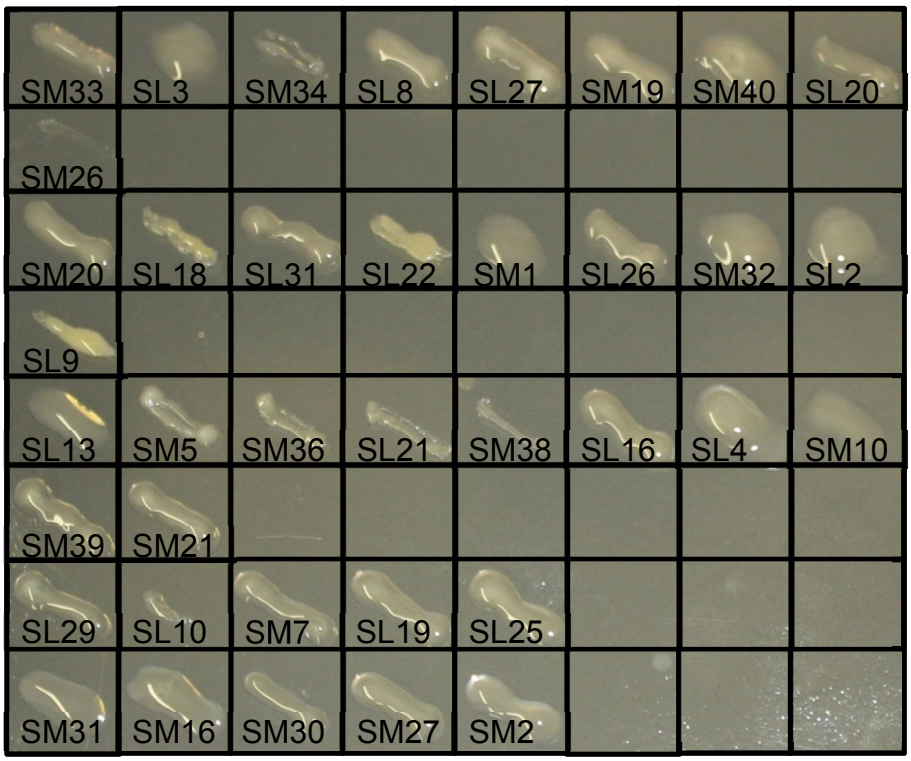


Figure 11. Relative exopolysaccharide production of isolates on semi-defined media supplemented with sucrose (2%)

2.6. Discussion

Diverse exopolysaccharide (EPS) producing bacterial strains were isolated from milled sugarcane produced at the South African Sugarcane Research Institute (SASRI). Monosaccharide analysis of EPS suggests the production of 24 homopolysaccharides consisting of glucose (i.e. glucans). The remainder are comprised of glucose and mannose, fructose as well as galactose (i.e. heteropolysaccharides) (Table 2). A number of species reported in this study such as *Leuconostoc* spp., *Enterobacter* spp., and *Bacillus* spp. have been previously associated with sugarcane (Eggleston, 2002; Solomon, 2009; Eggleston and Harper, 2005), however, the production of EPS in sugar mills is mainly attributed to *Leuconostoc* and *Weisella* species (Solomon, 2009; Leathers and Bischoff, 2011). The nearest relative of all the isolates were investigated in literature to determine if they have been previously reported as producing EPS (Table 1).

The large proportion of glucose based EPS was not surprising, considering the widespread association of dextran with sugarcane deterioration (Solomon, 2009; Egan, 1965; Soetaert *et al.*, 1995; Katina *et al.*, 2009; Eggleston, 2002). The presence of glucans that are partially susceptible to dextranase treatment may be the result of more than one type of dextran being produced. Dextran producing strains are known to produce more than one glucansucrase (Zahnley and Smith, 1995; Remaud-Simeon *et al.*, 2000; Robyt, 1996). Currently deterioration of harvested cane is routinely tested weekly for dextran after milling (Solomon, 2009).

The fraction of EPS other than dextran has not yet been linked to processing complications. There was a significant proportion of mannose containing EPS, which accounted for a quarter of the polysaccharides isolated (Table 2). This type of polysaccharide is produced by *Xanthomonas* species, which have been isolated in other studies (Sutherland 2001, 1994; Solomon, 2009), however, this species was not isolated in this study. The isolates (SL16, SL19 and SM26) produced a glucose and fructose containing EPS (Table 2). The closest relatives to isolate SL16 and SL19 have been shown to produce levan in previous literature (Table 1). The closest

relatives to SL16 are known to produce glucose based EPS, whereas SL19 are not (Table 1). *Enhydrobacter aerosaccus,* closest relative to isolate SM26 has not been shown in previous literature to produce an EPS (Table 1). The polysaccharide containing only glucose and galactose produced by SL22 which was *Escherichia* sp. have been shown to produce glucogalactans, but not in association with sugarcane (Table 1). The diversity of EPS isolated in this study may indicate either an over or under estimation of polysaccharide concentrations during routine testing.

The EPSs isolated in this study were tested for its *in vitro* susceptibility to dextranase. The glucans isolated showed that 16 of the 24 isolates in this study are dextranase susceptible the remainder are only partially susceptible (Table 2; Figures 6-8). The glucans in this study where assumed to be dextran like, however the partially susceptible EPSs indicate that there is a population of dextranase resistant glucans. *Chaetomium erraticum* dextranase is an endo hydrolytic enzyme that hydrolyses α-(1,6) glycosidic linkages in dextran. The susceptibility of the EPS is indicated by mobile oligosaccharides in comparison to an undigested control, partial susceptibility was indicated by a large immobile spot at the origin in digested samples in addition to motile oligosaccharides, and EPS that were not susceptible did not migrate.

The *in* vitro enzymatic hydrolysis of the heteropolysaccharides had an effect on 13 out of 14 polysaccharides (Table 2). The mannose containing EPS showed 8 of the 10 were only partially hydrolysed (Table 2; Figure 6-8). The EPS containing fructose may indicate the presence of both a dextran and fructan which would result in partial susceptibility to treatment (Table 1; Table 2; Figure 6-8). The glucose and galactose containing EPS showed complete resistance to dextranase treatment (Table 2; Figure 6).The sensitivity of the EPS containing other monosaccharides is associated with the presence of internal α-(1,6) glucosyl linkages within the polysaccharide structure.

Bacterial growth on the SDM supplemented with sucrose resulted in EPS formation in 37 of the 38 isolates (Figures 9-11). The isolates did not produce EPS comparably SM26, this may be the result of slower growth. The production of EPS on glucose and fructose was restricted to SL9, SL18 and SL22, these isolates were selected as string test positives (Table 2). Production of EPS on monosaccharides indicates the presence of a Leloir based polysaccharide production pathway (Freitas *et al.*, 2011; Sutherland, 1979). The isolate SM2 produced an EPS on both sucrose and glucose, however, the polysaccharide accumulation on sucrose was significantly greater. The differences between the EPS produced on sucrose and glucose or fructose shows a distinct sucrose linked polysaccharide formation. The incubation of these isolate for only 16 h is significantly less than the average storage time for the harvested cane. The sucrose linked polysaccharide accumulation indicates the intrinsic problems that faced by the sugar industry.

This study of the culturable EPS-producing bacteria isolated from blended sugarcane revealed an unexpectedly high diversity of microorganisms. The polysaccharides were isolated and monomer composition analysed revealing complexity that has not been previously reported. The analysis of these EPS and diversity of the EPS-producing bacteria allows a greater understanding of sugarcane deterioration and the detrimental polysaccharide accumulation. The dextranase treatment showed that 37 of the 38 isolates EPS was hydrolysed to some extent indicating that the treatment will have a beneficial effect on viscosity, however, there is an unaffected fraction. The value of this reduction in viscosity is offset by the production of oligosaccharides which negatively affect crystallisation of sucrose (Abdel-Rahman *et al.*, 2008). The most effective approach to significantly reduce EPS would be the use of good sanitation practises in combination with dextranase to minimize the build-up of problematic EPS, treat pre-formed dextran and reduce processing problems.

2.7. Acknowledgements

We would acknowledge the South African Sugarcane Research Institute (SASRI) for their support as well as Dr Sandy Snyman for her technical expertise and Dr Derek Watt. The authors would like to acknowledge Carl van Heerden and the Central Analytical Facility based at Stellenbosch University. We would also like to recognise Dr Jan Bekker for his contribution to this project. We would like to thank the NRF Innovation fund as well as the Institute for Plant Biotechnology for contributions to the project.

2.8. Author contributions

In this study I was responsible for the isolation of bacteria, extraction of genomic DNA, purification of the EPS, relative polysaccharide production, GC-MS and GC-FID sample preparation, GC analysis, dextranase sensitivity testing and TLC. Charl Marias was responsible for the 16S PCR amplifications in this study. Prof Karin Jacobs kindly assisted with the alignment of the sequences and drawing of the phylogenetic trees.

Chapter 3

General discussion

The post-harvest deterioration of sugarcane is problematic for the sugar industry and has attracted widespread attention in recent years (Solomon, 2009). There are a few strategies that have been developed to reduce the buildup of problematic deterioration products. The most effective remains the management of the cane during and after harvest. The current strategies for treatment of bacteria involved with deterioration requires approaches that influence all the species involved. The current strategy to minimize polysaccharide accumulation relies on the minimization of the "cut-to-crush' delay to reduce the overall amount of deterioration during storage that can subsequently interfere with downstream processing (Saxena *et al.*, 2010; Yusof *et al.*, 2000; Morel du Boil, 1995; Solomon, 2009). The time lag between harvesting and milling is therefore of critical importance to achieve maximum sucrose recovery (Solomon, 2009). Other factors, such as harvesting style or time of harvesting, influence the load of bacterial contamination and speed of deterioration (Singh and Solomon, 2003). Currently, management of harvesting and milling to reduce the time lag is reducing the amount of deterioration products to manageable parameters.

Previous literature suggests that the minimization of dextran levels in the sugar factory can be done by control of microorganisms in cut cane (Abdel-Rahman *et al.*, 2008). Biocidal chemicals have been used to reduce bacterial load after harvesting/during milling, these include halogen compounds, ammonium biflouride, formaldehyde, quaternary ammonium compounds and thiocarbamates (Solomon, 2009). Electrolyzed saline has been shown to be an effective anti-infective agent by denaturing proteins on bacterial cell walls though hypochlorus acid and free chlorine radicals (Solomon, 2009). The use of biocide has shown to have a significant effect in reducing any deterioration by slowing bacterial growth in harvested sugarcane

(Eggleston, 2002). These broad spectrum approaches to bacterial management have been effective but can also be highly detrimental to the environment. A greater understanding of the microorganisms that are the causative agent in the accumulation of harmful products will assist in the development of more targeted control mechanisms. This first look at the diversity of organisms involved in this process can lay the groundwork for future studies of this nature.

With the analysis of the polysaccharides and the development of a culture based technique to isolate the EPS produced by the isolates, new methodology can be developed for testing deterioration. This technique is advantageous because it allows for the scalable production of polysaccharides from the bacteria for research as well as industrial applications. The monosaccharide composition of the polysaccharides isolated indicated that there is a little described abundance of EPS that is produced during sugarcane deterioration. The concentration of dextran in milled sugarcane juice is the routine measure of both quality and the level of deterioration (Eggleston, 2002). The determination of cane deterioration is based on the testing for products that are cost effective and correlate to dextran concentrations. The use of ethanol as a measure of deterioration was shown to be inaccurate and unsuitable (Eggleston, 2002). The haze test was developed to test for the amount of dextran in the cane juice with partial ethanol precipitation of high molecular weight polysaccharides with 50% ethanol (Clarke *et al.*, 1987; Basedow and Ebert, 1979). The method is known to overestimate dextran concentrations due to absorption increases as a result of other soluble polysaccharides and inorganic components of sugarcane juice. The haze test was improved by removal of contaminating soluble polysaccharides with amylase to break down amylose which is partially soluble and contains α-(1,4) glucosyl linkages (Eggleston and Monge, 2005). This technique is still used in sugar mills to determine dextran concentrations (Anon, 1994). In the context of this study the haze test will precipitate any high molecular weight polysaccharides in solution (Basedow and Ebert, 1979). The total EPS component is measured rather than only dextran and this technique is more accurate for determination of the total amount of EPS present in the sugarcane juice.

Treatment of dextran buildup has been used, although the practice is not universal (Promraksa *et al.*, 2009). *In vitro* treatment of the EPSs with dextranase had an effect on 37 of the 38 isolates, however, there were 21 polysaccharides which were only partially digested. The partial digestion indicates that the treatment is only partially effective for remediation of the EPS produced during deterioration. The use of dextranase has been shown to be an effective method of removing dextran that has already formed in the extracted juice (Abdel-Rahman *et al.*, 2008; Morel du Boil and Wienese, 2002). These approaches are impracticable or proved to be uneconomical if the enzyme was not used optimally (Eggleston and Monge, 2005). The effective utilization of dextranase requires the juice to be heated to the enzymes optimal temperature of 50^{o}C for at least 5 minutes (Eggleston and Monge, 2005). This requires an additional heated tank to be installed into current sugarcane factories and refineries. Dextranase treatment does reduce the effect of dextran based viscosity increases of the massecuite, however, there is a release of oligosaccharides which is problematic during crystal formation (Morel du Boil, 1991; Promraksa *et al.*, 2009; Eggleston *et al.*, 2011; Eggleston *et al.*, 2009). The results in chapter 2 show the production of oligosaccharides from dextranase treatment, in addition to variable hydrolysis of the EPS. Dextranase treatment has been largely abandoned by the industry due to its usage being uneconomical.

The isolates cultured in this study were also investigated for the production of EPS on different sugars and there was a clear correlation between polysaccharide production and the presence of sucrose (Figures 6-8). The EPS productions profile is anecdotal evidence that many of the strains contain a dextransucrase type enzyme. The isolates that are able to produce EPS on all of the sugars are likely produced via Leloir glycosyltransferases. These EPS are of interest due to their high viscosity (String test positive), the role of these types of polysaccharides on both the physiochemical properties of the massecuite and crystallisation. These species require further investigation as possible biofilm forming bacteria that serve as a reservoir for bacteria involved in deterioration during the harvesting season.

Future work

There is an array of future studies that can be performed on the basis of the work presented here. The different bacteria isolated in this study can be utilised by the sugarcane industry in a range of studies to improve management strategies, reduce sucrose losses, and increase profitability. The isolates can also be used to test biocides to evaluate their effectiveness, more accurate measures of polysaccharide accumulation and develop treatments to specifically interfere with EPS production. The uses of non-EPS producing bacteria that inhibit the growth of isolates in this study are candidates for bio control that can be implemented to reduce polysaccharide accumulation in harvested cane. The variety of bacterial species involved in sugarcane deterioration suggests the analysis of the population dynamics during deterioration. The association of *L. mesenteroides* with deterioration may be the result of its ability to outcompete other species.

Dextrans are a versatile and high value product which is utilised by the food and pharmaceutical industries. The majority of the bacteria which were isolated from this sucrose rich environment clearly produce dextran-like glucans which can be used for other applications. There is a range of commercial applications for dextrans of different molecular weights and I would recommend that each polysaccharide be fully characterised with regards to chain length, branching frequency and linkage distributions. Structural determinations can be achieved through methylation analysis and/or nuclear magnetic resonance.

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