



# **CRC SUGAR Industry Innovation through Biotechnology**

## **Research Project Final Report**

**ALTERNATIVE SUGARS: NEW OPTIONS FOR THE SUGAR INDUSTRY**

**Report prepared by Anne Rae and Jason Hodoniczky.**

**Project 2b8**

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## 1.0 SUMMARY

Sugarcane has a highly effective carbohydrate biosynthetic and storage metabolism that has facilitated its use for the production of sucrose. Sugars are increasingly seen as low-cost, renewable organic resources which can be modified to produce food ingredients and industrial raw materials. For the sugar industry, alternative sugars offer a means of diversification in an area close to the existing core business. However a major restriction to development of alternative products has been ownership of enabling intellectual property by third parties. This project aimed to identify alternative sugars with desirable commercial properties and capture the IP to enable their production.

The initial phase of the project was a scoping study to identify novel, naturally-occurring sugars and enzyme systems that may be involved in their manufacture by collating information from the literature and patent databases. Sugars that occur naturally in sugarcane and closely related species were also examined for potential as higher value products. Preferred candidates were simple modifications of sucrose where the gene sequences encoding the enzymes were available and no prior IP existed. Four sugars with potential applications as alternative sweeteners or chemical feedstocks were identified.

Two of the candidate sugars could be either purchased directly or made by chemical synthesis from a purchased precursor. The remaining two candidate sugars were not available commercially and could not be synthesised easily. We proposed to make these sugars by cloning and expressing the genes that encode the enzymes from their native sources and then using the enzymes to synthesise the novel sugars *in vitro*. Two enzymes were expressed and characterised. Although neither of these enzymes carried out the predicted reactions, both enzymes were new; one is a dehydrogenase and the other is a glucosidase acting on gluco-oligosaccharides.

The potential value of any novel sugar depends on its physical and sensory properties. For application as an alternative sweetener, a novel sugar ideally needs to be as sweet as sucrose but offer health benefits, particularly low cariogenicity (tooth decay) and low calorie-yield. We developed methods that can be used in the laboratory to test industry-relevant properties of sugars, specifically sweetness, cariogenicity and digestibility. A set of commercially available sugars, including several alternative sweeteners, was used to test the assays and provide a comparison with the novel sugars.

Sweetness relative to sucrose and glucose was determined by a two-way preference ingestion assay with *Drosophila melanogaster* (fruit flies). Production of acid by the oral bacterium *Streptococcus mutans* was used as an assay to detect potentially cariogenic sugars. Calorie yield of sugars was measured by assays for digestibility by yeast invertase and rat  $\alpha$ -glucosidase/sucrase. We also tested whether the sugars were able to inhibit the digestion of sucrose, and whether the sugars could promote the growth of 'healthy' bacteria in the gut.

The results showed that two alternative sugars derived from sucrose have the properties required for an alternative sweetener. We also identified a disaccharide which is sweet-tasting and able to inhibit the digestion of sucrose. Further research will be required to develop an economic production system for these candidate sugars. The tests developed in this project also identified some interesting relationships between sugar structure and sensory or nutritive properties. Further analysis of these relationships may allow design of new sweeteners with optimal properties.

The outcome of this work is an improved ability to develop new sugar derivatives as alternative sweeteners. The information and tools developed by the project will assist future efforts to exploit new options for diversification in the sugar industry.

## **2.0 BACKGROUND**

Naturally occurring sugars are important as food ingredients, as feedstocks for fermentation processes such as ethanol generation, and as industrial raw materials. In the food industry, sugars add not only sweetness, but also colour, texture and preservation qualities. Sugars may be modified chemically to produce surfactants, emulsifiers and preservative coatings. With the projected decline in petrochemical-based materials, sugars are increasingly seen as low-cost, renewable organic resources for the chemical industry.

Sugarcane has a highly effective carbohydrate biosynthetic and storage metabolism that has facilitated its use for the production of sucrose. The price of sucrose has fluctuated greatly in recent years and there is a desire in the industry to buffer these effects through diversification. Alternative sugars offer a means of diversification in an area close to the existing industry's core business. For the Australian industry to compete in the alternative sugar product area we propose that it will be necessary to:

- (a) identify sugars with potential functionality that matches a commercial opportunity in the market
- (b) establish an IP position that will allow the protection and development of a process for the economical production of the sugar in either enzymatic, microbial or plant-derived production systems.

The aim of this project was to identify novel sugars with desirable commercial properties and capture the IP to enable production. The project is well-aligned with the Program 2 aim of developing technologies for delivery of high-value materials from sugarcane. One of the major restrictions to development of sugarcane as a biofactory has been ownership of enabling intellectual property by third parties. This position will create new options for the sugar industry for diversification in the area of novel sugars.

## **3.0 OBJECTIVES**

The aim of the project was to identify and capture market opportunities for production of novel sugars. The specific aims of the project were:

- (i) Identify sugars of potential commercial interest AND
- (ii) Identify a novel source of the enzymes and clone the genes

Achieved. The first two objectives were achieved by a scoping study in the initial part of the project. Novel naturally-occurring sugars and enzyme systems that may be involved in their manufacture were sought by collating information from the literature and patent databases. Preferred candidates were simple modifications of sucrose where the gene sequences encoding the enzymes were available and no prior IP existed. Four candidate sugars that met these criteria were identified: three sugars with potential applications as alternative sweeteners and one sugar as a potential chemical feedstock. Genes that were predicted to synthesise two of the sugars were cloned.

(iii) Demonstrate production of the sugar

Not achieved. One of the candidate sugars was purchased and a second was made under contract by a chemical synthesis company. The other two candidate sugars were not available commercially, and therefore we attempted to make them enzymatically. Two enzymes for synthesis were cloned and expressed in *E.coli*. Although the enzymes were active, they did not make the predicted sugars *in vitro*.

(iv) Test the properties of novel sugars relevant to potential applications

Achieved. The potential value of a novel sweetener depends on its physical and sensory properties, principally sweetness, digestibility and cariogenicity. Tests for these properties were developed and used to determine which sugars matched the characteristics of commercial sweeteners.

(v) Protect the IP for exploitation

Achieved. The IP produced by the project was examined carefully against the criteria of novelty and potential market value. Although some of the candidate sugars had the properties of a sweetener, no economic production system could be identified, making it unlikely that these new sugars would be competitive in the marketplace. Therefore, patent protection was not sought.

(vi) Prepare a plan for further research and commercialisation

Achieved. Some outcomes from this work have been approved for publication. One paper is already published, one submitted to a journal and one is still being prepared. The information on candidate sugars from the initial scoping study has not been disclosed and may become the subject of further research if opportunities arise.

## 4.0 METHODOLOGY

### 4.1 Identification of candidate sugars

The initial phase of the project was a desktop exercise to identify novel sugars and enzyme systems that may be involved in their manufacture. Preferred candidates were simple modifications of sucrose where the gene sequences encoding the enzymes were available and no prior IP existed. We examined naturally occurring sugars in the categories of sucrose isomers, sucrose-based oligosaccharides, sugar alcohols and rare sugars.

The criteria for selecting candidate sugars were:

- i. novel or rare sugar, not currently in commercial production
- ii. potential applications as low calorie sweeteners or as stereo-specific starting material for chemical synthesis of high value products
- iii. experimental tools such as gene sequences and a source of DNA are available

One additional candidate came from a project in the first phase of the CRC where sugarcane plants engineered to make sorbitol were also found to contain a novel sugar identified as gentiobitol (Fong Chong et al., 2007, 2009). This sugar was assessed for its potential value as a sweetener.

For each candidate sugar, a detailed assessment was prepared, covering background, properties, feasibility, IP position and market potential.

## 4.2 Functionality of candidate sugars

An industry partner, who requested confidentiality, provided information on market trends and on functional requirements for new sweetener products. Novel sugar products ideally need to be as sweet as sucrose but offer health benefits, particularly low cariogenicity and low calorie-yield.

We developed methods that can be used in the laboratory to test industry-relevant properties of sugars, specifically sweetness, digestibility and cariogenicity. New collaborations with Professor Carol Morris at SCU and with Dr Elizabeth McGraw at UQ were important in developing these methods.

The details of these methods are included in two draft publications (see Section 11). The methods are described briefly below.

To test the assays and provide a comparison with the novel sugars, we have used a set of commercially available sugars, listed in Table 1. A series of sugar alcohols, sucrose isomers and other di- and trisaccharides were sourced for comparison to the candidate sugars. The set included several commercial sweeteners.

**Table 1 Summary of sugar structures used for comparison**

MW	sugar name	structural information
<b>sugar alcohols</b>		
152.15	xylitol	reduced xylose
182.17	sorbitol	reduced glucose
344.31	maltitol	reduced maltose
344.32	gentiobitol	reduced gentiobiose
506.45	maltotriitol	reduced maltotriose
<b>sucrose isomers</b>		
342.3	sucrose	(glc-1,2-fru)
342.3	turanose	(glc-1,3-fru)
342.3	leucrose	(glc-1,5-fru)
342.3	palatinose	(glc-1,6-fru)
<b>disaccharides</b>		
378.33	trehalose.2H <sub>2</sub> O	(glc-1,1-glc)
342.2	kojibiose	(glc-1,2-glc)
360.31	maltose.H <sub>2</sub> O	(glc-1,4-glc)
342.3	gentiobiose	(glc-β1,6-glc)
342.3	melibiose	(gal-1,6-glc)
312.3	isoprimeverose	(xyl-1,6-glc)
<b>trisaccharides</b>		
504.44	melezitose	(glc-1,2-fruc-1,3-glc)
504.44	panose	(glc-1,6-glc-1,4-glc)
504.44	maltotriose	(glc-1,4-glc-1,4-glc)
504.44	erlose	(glc-1,4-glc-1,2-fru)
504.44	1-kestose	(glc-1,2 -fru-β1,2 -fru)
594.51	raffinose.5H <sub>2</sub> O	(gal-1,6-glc-1,2-fru)

candidate oligosaccharide

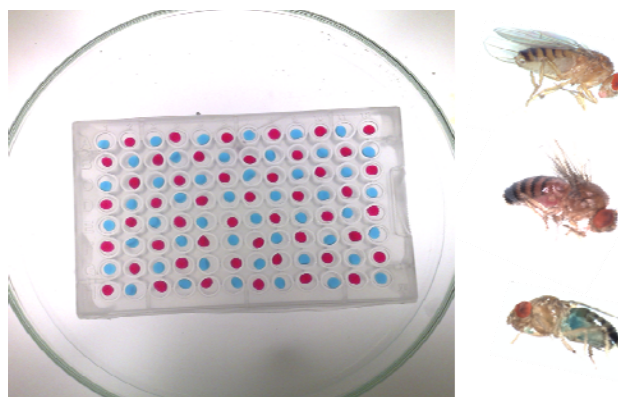
all structures alpha linked unless indicated



#### 4.2.1 Assay for sweetness

There is no laboratory assay for sweetness and the taste profiles of new food products are determined by trained panels of human testers. Since the novel sugars, by definition, did not have food safety approval, human taste tests could not be used. However, tests in animal model systems can be a good substitute and are also useful when applying for approval from the regulatory authority, Food Standards Australia and New Zealand (FSANZ). It is important that the model animal used has similar preferences for sweet compounds to the human taste profile. *Drosophila melanogaster* (fruit fly) was identified as a species which has sweet taste preferences closer to humans than many other animals, including other mammals (Gordesky-Gold et al. 2008). We have established a two-way preference ingestion assay which enabled sweetness, relative to sucrose, to be determined. In developing this assay we collaborated with Dr Elizabeth McGraw at the University of Queensland, to access materials and methods for handling *Drosophila*. Initially a lab strain of *Drosophila melanogaster* was used. A wild strain was also captured locally and bred in case the lab strain had lower discriminating ability.

In the assay (Figure 1), 96-well plates covered with Parafilm were prepared with droplets of 0.5% agarose containing a test sugar and a coloured dye (red or blue food dye). The droplets were presented in an alternating pattern and assays were prepared in duplicate with dye colours reversed to rule out possible colour preferences. Flies were kept on a 12 h light-dark cycle and were starved for 24 h prior to the assay. Approximately 50 flies per assay were then allowed to feed on the sugar solutions in darkness for 2 h. The flies were then killed by placing the dishes at -20°C for 48 h and the colour of each fly's abdomen (Figure 1) was scored as red, blue, purple (feeding on both sugars) or clear (indicates no feeding). The results were expressed as a preference index (PI) which equals the number of flies in red/blue + 0.5 times the number of purple flies divided by the total number of flies feeding. Each assay was performed several times with fresh flies.



**Figure 1** Set-up and scoring of bioassay for sweetness based on two-way preference by fruit flies (*Drosophila melanogaster*). On the left is a 96 well plate covered with parafilm and prepared with alternating sugar solutions in 0.5 % agarose containing either a red or blue dye. After feeding for two hours, flies were scored by the colour of their abdomen; shown are magnified images of flies with clear, red and blue abdomens.

#### 4.2.2 Assay for cariogenicity

Cariogenicity (tooth decay) is caused by a combination of microbial activities in the mouth. Early and late-colonising bacterial species use sucrose obtained from food for growth and produce dextrans, which form plaque, and acids, which attack tooth enamel. An oral isolate of *Streptococcus mutans* was obtained from the UQ Culture Collection and maintained at 37°C in brain-heart infusion (BHI) medium. Cells were resuspended in a medium that replicates saliva conditions and incubated with a sugar solution for 90 min at 37°C. The change in pH of the medium was then measured. A fall in pH indicates that *S. mutans* is able to use the sugar as a substrate for growth and produce acid.

#### 4.2.3 Assays for digestibility

Digestibility of sugars by oral and intestinal enzymes is an indicator of whether they are calorogenic. In developing these assays, collaboration with Professor Carol Morris and researchers in project 2b3 ("Bioactive Natural Products from Sugarcane") was very valuable in defining the most appropriate enzymes and methods.

Assays were developed with yeast invertase and rat  $\alpha$ -glucosidase/sucrase as models for human salivary invertase and human intestinal glucosidase/sucrase respectively, dominant enzymes that metabolise sucrose. The assays were tested on the panel of reference sugars described above as well as on the two candidate sugars, turanose and gentiobitol. Products of invertase digestions were monitored by HPLC.

Intestinal glucosidase reactions were performed at 37°C and stopped by the addition of 1 M Tris. An aliquot was then used for the determination of glucose release using a hexokinase assay monitored by absorbance at 340 nm and calculated from a standard curve. As a control to demonstrate that glucose release was due to glucosidase activity, the specific inhibitor acarbose was included in the reaction (0.4 mg/mL).

In addition to testing digestibility by glucosidase, we tested whether the sugars were able to inhibit the digestion of sucrose or isomaltose. The assay was performed as above, but with the addition of 15 mM isomaltose or 55 mM sucrose, both of which are digested rapidly by glucosidase. Acarbose was used in control reactions, as above. Percentage inhibition was calculated by comparison to digestion of either isomaltose or sucrose alone.

Sugars that are not digested by intestinal enzymes may support the growth of beneficial ("probiotic") bacteria in the gut. Plant & Food Research (New Zealand) have been contracted to assay the effect of a number of sugars on growth of *Bifidobacterium lactis* HN019 (DR10™) & *Lactobacillus rhamnosus* HN001 (DR20™). These assays are in progress and results should be known by the end of June 2010.

### 4.3 Enzymatic synthesis of sugars

Gene sequences were synthesised by GeneArt Ltd. then subcloned into a protein expression vector. The initial vector system chosen utilised the T7 promoter with a 6 x His tag to produce high level expression of the protein of interest in *E. coli*. The tag allows purification on a nickel column and identification of the enzyme by western blots using a Ni-NTA-alkaline phosphatase detection system. A vector that includes the Trx sequence, which is known to increase solubility of cloned proteins, was also tested. The vectors were initially expressed in *E. coli* strain BL21 (DE3) and strains were either auto-induced or induced by the addition of IPTG (0.3 mM) for 1-3 h at room temperature. Proteins in both the soluble and insoluble fractions were

recovered, then separated on SDS-polyacrylamide gels, transferred to PDVF membrane and detected with the Ni-conjugate.

Insoluble inclusion bodies were purified and refolding conditions were tested using a kit from Athena Enzyme Systems (QuickFold™). The system provides 15 combinations of refolding reagents in a factorial matrix design to identify key buffer components resulting in soluble protein.

The identity and viability of novel glucosidase enzymes were tested in an assay with the substrate, nitrophenol-glucoside. In this assay, cleavage of the glucoside unit releases nitrophenol which is monitored by an increase in absorbance at 595 nm. Purchased yeast glucosidase was used as a positive control.

Affinity purification using the Ni tag, 'Talon' affinity column was tested and the fractions analysed on SDS-polyacrylamide gels as above. A large-scale purification was carried out by the UQ Protein Expression Facility (PEF).

## **5.0 RESULTS**

### **5.1 Selection of candidate sugars**

Candidate sugars were selected according to the criteria described above (Section 4.1). The three candidates identified by literature searches are shown in Table 2 and described below. A fourth candidate, gentiobitol, was identified in another CRC project (see Section 4.1). Additional sugars that fulfill some of the criteria were also identified. In some cases, lack of tools such as sequences of the genes that synthesise the sugars prevented further work, or the IP position may have been weaker. These sugars could become viable candidates in the future if further information becomes publicly available.

Sugars that occur naturally in sugarcane and closely related species were also examined for potential as higher value products. There was little information in the literature on the occurrence of rare sugars in sugarcane. In order to address this question, a CRC-funded vacation scholar, Louise Ryan, worked with the project for 6 weeks to survey the sugars present in 7 species. Donna Glassop was also involved in the analysis and identification of sugars, using her experience from the metabolomics work in CRC project 1ai "Genes for enhanced sucrose accumulation" (Glassop et al. 2007). The results showed that many soluble sugars are present in sugarcane and closely-related species but at such low concentrations that their extraction would not be commercially viable. The results were published: Glassop D., Ryan L.P., Bonnett G.D. and Rae A.L. (2010) The complement of soluble sugars in the *Saccharum* complex. *Tropical Plant Biol.* 3:110–122. (Appendix 1).

**Table 2 Brief description of the three candidates selected from literature search**

Sugar	Enzyme	Source	Application
<b>1 <math>\alpha</math>-1,2-glucosyl sucrose</b>	glucosyl hydrolase/transferase	cyanobacteria ( <i>Anabaena/Nostoc</i> )	Non-digestible sweetener
<b>2 Turanose</b> (sucrose isomer)	$\alpha$ -Glucosidase	Honeybee ( <i>Apis mellifera</i> )	Low calorie sweetener
<b>3 3-ketosucrose</b>	Glucoside 3-dehydrogenase	<i>Agrobacterium tumefaciens</i>	Chemical feedstock

#### 1. $\alpha$ -1,2-Glucosyl sucrose

Glucans containing  $\alpha$ -1,2-linkages are considered highly desirable as sweeteners because they are indigestible and acariogenic. Most commercially available gluco-oligosaccharides are produced by the dextranase enzyme from *Leuconostoc* and contain a mixture of  $\alpha$ -1,2- and  $\alpha$ -1,6-linkages. Enzymes that catalyse solely  $\alpha$ -1,2-linkages have not previously been described. However, the trisaccharide, formed by the addition of glucose to sucrose was recently identified as a product of the cyanobacterium *Nostoc* (syn. *Anabaena*). The novel sugar is probably synthesised by the activity of a glucosyl hydrolase/transferase enzyme. The availability of the full genome sequence of *Nostoc* allowed us to identify and clone the genes that may encode this enzyme.

#### 2. Turanose

Honey contains a mixture of sweet-tasting, low calorie or low cariogenic compounds derived from sucrose, including the sucrose isomer, turanose and oligosaccharides such as erlose and theanderose. These are thought to be synthesised by an  $\alpha$ -glucosidase/transferase enzyme in the honeybee crop. The sequence of the enzyme is available and may provide a novel means of making these sugars.

#### 3. 3-Ketosucrose

This sugar was originally identified in extracts of *Agrobacterium*. The reports generated great interest because the introduction of a polymerisable double bond on the sugar molecule enables stereoselective addition of new functional groups. For example, derivatisation with vinyl side groups can be used to produce biodegradable polymers and latexes. Although the *Agrobacterium* genome sequence has been completed, it was not possible to identify the gene encoding the dehydrogenase enzyme due to lack of homologues for comparison. Recently, genes encoding glucoside-3-dehydrogenases in other species have been identified, enabling comparisons with *Agrobacterium*. This enzyme may open the market for new chemical products derived from sucrose.

#### 4. Gentiobiitol

Gentiobiitol is a disaccharide alcohol which is probably synthesised by transfer of glucose onto sorbitol by a  $\beta$ -glucosidase/transferase enzyme. The sugar is not in commercial production and the work by Fong Chong et al. (2010) is the first report of its biological synthesis. Disaccharide alcohols such as maltitol and isomaltitol are commercially produced for applications as low calorie sweeteners. Gentiobiitol would be expected to share some properties with these sugars, except that it may not be sweet, as gentiobiose is known to taste bitter.

## 5.2 Strategies for synthesis of candidate sugars

The production strategies used for the selected sugars are shown in Table 3. Amongst the candidate sugars, one could be purchased commercially (turanose) and one could be synthesised from a commercially-available sugar (gentiobiitol produced by the reduction of gentiobiose). The other two candidate sugars, glucosylsucrose and ketosucrose were not commercially available and we proposed to synthesise these by cloning and expressing the genes that encode the enzymes from their native sources.

**Table 3. Summary of strategies for producing selected novel sugars**

Target Sugar	Potential application	Native source	Production Strategy
Turanose	Sweetener	Honey bees	Purchase
Gentiobiitol	Sweetener	"Sorbitolcane" produced by Fong Chong et al. in a previous CRC project	Chemical synthesis from gentiobiose
Glucosyl sucrose	Sweetener	<i>Anabaena/Nostoc</i> cyanobacteria	Enzymatic synthesis using gene cloned from <i>Nostoc</i>
Ketosucrose	Chemical feedstock	<i>Agrobacterium tumefaciens</i> bacteria	Enzymatic synthesis using gene cloned from <i>Agrobacterium</i>

### 5.2.1 Gentiobiitol

The chemical synthesis company, Epichem Ltd. was contracted to synthesise gentiobiitol from 5 g of gentiobiose as starting material. The product was treated to remove impurities and analysed by <sup>1</sup>H NMR spectroscopy. The product was delivered on 2 February 2009. Interestingly, the Epichem chemists found that they were unable to crystallise the gentiobiitol as the sugar was highly hygroscopic. This property would limit the applications of gentiobiitol as a sweetener. For example, gentiobiitol could not be used by the spoonful to replace sucrose, but could still be useful in manufactured products such as bottled drinks and baked goods, where its hygroscopic nature might be an advantage.

### 5.2.2 Glucosyl sucrose

The trisaccharide, glucosyl sucrose was detected in the cyanobacterium, *Nostoc*, and is thought to be synthesised from sucrose by the action of a glucosidase enzyme. Two genes encoding putative glucosidases (aG1 and aG2) were identified, synthesised, and cloned into *E.coli*. Although the majority of the protein was recovered as insoluble inclusion bodies, significant amounts were soluble. Partially purified aG1 and aG2 enzymes were obtained by affinity chromatography. Assays with the artificial substrate, nitrophenol-glucoside, confirmed that the enzymes had glucosidase activity.

Production of enzyme aG2 was scaled up by recloning into a high-expression vector, and a large-scale production and purification was carried out by the UQ Protein Expression Facility (PEF). The fractions were tested for activity against a range of substrates. The results suggested that while the enzyme is an active glucosidase, its

specificity is for longer chain glucosides such as malto-oligosaccharides and that it has little activity against sucrose in the present form. The detailed experimental results are shown in Appendix 2.

This enzyme may be active against sucrose under conditions that were not tested, or alternatively, a different enzyme may be responsible for synthesis of glucosyl sucrose in *Nostoc*. Since no activity against sucrose was detected and the enzyme described here was not stable, no further purification and analysis was performed.

### 5.2.3 Ketosucrose

Ketosucrose has previously been detected in the bacterium *Agrobacterium tumefaciens* and is thought to be synthesised from sucrose by the enzyme, glucoside-3-dehydrogenase (G3DH). When the sequence of the *Agrobacterium* genome was published it was not possible to identify the gene encoding G3DH due to a lack of well-defined homologues in other species. Since then, the G3DH gene has been identified in a number of bacterial species including *Halomonas* and *Gramella*. We used these sequences to identify the homologous gene in *Agrobacterium tumefaciens* and *Stenotrophomonas maltophilia*. The *A. tumefaciens* and *S. maltophilia* G3DH genes were synthesised and subcloned into a protein expression vector and expressed in *E.coli*.

A number of induction, expression and refolding strategies were tested. Soluble enzyme was recovered and assays with a model substrate suggested that the enzyme retained activity. However, after incubation of the enzyme with sucrose, we were not able to detect ketosucrose in the reaction products. The detailed experimental results are shown in Appendix 3.

In *Agrobacterium*, this enzyme is secreted into the periplasmic space which may indicate that it interacts with other proteins to achieve the conversion of sucrose to ketosucrose. Future work may be able to resolve this process.

## 5.3 Physical and sensory properties of candidate sugars

Assays were developed for sweetness, cariogenicity (tooth decay), digestibility and probiotic activity. These assays were used to test two of the candidate sugars as well as a panel of commercially available sugars. A summary of the results is presented here. The full methods and results will be described in two journal publications; the drafts of these papers are attached to this report as Appendix 4 and Appendix 5.

### 5.3.1 Sweetness

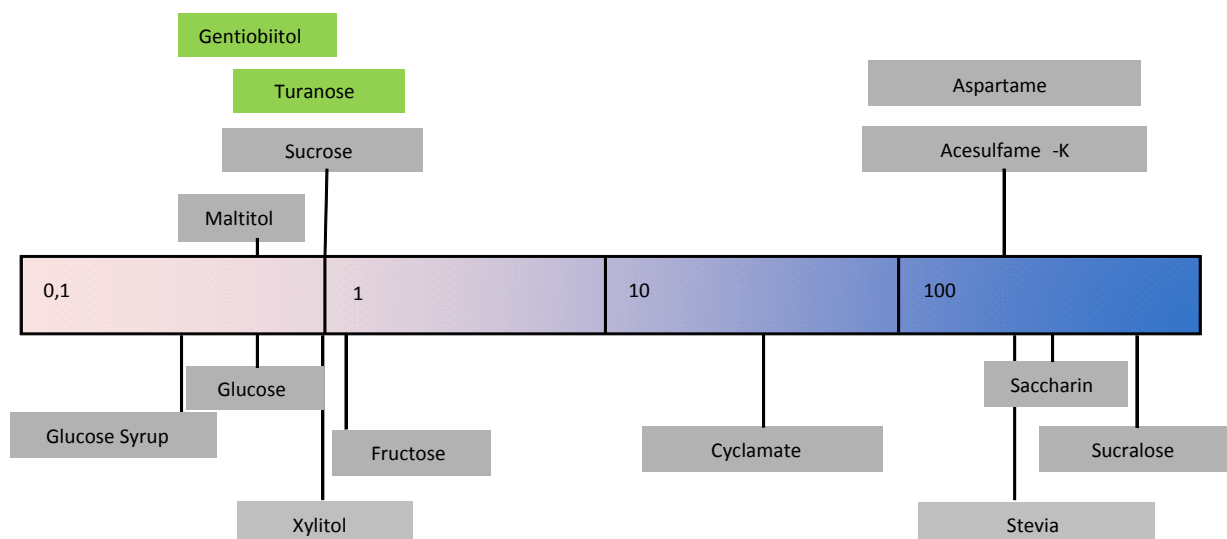
Sweetness relative to sucrose and glucose was determined by a two-way preference ingestion assay with *Drosophila melanogaster* (fruit flies). Two of the candidate sugars were tested as well as a panel of commercially available sugars.

Initially the accuracy of the assay was confirmed by comparing known sugars. Flies showed a strong and reliable preference for 5 mM or 2 mM sucrose solutions over water. The next series of assays confirmed that the flies could pick a sweeter solution reliably (10 mM sucrose compared to 2 mM sucrose). When the flies were presented with 10 mM fructose compared to 10 mM glucose, fructose was preferred. This was the expected result, as human taste tests rate fructose to be approximately two times as sweet as glucose. These results confirmed that the assay has good discriminating power and that differences were statistically significant.

The sweetness of two candidate sugars, gentiobitol and turanose has been tested against both glucose and sucrose. These results indicated that gentiobitol is not as

sweet as sucrose but has the same sweetness as glucose, on a molar basis. Turanose was found to have a similar sweetness to sucrose. The relative sweetness of the two candidate sugars is shown diagrammatically in Figure 2.

Analysis of the sweetness of a large panel of commercially available sugars highlighted some interesting relationships between structure and sweetness. The results showed that  $\alpha$ -linked sugars were generally more palatable than  $\beta$ -linked sugars. Conversion of a  $\beta$ -linked sugar to a sugar alcohol appeared to improve its palatability. Amongst the isomers of sucrose, turanose had a similar sweetness preference, while leucrose and palatinose were judged less sweet.

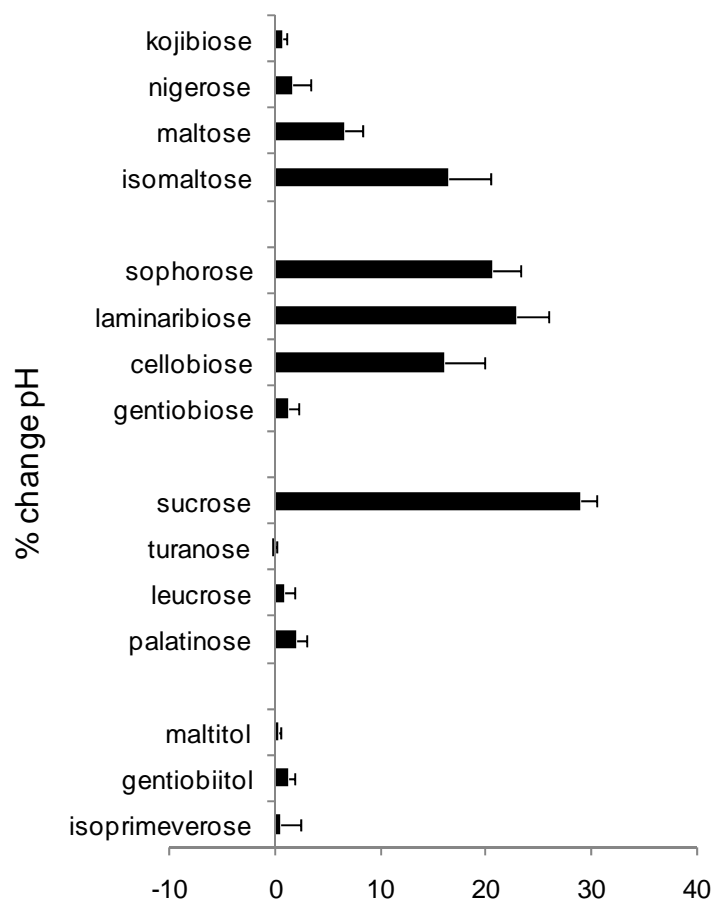


**Figure 2 Sweetness of gentiobiitol and turanose relative to a range of commercial sweeteners including sucrose.**

### 5.3.2 Cariogenicity

Dietary sugars are used by oral bacteria and produce acid that contributes to tooth decay. Production of acid by the oral bacterium *Streptococcus mutans* was used as an assay to detect potentially cariogenic sugars. The results (Figure 3) showed that *S. mutans* produced significant amounts of acid when incubated with sucrose, as expected. The starch-derived glucobioses and all of the  $\beta$ -linked glucobioses except for gentiobiose were also used by the bacteria. However isoprimeverose (a disaccharide derived from xyloglucans) and the isomers of sucrose were not used by the bacteria.

The candidate sugars turanose and gentiobiitol were not used by *S. mutans* indicating that they are likely to be non-cariogenic. These two sugars behaved in a similar way to the commercial sweeteners palatinose (= isomaltulose) and maltitol.



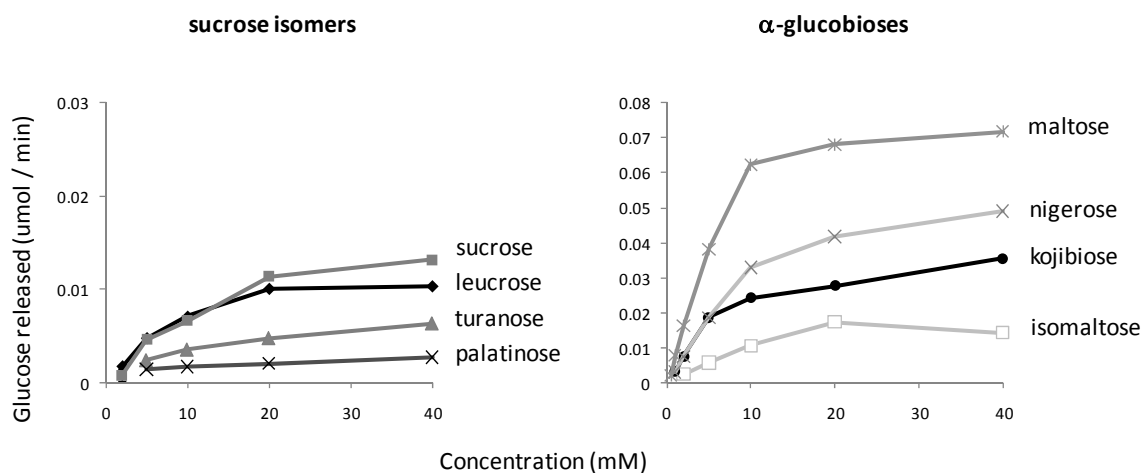
**Figure 3** Change in pH following growth of *Streptococcus mutans* on various sugar substrates. Results are expressed as % change in pH compared to the pH at time zero. No change in pH indicates that the sugar would not contribute to tooth decay. Error bars represent standard error.

### 5.3.3 Digestibility and probiotic activity

Assays were developed with yeast invertase and rat  $\alpha$ -glucosidase/sucrase as models for human oral and intestinal digestion respectively. The assays were tested on the panel of reference sugars described above as well as on the two candidate sugars, turanose and gentiobiitol. Products of invertase digestions were monitored by HPLC and the results showed that only erlose, raffinose and 1-kestose were digested. These sugars showed complete breakdown within 30 min, similar to the breakdown of sucrose as the positive control (data not shown).

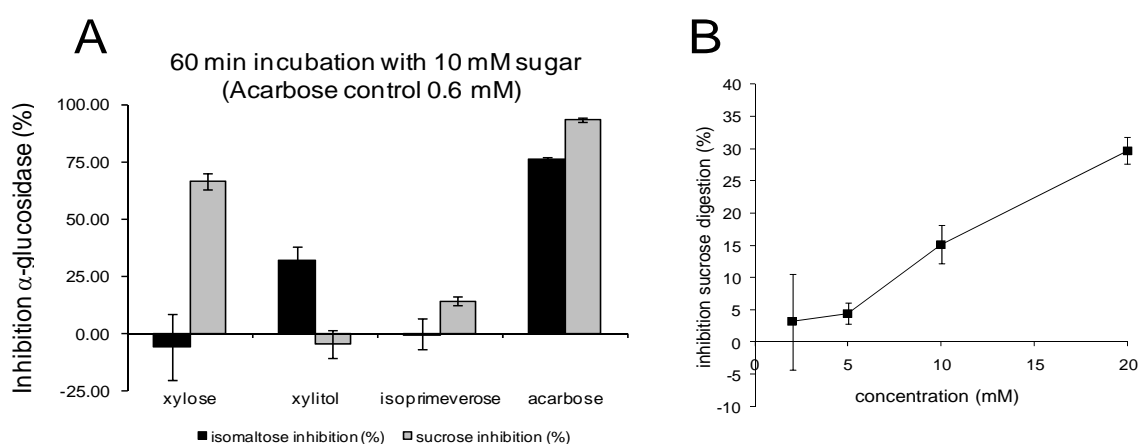
The assay with rat intestinal enzymes determined the digestibility of the candidate and reference sugars (Figure 4). The enzyme showed different activities on the three sucrose isomers tested. Leucrose was digested at a similar rate to sucrose, while turanose was only partially digested and palatinose was digested minimally. Most of the  $\alpha$ -glucobioses were digested. Gentiobiitol and the related disaccharide alcohol, maltitol were not digested. The results suggest that gentiobiitol would be non-calorigenic while turanose would release calories more slowly than sucrose.





**Figure 4 Activity of rat glucosidase on various sugar substrates**

In addition to testing digestibility by glucosidase, we tested whether the sugars were able to inhibit the digestion of sucrose or isomaltose. Products such as acarbose which inhibit sucrose digestion have applications as pharmaceutical products to help control diabetes. Neither of the candidate sugars showed significant inhibition of sucrose digestion. However, several of the reference sugars showed an unexpected inhibitory activity. Isomaltose digestion was inhibited by xylitol, while sucrose digestion was inhibited by xylose and by isoprimeverose, a disaccharide (Xyl-Glc) derived from xyloglucan (Figure 5A). A dose-response curve for isoprimeverose was obtained (Figure 5B). Isoprimeverose was purchased for use as one of the reference sugars and it has not been well characterised. The activity of isoprimeverose in inhibiting sucrose digestion has not previously been described. The results suggest that isoprimeverose and other xyloglucan derivatives may be worth investigating as a dietary additive to reduce sugar uptake.



**Figure 5 (A) Inhibition of digestion of sucrose and maltose by alternative sugars. (B) Dose-response curve for the activity of isoprimeverose on inhibition of sucrose digestion. Error bars represent standard deviation.**

In the human gut, sugars that are not digested may offer additional health benefits by supporting the growth of probiotic bacteria which produce short-chain fatty acids. As the two candidate sugars were shown to be poorly digested by mammalian enzymes, assays for growth of probiotic bacteria using these sugars as substrates are being carried out. The results of these assays should be known by the end of June 2010.

### 5.3.4 Summary

A summary of the results from analysis of the candidate sugars is shown in Table 4. Gentiobiitol was sweet tasting. It did not support the growth of oral bacteria. It was not digested by invertase or by glucosidase. Turanose was also sweet tasting and was also not utilised by oral bacteria. Turanose was not digested by invertase and was only slowly digested by glucosidase. These properties match the activities of the commercial sweeteners maltitol and palatinose (isomaltulose).

One of the reference sugars, isoprimeverose, also showed some interesting properties. In addition to being undigested, isoprimeverose was able to inhibit the digestion of sucrose to some extent. As the sweetness assay indicated that isoprimeverose is palatable, this sugar may have potential as a food supplement to improve glycaemic index.

**Table 4 Summary of the results from analysis of sugar properties.**

Sugar name	Sweet taste	Cariogenic	Invertase	Glucosidase	Inhibition
Sucrose	Yes	Yes	Digestible	Digestible	-
Xylitol	Yes	No	Not digestible	Not digestible	Yes
Isomaltulose	Yes	No	Not digestible	Not digestible	No
<b>+ 15 other reference sugars</b>					
Turanose	Yes	No	Not digestible	Not digestible	No
Gentiobiitol	Yes	No	Not digestible	Not digestible	No
Isoprimeverose	Yes	No	Not digestible	Not digestible	Yes

## 6.0 OUTPUTS

1. An assessment of opportunities for developing alternative sugars from sucrose based on technical feasibility, IP and market opportunities.
2. Expression and characterisation of two enzymes that were predicted to synthesise novel sugars from sucrose. Although neither of these enzymes carried out the predicted reactions, both enzymes were new; one is a dehydrogenase and the other is a glucosidase acting on gluco-oligosaccharides.

3. A bioassay for estimating the relative sweetness of novel sugars based on a behavioural assay with fruit flies.
4. Methods for analysing the oral and intestinal digestibility of novel sugars.
5. Demonstration that two alternative sugars derived from sucrose (gentiobitol and turanose) have the properties required for an alternative sweetener.
6. Identification of a disaccharide (isoprimeverose) which is sweet-tasting and able to inhibit the digestion of sucrose.

## **7.0 INTELLECTUAL PROPERTY:**

### **7.1 Project IP**

(i) The information in the initial scoping study on candidate sugars and the processes underlying their production represents IP of potential value to the CRC and includes confidential information from the commercial partner. This information has been protected as a trade secret.

(ii) Two potential sweeteners have been identified. This project IP was examined carefully against the criteria of novelty and potential market value. Although the candidate sugars had the properties of a sweetener, no economic production system could be identified, making it unlikely that these new sugars would be competitive in the marketplace. Therefore, patent protection was not sought and release of the information as journal papers has been approved.

(iii) A potential inhibitor of glucose release has been identified. However, patent protection was not pursued because the level of inhibition was below that of the commercial inhibitor, acarbose. This would probably require further investigation before a competitive product could be developed.

### **7.2 Sub-contracts**

Four subcontracts were entered into during the course of the project. All contracts were discussed and agreed with the Commercialisation Manager before signing.

(i) The chemical synthesis company, Epichem Ltd. was contracted to synthesise gentiobitol (Appendix 6). On the advice of the CRC lawyers, the contract was modified to ensure that (i) the CRC retained all of the compound synthesised, and (ii) the CRC was granted first rights to any new synthetic technologies developed in the process.

(ii) The UQ Protein Expression Facility was contracted to purify two enzymes (Appendix 7 and 8).

(iii) Plant & Food Research (New Zealand) have been contracted to perform probiotic assays (Appendix 9).

## **8.0 ENVIRONMENTAL AND SOCIAL IMPACTS:**

There were no environmental or social impacts from conducting the project. Although production of alternative sugars in transgenic sugarcane has been suggested, future implementation of this technology is more likely to involve *in vitro* production

systems such as microbial bioreactors, as these would be cheaper and faster to implement than production in a transgenic plant.

## 9.0 EXPECTED OUTCOMES

The outcome of this work is an improved ability to exploit new options for diversification in the sugar industry. Although the project has not produced a new commercial product, the information and tools developed by the project will assist future efforts to develop new sugar derivatives as alternative sweeteners.

## 10.0 FUTURE NEEDS AND RECOMMENDATIONS

The research described here is at a very early stage with respect to producing a commercial sweetener. The scoping study showed that candidate sugars and enzymes of synthesis can be identified. However the cloning and expression of those enzymes showed that reproducing native enzyme activities *in vitro* can be very difficult. Techniques for regulating enzyme action and modifying enzyme specificity and kinetics have been described and could be applied to these enzymes if the product was valuable enough.

Turanose was identified as a potential alternative sweetener. In further work, the enzymes that are predicted to synthesise turanose from sucrose in honey bees should be cloned and tested. During the scoping study, several other sugars with potential as alternative sugars were identified, but the sequences of enzymes that may synthesise these sugars were not available at that time. As more complete genome sequences and comparative analyses become available, these sugars may become more attractive prospects for *in vitro* production.

The tests developed in this project identified some interesting relationships between sugar structure and sensory or nutritive properties. Further analysis of these relationships may allow design of new sweeteners with optimal properties.

## 11.0 PUBLICATIONS ARISING FROM THE PROJECT

1. Glassop D., Ryan L.P., Bonnett G.D. and Rae A.L. (2010) The complement of soluble sugars in the *Saccharum* complex. *Tropical Plant Biol.* 3:110–122. DOI 10.1007/s12042-010-9049-y (Appendix 1)
2. Hodoniczky J., Robinson G.J., McGraw E.A. and Rae A.L. Fruit fly bioassay to distinguish 'sweet' sugar structures. Submitted to *J. Agric. Food Chem.* (Appendix 4)
3. Hodoniczky J., Clayton, D., Morris, C. and Rae A.L. Oral and intestinal digestion of carbohydrates in structurally relevant terms. Manuscript in preparation. (Abstract included as Appendix 5)

## 12.0 ACKNOWLEDGMENTS

We wish to thank Dr Donna Glassop (CSIRO Plant Industry) and Ms Louise Ryan (CRC Vacation Scholar) for a survey of the range of sugars present in sugarcane and related species. We thank Dr Elizabeth McGraw (University of Queensland) for advice on the experiments with fruit flies and for access to specialist equipment for growing and handling the flies. We also thank Professor Carol Morris and members of her research group (Southern Cross University) for advice and assistance in the assays for digestibility.

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Fong Chong B, Bonnett GD, Glassop D, O'Shea MG and Brumbley SM. (2007) Growth and metabolism in sugarcane are altered by the creation of a new hexose-phosphate sink. *Plant Biotechnology Journal* 5, 240–253 doi: 10.1111/j.1467-7652.2006.00235.x

Fong Chong B, Abeydeera WP, Glassop D, Bonnett GD, O'Shea MG and Brumbley SM. (2010) Co-ordinated synthesis of gentiobitol and sorbitol, evidence of sorbitol glycosylation in transgenic sugarcane. *Phytochemistry*. 71(7):736-41.

Glassop D., Roessner U., Bacic A., and Bonnett G.D. (2007) Changes in the sugarcane metabolome with stem development. Are they related to sucrose accumulation? *Plant Cell Physiol*. 48(4): 573–584. doi:10.1093/pcp/pcm027

Gordesky-Gold, B., Rivers, N., Ahmed, O.M. and Breslin, P.A.S. (2008) *Drosophila melanogaster* prefers compounds perceived sweet by humans. *Chemical Senses* 33, 301-309.

### **14.0 APPENDIX 1**

Glassop D., Ryan L.P., Bonnett G.D. and Rae A.L. (2010) The complement of soluble sugars in the Saccharum complex. *Tropical Plant Biol.* 3:110–122. DOI 10.1007/s12042-010-9049-y

### **15.0 APPENDIX 2**

Enzymatic synthesis of glucosyl sucrose

### **16.0 APPENDIX 3**

Enzymatic synthesis of ketosucrose

### **17.0 APPENDIX 4**

Hodoniczky J., Robinson G.J., McGraw E.A. and Rae A.L. Fruit fly bioassay to distinguish 'sweet' sugar structures. Manuscript submitted to *Journal of Agricultural and Food Chemistry*.

### **18.0 APPENDIX 5**

Hodoniczky J., Clayton, D., Morris, C. and Rae A.L. Oral and intestinal digestion of carbohydrates in structurally relevant terms. Manuscript in preparation.

### **19.0 APPENDIX 6**

Contract with Epichem Ltd.

### **20.0 APPENDIX 7**

Contract #1 with UQ Protein Expression Facility

### **21.0 APPENDIX 8**

Contract #2 with UQ Protein Expression Facility

## **22.0 APPENDIX 9**

Contract with Plant and Food Research (New Zealand)

# The Complement of Soluble Sugars in the *Saccharum* Complex

Donna Glassop · Louise P. Ryan · Graham D. Bonnett · Anne L. Rae

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**Abstract** The use of sugarcane as a biofactory and source of renewable biomass is being investigated increasingly due to its vigorous growth and ability to fix a large amount of carbon dioxide compared to other crops. The high biomass resulting from sugarcane production (up to 80 t/ha) makes it a candidate for genetic manipulation to increase the production of other sugars found in this research that are of commercial interest. Sucrose is the major sugar measured in sugarcane with hexoses glucose and fructose present in lower concentrations; sucrose can make up to 60% of the total dry weight of the culm. Species related to modern sugarcane cultivars were examined for the presence of sugars other than glucose, fructose and sucrose with the potential of this crop as a biofactory in mind. The species examined form part of the *Saccharum* complex, a closely-related interbreeding group. Extracts of the immature and mature internodes of six different species and a hybrid were analysed with gas chromatography mass spectrometry to identify mono-, di- and tri-saccharides, as well as sugar acids and sugar alcohols. Thirty two sugars were detected, 16 of which have previously not been identified in sugarcane. Apart from glucose, fructose and sucrose the abundance of sugars in all plants was low but the research demonstrated the presence of sugar pathways that could be

manipulated. Since species from the *Saccharum* complex can be interbred, any genes leading to the production of sugars of interest could be introgressed into commercial *Saccharum* species or manipulated through genetic engineering.

**Keywords** GC-MS · Metabolite analysis · Soluble sugar · Sugarcane

## Abbreviations

DM dry mass  
FM fresh mass  
GC gas chromatography  
MS mass spectrometry

## Introduction

Sugarcane (*Saccharum* hybrid) has a specialised metabolism that efficiently synthesises and stores sucrose at higher concentrations than most plants. Carbon is partitioned between sinks at the meristematic regions and the storage tissue in the stalk depending on developmental age and environmental influences. Whilst the major storage compound is the disaccharide sucrose, in immature tissues, the constituent monosaccharides, glucose and fructose are present at higher levels than sucrose (Hoepfner and Botha 2003). A variety of other soluble sugars has also been detected in analyses of stem tissue from sugarcane (Glassop et al. 2007). As the price of sucrose on world markets is volatile, there is increasing interest in the extraction of higher value products from sugarcane (Edye et al. 2006). The presence and therefore potential exploitation of soluble sugars other than sucrose in sugarcane and related species has not been thoroughly explored.

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*Saccharum* is a genus in the grass family Poaceae, tribe Andropogoneae (Daniels and Roach 1987). Modern sugarcane cultivars are derived predominantly from interspecific crosses between *S. officinarum* L. and *S. spontaneum* L.. Together with *Saccharum*, the genera *Erianthus*, *Miscanthus*, *Narenga* and *Sclerostachya* form the “*Saccharum* complex”, a closely-related interbreeding group (Mukherjee 1957). Genera within the *Saccharum* complex can be forced to interbreed with *Saccharum* and thus if high value sugars are present within any of these species the genes responsible for their synthesis could be introgressed into agronomically superior *Saccharum* hybrids.

An increased role has been ascribed to sugars in the regulation of metabolites. Trehalose is postulated to modulate hexokinase activity which is implicated in sugar sensing and plant development (Bosch 2005; Rolland et al. 2006; Zhang et al. 2006). There are also reported links between sugar levels and gene expression through complex signal transduction networks (Smeekens 2000; Koch 2004; Rolland and Sheen 2005; Felix et al. 2009). Other sugars (palatinose, turanose, cellobiose, gentiobiose, lactulose and leucrose) have been implicated in repressing gibberellin signalling in barley embryos (Loreti et al. 2000). Consequently identifying the range of sugars present in sugarcane may give an additional benefit through the study of gene-expression and metabolic regulation in sugarcane.

In this survey, we analysed the soluble sugars from a variety of species belonging to the *Saccharum* complex because identification of these less abundant sugars may indicate the existence of pathways of sugar biosynthesis other than those involving sucrose, fructose and glucose. The other motive of this work was to search for sugars that may be of higher economic value than sucrose. Together with our understanding of sugar metabolism, knowledge of alternative sugars may be useful in developing sugarcane as a biofactory and may illustrate areas for future manipulation.

## Results and Discussion

### Water Content

The three main components of sugarcane are soluble sugars, fibre and water. Significant differences ( $P \leq 0.05$ ) in water content were observed between species and stages of development. Water content in the immature internodes ranged between 72 and 90% with the highest water contents detected in commercial hybrid Q117 and *S. officinarum* (Table 1). The lowest water content in immature internodes was found in *S. spontaneum*. In the mature internodes, there was a rearrangement of the order of species with moisture contents ranging from 58 to 73%; *S. officinarum*

**Table 1** Percent moisture content of sugarcane internodes ( $\pm$  standard deviation). Letters represent least significant difference ( $P \leq 0.05$ ) between all samples (species and internode position), samples with the same letter are not significantly different

Species	Internode position	
	Immature	Mature
<i>S. spontaneum</i>	71.74 $\pm$ 7.05 <sub>bc</sub>	58.62 $\pm$ 6.06 <sub>a</sub>
<i>E. arundinaceous</i>	75.31 $\pm$ 1.13 <sub>cd</sub>	68.43 $\pm$ 2.79 <sub>b</sub>
<i>S. robustum</i>	79.17 $\pm$ 2.94 <sub>de</sub>	61.45 $\pm$ 2.25 <sub>a</sub>
<i>M. sinensis</i>	81.56 $\pm$ 2.22 <sub>e</sub>	70.80 $\pm$ 4.73 <sub>bc</sub>
<i>S. edule</i>	88.93 $\pm$ 0.69 <sub>f</sub>	73.07 $\pm$ 0.03 <sub>bc</sub>
<i>S. officinarum</i>	89.02 $\pm$ 1.21 <sub>f</sub>	73.03 $\pm$ 0.69 <sub>bc</sub>
comm.hybrid Q117	89.57 $\pm$ 0.19 <sub>f</sub>	70.34 $\pm$ 4.06 <sub>bc</sub>

and *S. edule* had the highest and *S. spontaneum* the lowest water content in mature internodes (Table 1).

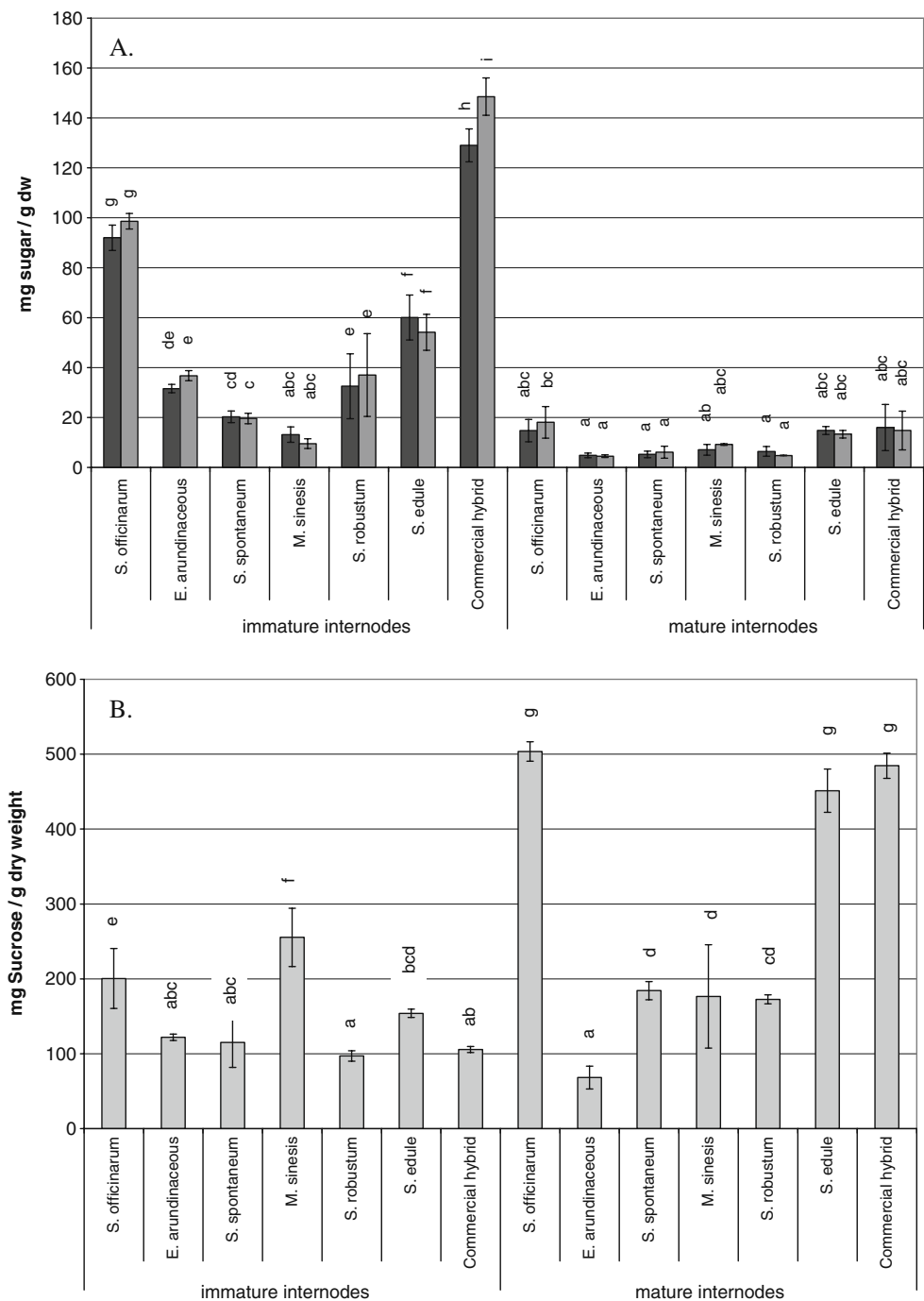
### Sucrose, Glucose and Fructose Content

Significant differences were observed for sucrose, glucose and fructose content between species and between internodes within a species (Fig. 1). Glucose and fructose concentrations were similar to each other within each sample, irrespective of species or internode maturity (Fig. 1a). The concentrations of these hexoses were higher in immature internodes than in mature internodes. The sucrose content in the mature internode was generally higher than in the immature internode for all species except *E. arundinaceous* and *M. sinensis*, where the sucrose content did not increase with maturity (Fig. 1b). *S. robustum* and *S. spontaneum* displayed a smaller increase in sucrose content between immature and mature internodes compared to the other species. The lower levels of sucrose in *S. robustum* and higher levels in *S. officinarum* are used as a taxonomic indicator of these two species (Whalen 1991; Irvine 1999).

Amongst the three main constituents of the sugarcane stem (fibre, water and sucrose) an association has been demonstrated between the amount of sucrose accumulated and the water content. Bonnett et al. (2006) and Keating et al. (1999) observed in commercial cultivars, that as sucrose concentration increases above 100 mg g<sup>-1</sup> fresh mass (FM), the water content decreases at a proportional rate of 1:1. In the current experiment, this observation was upheld for the commercial hybrid Q117, and was also seen in *S. officinarum* and *S. edule* (Fig. 2). *S. spontaneum*, *S. robustum*, *M. sinensis* and *E. arundinaceous* do not fit the above mentioned model because they have sucrose concentrations lower than 100 mg g<sup>-1</sup> FM (Fig. 2).



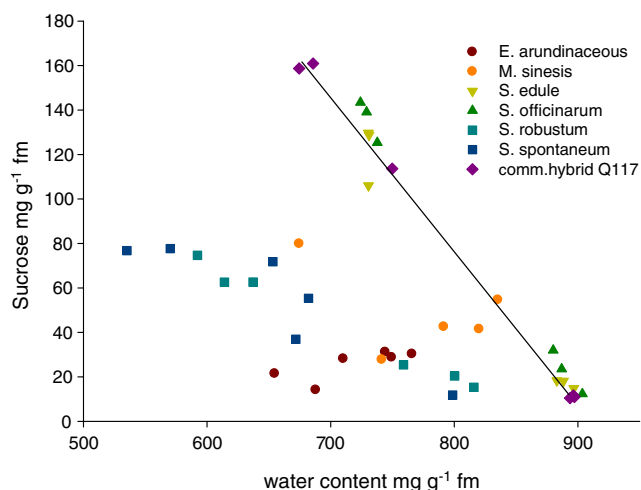
**Fig. 1** Mean sugar concentrations per g dry weight of immature and mature internodes of genotypes from the *Saccharum* complex. **a.** Fructose—dark shading and Glucose—light shading; **b.** Sucrose. Error bars represent standard errors ( $n=3$ ). Letters represent least significant difference ( $P\leq 0.05$ ) between all samples (species and internode position), samples with the same letter are not significantly different



### Other Sugars

In addition to the more abundant sugars associated with sugarcane, a number of sugars present in lower concentrations were detected (Table 2, relative abundance values are presented) and identified by combined gas chromatography mass spectrometry. Identification of sugars was limited by the availability of sugar mass spectras in the libraries, and it is possible that a wider range of sugars were

present but could not be identified. A total of 32 sugars were identified and their relative abundance measured. Only glucose, fructose and sucrose were quantified as they were present at a sufficient concentration to be detected by HPLC. The other sugars were present in such low concentrations that they could not be quantified or detected by HPLC. Bosch et al. (2003) measured five sugars in sugarcane (inositol, raffinose, maltose, xylose and trehalose) at concentrations ranging from 0.0005 to 0.5  $\mu\text{mole g}^{-1}$



**Fig. 2** Water content of individual replicate internode samples plotted against sucrose concentration on a fresh mass basis. The line represents the 1:1 trend of some sugarcane species to replace water at a rate of 1 g sucrose g<sup>-1</sup> water when sucrose concentrations above 100 mg g<sup>-1</sup> FM are achieved

FM. Xylitol, trehalose, arabinose, galactose and maltose were also detected via high performance anion exchange chromatography in progeny obtained from a cross between Brazilian cultivars SP80-180 and SP80-4966 (Felix et al. 2009).

Sixteen new sugars were identified in addition to those already reported in sugarcane and its relatives by MutalshaiKhov and Ismailov (1976), Bosch et al. (2003), Felix et al. (2009) and Glassop et al. (2007) (Table 2). In the samples of the species examined in this research neither theandrose nor 1-kestose were found, although they have previously been observed in fresh cane juice by Eggleston et al. (2004).

#### Components of Cell Wall Polysaccharides

A number of the sugars found can be attributed to intermediates in core metabolism, particularly cell wall synthesis and breakdown. For example, cellobiose, the disaccharide consisting of two glucose molecules joined by a  $\beta(1 \rightarrow 4)$  linkage, is likely to be a component of the cell wall polysaccharide, cellulose. Cellulose is ubiquitous in higher plant cell walls and its presence has been confirmed amongst  $\beta$ -D-glucans of sorghum, maize, barley, rye and wheat (Woolard et al. 1976). In the present study, cellobiose levels were not significantly different ( $P > 0.05$ ) between the species and internodes examined.

Similarly, laminaribiose, the  $\beta(1 \rightarrow 3)$  linked disaccharide of glucose, is a component of the structural and wound-induced glucan, callose, or the mixed-link glucans which have been described in grasses (Carpita 1996). Although not previously identified in sugarcane, laminaribiose is wide-

spread in plants and is a component of  $\beta$ -D-glucans from related grasses including sorghum, maize, barley, rye and wheat (Woolard et al. 1976). Mixed-linked glucans were visualised in the cell walls of rind, parenchyma, phloem and vascular parenchyma cells of immature Q117 internodes, though this particular glucan was not observed in xylem or bundle sheath cells (Fig. 3a,b). Callose was present in the sieve plates and wall deposits of phloem in Q117 mature internodes (Fig. 3c). Relative abundance values of laminaribiose were significantly different between species ( $P < 0.01$ ) and internodes ( $P < 0.01$ ); with higher values observed in *S. robustum* and *M. sinensis* for both internodes and mature *S. spontaneum* internodes than in the other species examined in this study.

#### Cell Wall Hemicellulosic Polysaccharides

In addition to  $\beta$ -glucans, cell wall polysaccharides from grasses typically include hemicelluloses and small quantities of pectins (Carpita 1996). It is possible that the xylose and arabinose detected are intermediaries in the metabolism of the hemicellulose polysaccharide, arabinoxylan, which is an important component of grass cell walls (Cobbett et al. 1992). Arabinose levels were significantly different between species ( $P \leq 0.05$ ) and internodes ( $P \leq 0.01$ ), while xylose levels were only significantly different between internodes ( $P \leq 0.01$ ). The relative abundance values for both xylose and arabinose were higher in immature internodes than mature internodes for all species examined. This may reflect higher demand for precursors of cell wall polysaccharides in young tissues.

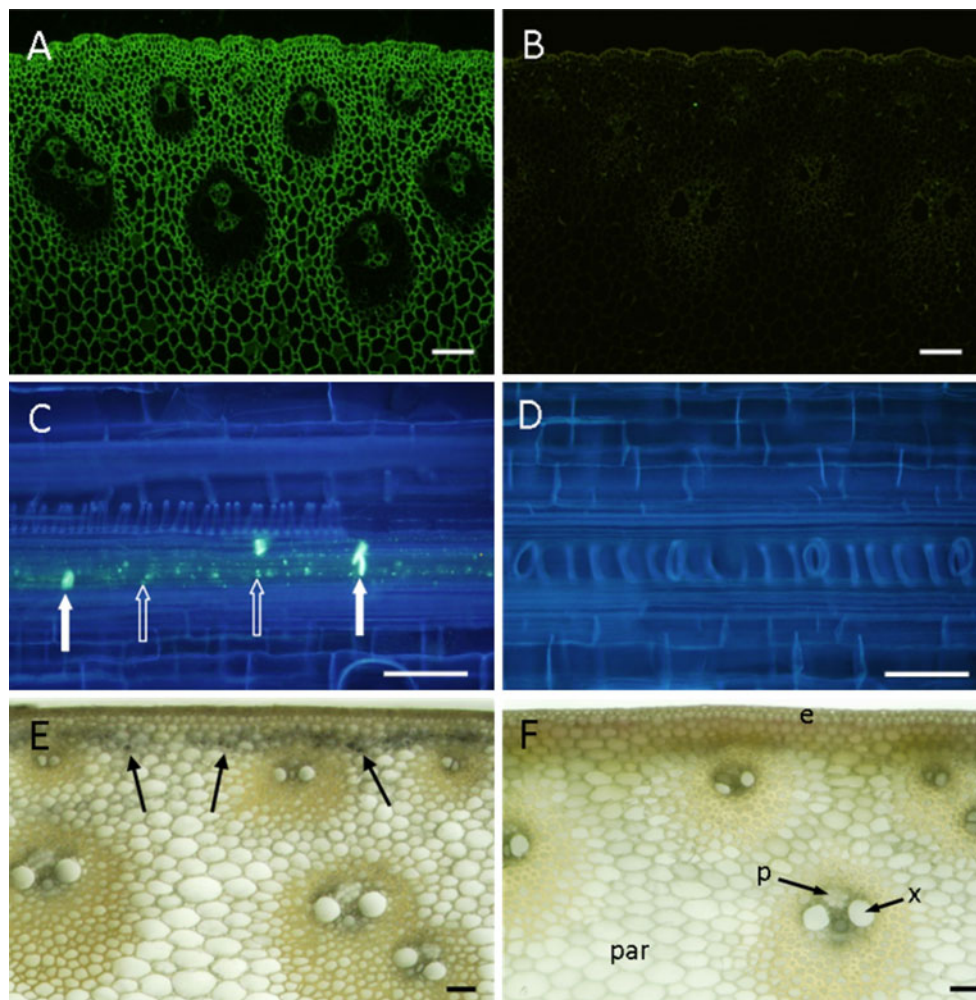
#### Cell Wall Pectic Polysaccharides

Arabinose is an important constituent of pectic cell wall polysaccharides such as arabinogalactans and rhamnogalacturonans, and is found in hydroxyproline-rich cell wall glycoproteins (Cobbett et al. 1992; Ralet et al. 1994; Burget et al. 2003). Together with the sugar acids, galactonic ( $C_1$  carboxylic acid), galacturonic ( $C_6$  carboxylic acid) and glucuronic acids, the galactose-arabinose disaccharide, which was identified by comparison to a private library of mass spectra (pers. comm., U. Roessner, University of Melbourne) is also linked to pectic polysaccharides (Nichols 1974). Within the species of the *Saccharum* complex examined here, the relative abundance values of the sugar acids were low, with significant differences between species (galacturonic acid,  $P \leq 0.01$ ) and internodes (galacturonic and glucuronic acids,  $P \leq 0.01$  for both), again suggesting that turnover of pectic cell wall precursors is higher in younger tissues. Galactonic acid had significant differences between species ( $P \leq 0.01$ ) and internodes ( $P \leq 0.05$ ) with *S. officinarum*, *E. arundinaceus* and *M. sinensis* containing



Table 2 (continued)

Saccharides	Stalk maturity									
	<i>Saccharum officinarum</i>	<i>Erianthus arundinaceus</i>	<i>Saccharum spontaneum</i>	<i>Miscanthus sinensis</i>	<i>Saccharum robustum</i>	<i>Saccharum edule</i>	<i>Saccharum hybridQ117</i>			
Mature intermode										
Cell wall										
cellobiose	0.04 (0.01)	0.05 (0.02)	0.08 (0.05)	0.04 (0.001)	0.23 (0.09)	0.02 (0.001)	0.10 (0.03)			
laminaribiose	4.76 (1.09)	2.42 (1.29)	20.30 (12.4)	18.38 (4.52)	35.9 (2.70)	3.76 (0.78)	3.82 (1.50)			
Cell wall hemicellulosic										
arabinose	6.86 (0.40)	6.05 (0.58)	10.75 (3.55)	14.23 (2.95)	8.18 (1.35)	9.83 (0.53)	6.15 (4.23)			
xylose	5.18 (1.39)	1.37 (0.11)	3.88 (2.14)	3.69 (1.18)	2.13 (0.99)	3.72 (0.44)	2.75 (2.29)			
Cell wall pectic										
galactonic acid	18.90 (4.43)	7.82 (0.22)	29.22 (2.09)	29.61 (1.06)	28.4 (11.6)	27.0 (0.69)	20.0 (10.4)			
galacturonic acid	4.51 (0.51)	1.06 (0.18)	1.98 (0.61)	3.14 (1.81)	5.58 (2.37)	6.03 (2.71)	6.38 (1.82)			
gal-ara	2.67 (0.37)	0.76 (0.22)	4.37 (0.94)	1.95 (0.24)	4.95 (0.32)	2.79 (0.53)	3.94 (0.91)			
galactose	71.97 (4.50)	263 (62.4)	77.7 (30.9)	156 (13)	51 (26)	61.7 (1.19)	157 <sup>c</sup> (1.99)			
glucuronic acid	4.52 (2.96)	2.62 (0.41)	2.69 (1.26)	4.68 (2.21)	0.90 (0.16)	2.18 (1.28)	2.35 (1.99)			
Metabolism										
gentiobiose	9.58 (0.46)	26.14 (4.57)	84.13 (42.3)	42.55 (18.9)	14.9 (4.39)	8.54 (1.76)	14.9 (1.23)			
ribose	3.51 (0.27)	1.88 (0.25)	2.44 (0.77)	6.15 (0.20)	1.96 (0.48)	2.72 (0.16)	2.16 (1.01)			
fructose 6 P	5.29 (0.70)	14.03 (0.38)	83.35 (67.9)	22.46 (6.40)	12.0 (2.65)	11.4 (1.99)	7.55 (5.38)			
glucose 6 P	10.79 (1.78)	15.25 (0.67)	25.94 (7.78)	32.68 (11.0)	20.5 (5.00)	20.5 (4.14)	11.0 (7.90)			
Physiological stress										
maltose	0.13 (0.04)	0.17 (0.03)	0.07 (0.02)	0.07 (0.03)	0.87 (0.49)	0.29 (0.07)	0.13 (0.03)			
maltotriose	19.15 (3.47)	4.54 (0.56)	90.70 (44.4)	61.31 (50.1)	18.6 (7.42)	34.1 (5.72)	72.2 (25.0)			
trehalose	9.48 (1.14)	39.67 (19.3)	76.99 (22.3)	276.7 (73.5)	11.3 (1.16)	6.56 (1.30)	110 (55)			
raffinose	7.33 (0.15)	10.20 (1.58)	3.37 (0.60)	6.59 (1.91)	1.40 (0.25)	21.3 (2.89)	13.4 (3.28)			
Alcohols/Polyols										
erythritol	0.1 <sup>c</sup>	0.06 (0.03)	1.05 (0.43)		1.46 (0.64)					
galactinol	0.82 (0.28)	17.27 (3.65)	0.24 (0.17)	10.82 (7.74)	0.38 (0.04)	0.11 (0.03)	0.37 (0.23)			
inositol IP	2.25 (0.10)	3.45 (0.31)	2.98 (0.60)	4.97 (2.20)	4.85 (1.52)	2.82 (0.24)	1.69 (0.46)			
mannitol	24.5 (10.0)	8.02 (1.57)	3.18 (1.77)	2.81 (1.03)	1.90 (0.76)	20.6 (7.83)	58.1 (55.4)			
myo-inositol	37 (4)	18.89 (1.60)	41.73 (11.9)	58.62 (17.1)	23.0 (3.80)	84.5 (4.46)	24.0 (15.9)			
pinitol	7.69 (0.26)	13.23 (1.87)	15.21 (1.16)	31.19 (9.26)	6.71 (1.17)	6.06 (0.59)	12.9 (3.79)			
sorbitol	3.75 (1.18)	6.34 (0.88)	6.05 (0.79)	12.03 (4.29)	6.32 (1.17)	3.90 (0.05)	6.74 (3.52)			
xylitol	0.19 (0.05)	0.16 (0.01)	0.06 (0.03)	0.33 (0.01)	0.25 (0.07)	0.20 (0.05)	0.10 (0.05)			
Rare plant saccharides										
melezitose	1.71 (0.05)	3.46 (0.50)	11.95 (2.56)	10.80 (0.42)	7.39 (2.10)	1.57 (0.45)	2.10 (0.36)			
turanose	1.81 (0.45)	5.82 (2.14)	15.02 (5.12)	4.63 (0.68)	5.70 (0.94)	1.27 (0.17)	12.6 (2.46)			



**Fig. 3** Detection of polysaccharides in stem tissue of commercial hybrid Q117 by histochemical staining and immunolabelling. **a, b** show sections of fixed tissue embedded in paraffin; **c–f** show free hand sections. **a**. In a section of internode 1 labelled with antibody to the (1–3),(1–4)- $\beta$ -glucan and an Alexa-Fluor 488 conjugated secondary antibody, green fluorescence was observed in cell walls of rind, parenchyma, phloem and vascular parenchyma cells, but not in the xylem or bundle sheath cells. **b**. In control experiments where the primary antibody was omitted, only weak background fluorescence was observed. **c**. Yellow fluorescence indicates the presence of callose

in sieve plates (*solid arrows*) and wall deposits (*open arrows*) in the phloem in a longitudinal section of internode 10 stained with the aniline blue fluorochrome. Blue autofluorescence of unlabelled cell walls is also visible. **d**. In an unstained section, only blue autofluorescence is visible. **e**. In a transverse section of the tenth internode stained with  $I_2$ -KI, starch is visualised as black staining in the chlorenchyma cells near the rind (*arrows*). **f**. In an unstained section, there is no dark staining in the chlorenchyma. Tissues present include epidermis (**e**), metaxylem vessels (**x**), phloem (**p**), and parenchyma (**par**). Bars: **a, b** 100  $\mu$ m; **c–f** 200  $\mu$ m

higher levels in mature than immature tissues. The relative abundance of galactose-arabinose disaccharide was not significantly different between species and internodes ( $P > 0.05$  for both), though *S. officinarum* had a higher relative abundance level in mature than immature tissues which was the opposite of the remaining species examined.

#### Sugars With Other Roles in Metabolism

The relative abundance values of ribose in the samples were low, but higher in immature internodes than mature internodes (significantly different,  $P \leq 0.01$ ). While no significant

difference between species ( $P > 0.05$ ) was observed there was a species by internode interaction ( $P \leq 0.05$ ). A small amount of free ribose is not surprising since it is a main component of RNA, ATP and other metabolites, and the presence of ribose has been detected in other plant tissues (Seymour et al. 1989).

Gentiobiose ( $\beta$ -1,6-glucosyl-glucose) has been identified in microorganisms, but it occurs rarely as a free sugar in plants, although there is evidence of  $\beta$ -glucosidases with transferase activity which could synthesise this sugar (Zhifang and Loescher 2003). Gentiobiose has been detected in plant tissue by GC separation methods (Seymour et al. 1989) and

it is found as an esterified side-chain in secondary metabolites such as crocin. Free gentiobiose was recently found in tomato fruit, where it was implicated in signalling during fruit ripening (Dumville and Fry 2003). Gentiobiose relative abundance values were significantly different between species ( $P \leq 0.01$ ) with higher levels observed in immature than mature tissue of *S. officinarum*, *M. sinensis*, *S. edule* and commercial hybrid Q117, similar to the difference seen between immature and mature tomato fruit.

#### Sugars Related to Adaptation to Stress

Maltose and maltotriose are likely to be derived from the  $\alpha(1 \rightarrow 4)$ -glucan, starch (Weise et al. 2004; Smith et al. 2005) which was shown to be present in small amounts in stems of the commercial variety Q117 (Fig. 3e). Maltose was detected at both stages of development in all species examined, with significant differences between species ( $P \leq 0.01$ ) and internodes ( $P \leq 0.05$ ). The presence of maltose was associated with tissue maturation in tomatoes (Roesnner-Tunali et al. 2003). The trisaccharide maltotriose was present in both stages of internode development, with all genotypes except *E. arundinaceus* exhibiting a higher relative abundance level in the mature internodes than the immature internodes; significant difference is noted between species and internodes ( $P \leq 0.01$  for both) with no significant interaction ( $P > 0.05$ ).

In some plant species including several pteridophytes, members of the Apiaceae and the xerophyte *Myrothamnus flabellifolius*, trehalose is the primary carbohydrate for translocation and storage, and it has also been implicated in sugar sensing and responses to abiotic stress (Goddijn et al. 1997; Goddijn and van Dun 1999; Avonce et al. 2005). In previous studies, trehalose was positively correlated with sucrose in the sugarcane hybrid Q117 (Glassop et al. 2007), but negatively correlated in the hybrid cultivars N19 and US6656-15 (Bosch et al. 2003). Trehalose and trehalose-6-phosphate are thought to mediate carbon metabolism and partitioning through modulation of hexokinase activity in a similar manner to that observed in yeast (Goddijn and Smeekens 1998; Müller et al. 1999; Eastmond et al. 2003; Schluempmann et al. 2004; Avonce et al. 2005). McCormick et al. (2008) observed a decreased ratio of trehalose-6-phosphate synthase : trehalose-6-phosphate phosphatase (TPS:TPP) in sugarcane leaves with reduced photosynthesis due to cold-girdling. Transgenic sugarcane overexpressing TPS from the fungus *Grifola fondosa* accumulated trehalose which conferred increased drought tolerance, though no effects on carbon partitioning/accumulation were reported (Zhang et al. 2006).

Amongst the samples examined in this study, there was no clear association between sucrose content and trehalose. There were significant differences between species ( $P \leq$

0.01). In *E. arundinaceus* and commercial hybrid Q117 the differences in relative abundance values between immature and mature internodes were small but there was more trehalose in the immature internodes, while there was no large difference between relative abundance values of immature and mature internodes for *S. officinarum*, *S. robustum*, *S. edule* and *S. spontaneum*. *M. sinensis* had a higher relative abundance of trehalose in the mature internode than immature internode. In our study, the highest levels of trehalose were found in *M. sinensis* which had relative abundance values in the mature tissues that were double the levels in other samples.

Raffinose ( $\alpha$ -1,6-galactosyl-sucrose) and its homologous series of galactosyl sucroses are synthesised from sucrose by the transferase activity of various  $\alpha$ -galactosidases, using galactinol as the galactosyl donor (Kandler and Hopf 1980). Relative abundance levels for galactinol in immature internodes were higher than mature internodes for all species examined; with significant differences between species, internodes and species  $\times$  internodes interactions ( $P \leq 0.01$  for all parameters). It has been suggested that raffinose and the raffinose-family of oligosaccharides are involved in stress tolerance and they are commonly found in seeds as a desiccation tolerance agent (Claeysen and Rivoal 2007). In the present study *M. sinensis*, *S. robustum* and *S. spontaneum* had higher relative abundance values of raffinose in the immature than mature internodes, the opposite of what was observed in the remaining species; with significant differences between species and a significant species  $\times$  internode interaction ( $P \leq 0.01$  for both).

Sugar alcohols or polyols are thought to play an important role as osmoregulators, associated with osmotic stress caused by temperature, drought, salinity or high sugar concentrations (Bialeski 1982; Pommerrenig et al. 2007), possibly by scavenging hydroxyl radicals and preventing oxidative damage (Smirnoff and Cumbes 1989; Loescher and Everard 1996; Nishizawa et al. 2008). In some species, polyols, like mannitol (Apiaceae, Oleaceae, Rubiaceae and Scrophulariaceae), and sorbitol (woody Rosaceae, Spiridaceoideae, Pyroideae and Prunoideae), can be synthesised and translocated from leaves in addition to sucrose (Bialeski 1982; Nadwodnik and Lohaus 2008). These sugar alcohols may represent as much as 30% of the carbon fixed by polyol translocating plants (Bialeski 1982; Loescher and Everard 1996; Nadwodnik and Lohaus 2008).

In our study, the relative abundance of mannitol in all genotypes, except the commercial cultivar Q117, were slightly higher in immature than mature internodes; in Q117 relative abundance levels in the immature internodes were more than 3 times higher than in mature internodes; with significant differences between species ( $P \leq 0.01$ ), internodes ( $P \leq 0.01$ ) and a significant species  $\times$  internode

interaction ( $P \leq 0.05$ ). Mannitol has been detected in more than 100 vascular plants (Nadwodnik and Lohaus 2008) and it is a major translocatable sugar in some species (Claeysen and Rivoal 2007; Trip et al. 1965) where it may play a similar role to sucrose in transferring photosynthate to various sinks (Bielecki 1982). Studies in transgenic plants have also suggested that mannitol may have a role in resistance to salt stress (Abebe et al. 2003; Zhifang and Loeschner 2003).

Within the commercial cultivar Q117, sorbitol was only detected in mature internodes. While low relative abundance levels were observed in both internodes for the remaining species there are significant differences between species, internodes and species  $\times$  internode interactions ( $P \leq 0.01$  for all parameters). The presence of sorbitol in the woody Rosaceae family is well documented, with concentrations ranging from 15 to 80% of the soluble carbohydrate content depending on tissue/organ (Bielecki 1982; Nadwodnik and Lohaus 2008). *Plantago coronopus* plants undergoing salt stress had increased sorbitol concentrations that may play a role as an osmoregulator (Gorham et al. 1981).

The cyclic polyol, myo-inositol, and its monomethylated derivative, pinitol, were present in all the *Saccharum* complex species. Differences in myo-inositol relative abundance values are significant for species, internodes and species  $\times$  internode interaction ( $P \leq 0.01$  for all parameters). The abundance of myo-inositol is higher in immature tissue than mature tissue for all species except *E. arundinaceus* where the values are similar. Relative abundance values for pinitol were significantly different for species and internodes ( $P \leq 0.01$  for both) with slightly higher values in immature than mature internodes for all species. Pinitol was found to be present in a small number of Proteaceae species; when present normally in concentrations higher than inositol (Bielecki and Briggs 2005). In alfalfa, pinitol levels increased under salt stress suggesting that pinitol was acting as a compatible solute (Fougere et al. 1991). Pinitol has also been linked to drought or salt tolerance in *Sesbania aculeate* (Ashraf and Harris 2004), *Mesembryanthemum crystallinum* (Paul and Cockburn 1989) and soybean (Streeter et al. 2001).

The four-carbon polyol, erythritol was not detected in *S. edule* or in the mature internodes of commercial hybrid Q117 and *M. sinensis*, but was found in all of the other samples; with significant difference between species ( $P \leq 0.01$ ) and a significant species  $\times$  internode interaction ( $P \leq 0.05$ ). Erythritol has been identified in a range of plants including some grasses and *Primula* (Stacey 1974; Bielecki 1982).

Differences in relevant abundance values of xylitol were significant for species and internodes ( $P \leq 0.01$  for both) with greater differences observed in immature internodes of *S. edule*, *S. officinarum* and *S. robustum* than mature

internodes. The remaining species had only a slightly higher abundance of xylitol in immature than mature internodes. Xylitol detection in plants as a product of plant metabolism has not been convincing due to the method of analysis and the presence of xylitol has often been attributed to fungal pathogens degrading xylans (Bielecki 1982). The propagation of the *Saccharum* species in field conditions cannot guarantee that the cane was not colonised by fungi, though no infestation was observed. The presence of xylitol in sugarcane needs to be further examined to ensure that it is a sugarcane metabolite and not a fungal metabolite.

Sugar alcohols, including sorbitol, mannitol, erythritol and xylitol are commercially produced for applications as low calorie sweeteners. The results suggest that sugar alcohols naturally occur within species of the *Saccharum* complex and although they are in small quantities there may be potential to increase the yields of these compounds. The effect of accumulating an alternative sugar product in the tissue would need to be examined carefully. Transgenic sugarcane plants expressing the sorbitol-6-phosphate dehydrogenase gene, from *Malus domestica*, were able to accumulate sorbitol in the leaf lamina ( $\sim 120 \text{ mg g}^{-1} \text{ DM}$ ) and stalk pith ( $\sim 10 \text{ mg g}^{-1} \text{ DM}$ ) and although this was not lethal there was a negative effect on growth and metabolism (Fong Chong et al. 2007).

#### Sugars Rarely Found in Plants

The detection of turanose and melezitose in the samples may indicate that there was some insect exudate present on the material. Turanose is an isomer of sucrose that is reportedly not synthesised, cleaved or transported by plant enzymes (Sinha et al. 2002), while melezitose has only rarely been reported in plants (Farrant et al. 2009). There was no significant difference ( $P > 0.05$ ) for turanose, but a higher abundance of turanose in immature internodes of *S. spontaneum* and *M. sinensis* than in mature internodes, which was the opposite of that observed for the remaining species. The amounts of the trisaccharide melezitose was significantly different between species and internodes ( $P \leq 0.01$  for both), with higher abundance in the immature than mature tissue for all species except *S. edule* that showed no difference between internodes. Both turanose and melezitose have been detected in honey and are thought to be formed by the action of honeybee or aphid glucosidases which also have some transferase activity, particularly at high concentrations of sucrose (Da Costa Leite et al. 2000; Hogervorst et al. 2007). It is possible that small amounts of turanose and melezitose are formed in plants such as sugarcane, as a side reaction similar to the 1-kestose production from sucrose by invertase from some organisms at high sucrose concentrations (Pollock and Cairns 1991).

## Conclusion

Apart from glucose, fructose and sucrose, the quantities of soluble sugars detected were low, but it is clear that a wider range of sugars is made by species in the *Saccharum* complex than has previously been described. Some of the sugar alcohols found in these species would have value as alternative products if the concentrations could be increased. It may be possible to selectively breed or genetically modify particular species to increase the production of valuable sugars for harvest. The ability to use sugarcane as a bio-factory for alternative sugar production has been demonstrated by Wu and Birch (2007) with the production of transgenic sugarcane synthesising isomaltulose. The positive results of Wu and Birch (2007) demonstrate that total sugar content of sugarcane may be increased and introduction of other sugars could be a way of increasing value of sugarcane whilst maintaining sucrose content.

The roles of the reported complement of sugars in sugarcane need to be further researched. Carbon partitioning and accumulation can be affected by a large number of molecules with a signalling role, including sugars. Sugar sensing and signalling can modulate plant growth, development and response to stress. Understanding these pathways will be important in designing strategies to optimise the production of both sucrose and alternative products with higher value from sugarcane.

## Materials and Methods

### Plant Tissue

Samples of stalk internode were collected from an immature internode (internode 4) and a mature internode (internode 7–15) from plants grown in soil at the CSIRO Davies Laboratory in Townsville, Australia (Lat. 19° 15'S, Long. 146°46'E) in the late afternoon 18th October 2006. Plants were regularly watered and grown as single isolated plants in a germplasm garden in the open under the same conditions. Internodes were numbered from the top of the stem towards the base in accordance with Moore (1987); internode 1 is immediately below the node to which the first fully expanded leaf subtends. Triplicate samples were collected from a multistem plant of the following species: *Erianthus arundinaceus* (IJ76-394), *Miscanthus sinensis* (NC-100), *Saccharum spontaneum* (Mandalay), *S. edule* (NG57-78), *S. robustum* (NG-289), *S. officinarum* (Goru) and *Saccharum* hybrid (cv. Q117). Internode samples were quickly frozen in liquid nitrogen after harvesting and stored at  $-80^{\circ}\text{C}$ . Frozen tissue was ground using liquid nitrogen and a mortar and pestle. Ground tissue was used for measuring variables as detailed below.

### Moisture Content Measurements

Moisture content was determined after weighing samples of each internode (fresh mass—FM) and then drying to constant weight (dry mass—DM).

### Glucose, Fructose and Sucrose (GFS) Extraction

Stem samples (<2 g) were weighed into 15 mL tubes. Distilled water (9.9 mL) was added to each tube (Hamilton diluter). Samples were incubated in a waterbath overnight at  $70^{\circ}\text{C}$ ; the solution was decanted and stored in a 50 mL tube. Another 9.9 mL water was added to the original sample, and the samples were incubated overnight at  $70^{\circ}\text{C}$  again. The second solution was mixed with the first, and an aliquot was taken for HPLC analysis. HPLC analysis was performed as described in Glassop et al. (2007).

### Metabolite Extraction and GC-MS

Aqueous extractions were performed to prepare samples for analysis of sugars by combined gas-chromatography/mass spectrometry (GC-MS). Aliquots of 60  $\mu\text{L}$  were dried for GC-MS analysis. Sugars were identified by comparison to a library of standards using methods developed by Glassop et al. (2007). The following changes were made to the GC-MS protocol, in order to increase the separation of sugars to avoid co-elution. The oven temperature initiated at  $70^{\circ}\text{C}$ , increased to  $160^{\circ}\text{C}$  at  $6^{\circ}\text{C}/\text{min}$ , a second temperature increase to  $226^{\circ}\text{C}$  at  $2^{\circ}\text{C}/\text{min}$ , a final temperature increase to  $330^{\circ}\text{C}$  at  $6^{\circ}\text{C}/\text{min}$  and hold for 10 min. The MS scanned from mass 70 to 600  $m/z$  every 0.4 s (with an interscan time of 0.05 s), starting at 7.5 min and ending at 73 min.

### Peak Identification and Determination of Relative Abundance Method Development

One chromatogram of each cultivar and each internode was examined in detail, using characteristic mass to charge ratio's ( $m/z$ ), to identify which sugars were present. These sugars were then incorporated into an automated method that identified their presence by confirming  $m/z$ , retention time and comparison with mass spectra from publicly and privately available libraries specific for each sugar. The automated method was then used to identify and measure the peak areas from the chromatograms of all samples. This procedure resulted in the measurement of 28 sugars via GC-MS. Peak area is equivalent to metabolite concentration; since calibration curves were not performed for each metabolite the peak area is used as a relative abundance value. Differences in relative abundance values are indicative of differences in concentration. However the peak area of one metabolite cannot be compared to another metabolite



due to different chemistries which affect sensitivity. Glucose, fructose and sucrose were not measured from the GC-MS chromatograms but by HPLC because the levels of abundance of these three sugars were outside the detection range of the GC-MS chromatograms, which were designed to ensure detection of sugars present at low concentrations. HPLC techniques are well established for GFS measurements.

#### Normalisation of Results

Peak areas obtained from the quantification method were divided by peak area of ribitol (internal standard) and fresh or dry weights of samples extracted to get relative abundance value /g fresh or dry mass respectively, in accordance with methods presented in Glassop et al. (2007).

#### Histochemistry and Immunolabelling

For histochemical staining, thin sections of sugarcane stalk tissue were cut by hand with a razor blade. Callose ((1-3)- $\beta$ -glucan) was detected by fluorescence of sections under UV illumination after staining with the aniline blue fluorochrome (Evans et al. 1984; Biosupplies Australia Pty. Ltd., Parkville Vic. 3052, Australia) according to the manufacturer's protocol. Starch was detected by staining with I<sub>2</sub>-KI and visualised as black deposits by bright field microscopy. In control experiments, similar tissues sections that had not been stained with histochemical reagents were viewed under UV or bright field illumination.

For immunolabelling, stalk tissue was fixed in paraformaldehyde-glutaraldehyde, embedded in paraffin and sectioned as described in Rae et al. (2005). A mouse monoclonal antibody with specificity for (1-3),(1-4)- $\beta$ -glucan (Meikle et al. 1994) was purchased from Biosupplies Australia Pty. Ltd. Sections were labelled with 10  $\mu$ g/ml antibody following the method of Rae et al. (2005) except that the secondary antibody was chicken anti-mouse IgG conjugated to Alexa Fluor 488 used at a final concentration of 2  $\mu$ g/ml. In control sections, the primary antibody was omitted.

All sections were examined under a Zeiss Axioskop microscope and images recorded with an Olympus DP-70 camera.

#### Statistical Analysis

Metabolomic experiments yield values that vary from single digits to numbers in the hundreds and thousands; statistical analysis of these data sets with this variation can lead to invalid conclusions. Transformation of the data is common; log 10 transformation was used in this experiment, in order to overcome the degree of variation

(Goodacre et al. 2007). As zeros are present in the data set a set value is added to all values. The set value was determined from the smallest peak area greater than zero and dividing this value by 1,000. Log 10 transformation was performed on these values. The transformed values were analysed with ANOVA, Genstat. Genstat was also used for the ANOVA and least significant difference analysis of moisture content and GFS data.

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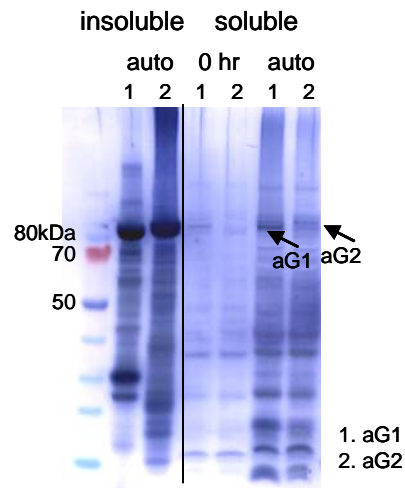
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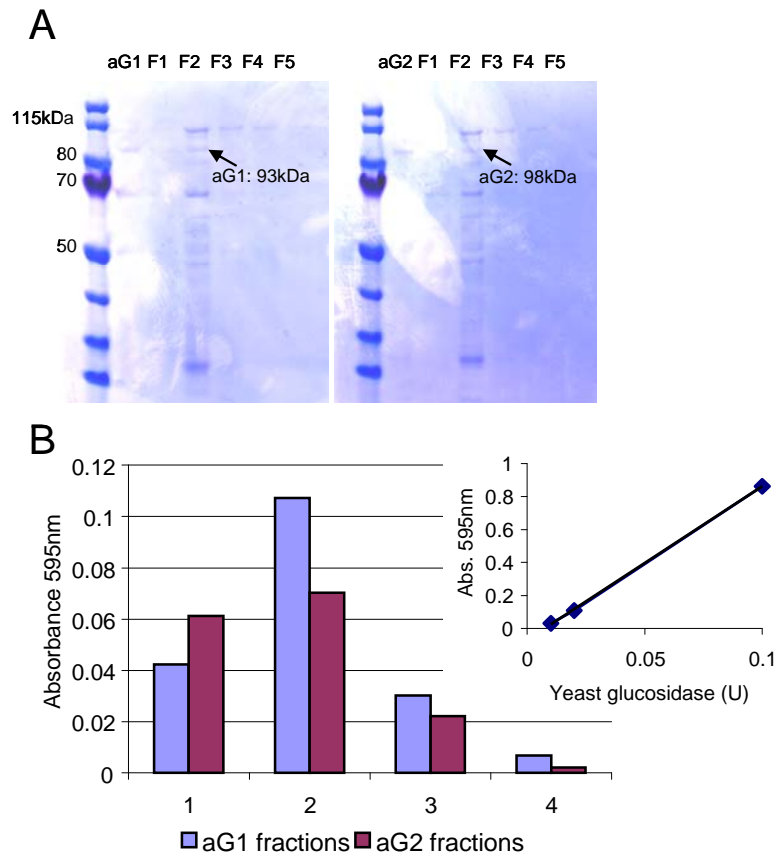
## APPENDIX 2 – ENZYMATIC SYNTHESIS OF GLUCOSYL SUCROSE

The trisaccharide, glucosyl sucrose was detected in the cyanobacterium, *Nostoc*, and is thought to be synthesised from sucrose by the action of a glucosidase enzyme. Two genes encoding putative glucosidases (aG1 and aG2) were identified, synthesised, and cloned into *E.coli*. The vector system chosen utilises the T7 promoter with a 6 x His tag to produce high level expression of the protein of interest in *E. coli*. The tag allows purification on a nickel column and identification of the enzyme by western blots using a Ni-NTA-alkaline phosphatase detection system. Initially, the genes aG1 and aG2 were expressed by autoinduction in BL21 (DE3) cells. Although the majority of the protein was recovered as insoluble inclusion bodies, significant amounts were soluble (Figure a1-1).



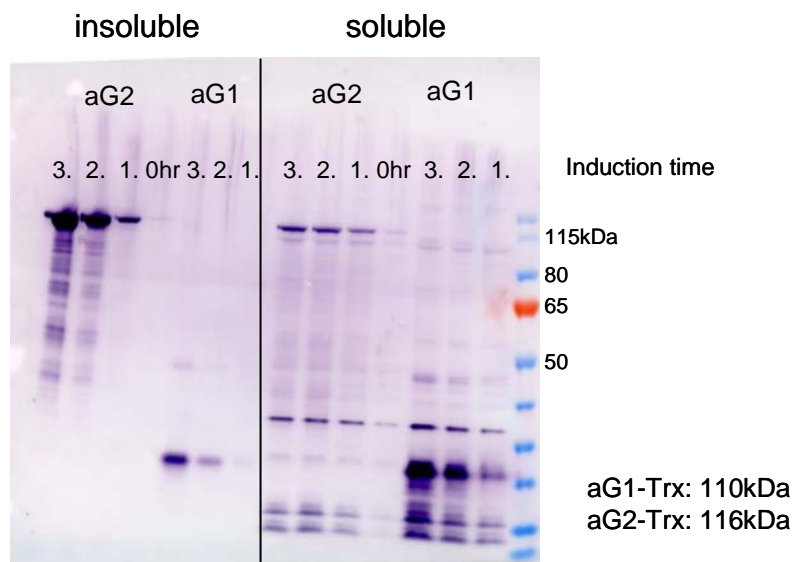
**Figure A1-1 Analysis of enzyme production in *E. coli* cultures that express the *Nostoc* genes aG1 or aG2. After overnight autoinduction, some soluble protein at the correct molecular weight (arrows) was detected on a protein blot developed with Ni-NTA alkaline phosphatase.**

Methods for purifying the enzymes were then tested. Following affinity-purification using the Ni tag, partially purified aG1 and aG2 enzymes were recovered (Figure A1-2A). The identity and viability of these enzymes were tested in an assay with the substrate, nitrophenol-glucoside. In this assay, cleavage of the glucoside unit releases nitrophenol which is monitored by an increase in absorbance at 595 nm. Purchased yeast glucosidase was used as a positive control. Figure A1-2B shows that the fractions recovered from the affinity purification contained glucosidase activity. These experiments confirmed that the gene predictions were correct and that the enzymes had the desired activity after expression and purification.



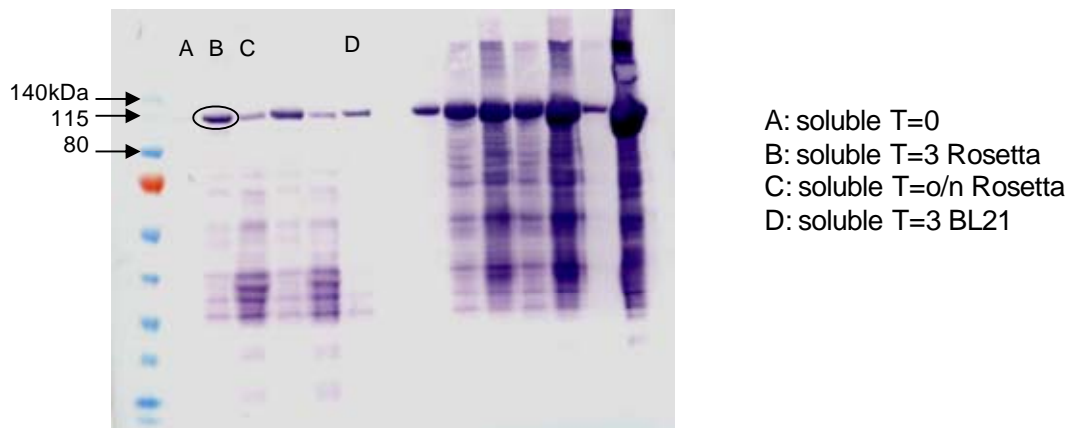
**Figure A1-2 (A) Protein blot showing partially purified aG1 and aG2 enzymes in the eluted fractions F1 to F5 following separation on a Talon affinity column. (B) Assay for glucosidase activity in fractions from the affinity column. Results are expressed as absorbance at 595 nm, equivalent to release of nitrophenol from the substrate. Maximum activity was obtained in fraction 2, corresponding to the presence of the bands shown in (A). Inset shows a linear relationship between absorbance and the amount of the control enzyme, yeast glucosidase.**

Further experiments were then carried out to increase the ratio of soluble to insoluble enzyme produced by this system. For enzyme aG2, this was achieved by re-cloning into a vector that includes the Trx sequence, which is known to increase solubility of cloned proteins (Figure A1-3). This approach was not successful with aG1, which appeared to be degraded.



**Figure A1-3 Protein blot showing production of aG1 and aG2 enzymes following recloning with the Trx tag and induction with IPTG (0.3 mM) for 1-3 h at room temperature. Increased amounts of soluble aG2 were obtained however aG1 appeared to be degraded into smaller fragments.**

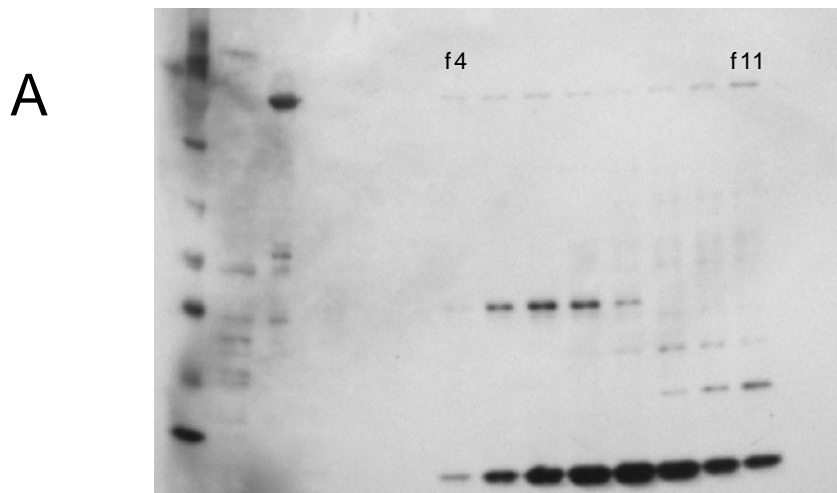
Larger scale expression of enzyme aG2 in the Trx vector was then tested in two different cell lines (Figure A1-4) to determine the optimum conditions. Prolonged expression resulted in degradation of the protein, suggesting that protease inhibitors would be required. A large-scale production and purification was carried out by the UQ Protein Expression Facility (PEF) using vectors and conditions described here.



**Figure A1-4 Western blot using Trx monoclonal antibody on soluble (left) and insoluble (right) fractions following expression of vector aG2 pET32a induced with 0.3 mM IPTG for 3 h at room temperature. The protein has a predicted molecular weight of 115.7 kDa and predicted pI of 5.77.**

Fractions from the large scale purification performed by PEF were separated on a SDS-polyacrylamide gel transferred to a PVDF membrane and labelled with the Trx antibody. The results (Figure A1-5A) showed that the aG2-Trx fusion protein was recovered in fractions #4-11, however, a large amount of low molecular weight degradation product was also detected. The activity of fractions #6-11 was tested with the artificial substrate, nitrophenyl-6Glc, which contains a short chain of 6 glucose units. Fractions #8-10 were active against this substrate (Figure A1-5B). The activity of fraction #9 was shown to increase in the presence of divalent cations (Figure A1-5C), which is characteristic of glucosidases. Yeast glucosidase was used as a control in these experiments. Unfortunately the enzyme appeared to be very unstable, as activity was abolished when trying to remove imidazole by dialysis.

Incubation of the enzyme with sucrose and maltose suggested that there was no activity with these substrates, as no glucosyl-sucrose could be detected by HPLC. However, the enzyme was shown to be active against maltotriose. The results suggest that this enzyme is indeed a glucosidase, but that it has specificity for longer chain glucosides such as malto-oligosaccharides and has little activity against sucrose in its present form. As the enzyme was not active on sucrose and was unstable no further expression & purification was carried out.



**B**

A405	0.008	0.067	0.091	0.09	0.084	0.074
(blank subtracted)						
fraction	6	7	8	9	10	11

**C**

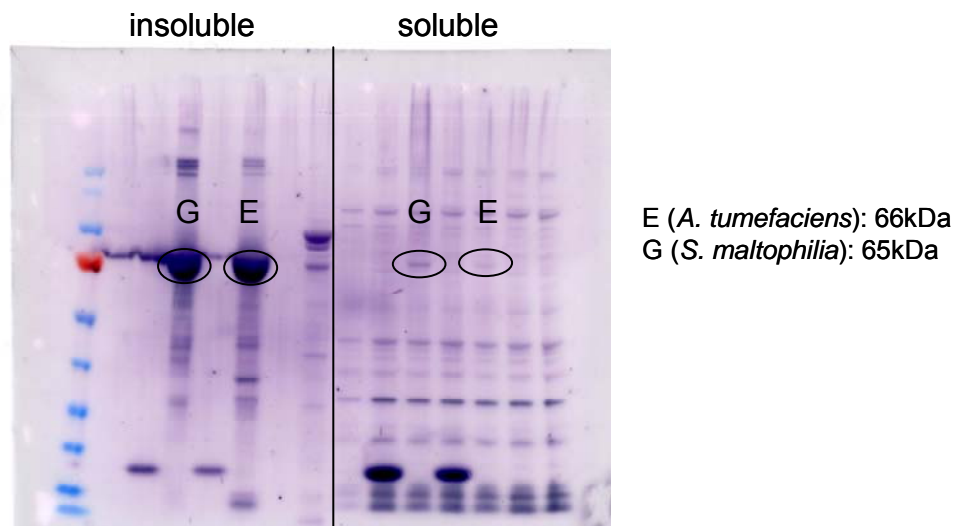
	room temperature			37 C incubation			
yeast glc	f9+DTT	f9+Mn2+	f9+Mg2+	yeast glc	f9+DTT	f9+Mn2+	f9+Mg2+
1.614	0.02	0.028	0.039	1.595	0.044	0.086	0.064

**Figure A1-5 Purification and assay of glucosidase. (A) Western blot using Trx monoclonal antibody on fractions from affinity purification of aG2-Trx protein showed recovery of enzyme in fractions 4-11. (B) Activity of fractions against the substrate nitrophenyl-6xGlc, showed highest activity in fractions 8-10. (C) Activity of fraction #9 increased in the presence of the divalent cations Mn<sup>+</sup> and Mg<sup>+</sup>.**

### APPENDIX 3 - ENZYMATIC SYNTHESIS OF KETOSUCROSE

Ketosucrose has previously been detected in the bacterium *Agrobacterium tumefaciens* and is thought to be synthesised from sucrose by the enzyme, glucoside-3-dehydrogenase (G3DH). When the sequence of the *Agrobacterium* genome was published it was not possible to identify the gene encoding G3DH due to a lack of well-defined homologues in other species. Since then, the G3DH gene has been identified in a number of bacterial species including *Halomonas* and *Gramella*. We used these sequences to identify the homologous gene in *Agrobacterium tumefaciens* and *Stenotrophomonas maltophilia*. The *A. tumefaciens* and *S. maltophilia* G3DH genes were synthesised and subcloned into a protein expression vector. The vector system chosen utilises the T7 promoter with a 6 x His tag to produce high level expression of the protein of interest in *E. coli*. The tag allows purification on a nickel column and identification of the enzyme by western blots using a Ni-NTA-alkaline phosphatase detection system.

The gene was initially expressed in *E. coli* strain BL21(DE3). Analysis of the proteins in both the soluble and insoluble fractions on SDS-polyacrylamide gels indicated that the protein was being produced but not folded correctly, resulting in the production of insoluble inclusion bodies. This is a common problem in protein expression studies and a variety of strategies have been used by other researchers to overcome the problem. Growing the cultures under different temperatures or with different conditions for induction was tested but did not improve solubility of the protein. We also tested expression of the plasmid in a range of host strains containing various chaperones (plasmid set from Takara Ltd.) to assist with folding but this was not successful. Advice from Dr Ulrike Kappler suggested that folding may be assisted by secretion of the protein into the periplasmic space, so the G3DH genes were re-cloned into an expression vector containing a periplasmic export signal (plasmid pET22b). This strategy resulted in the best recovery of soluble protein, however significant amounts remained in inclusion bodies in the insoluble fraction (Figure A2-1).

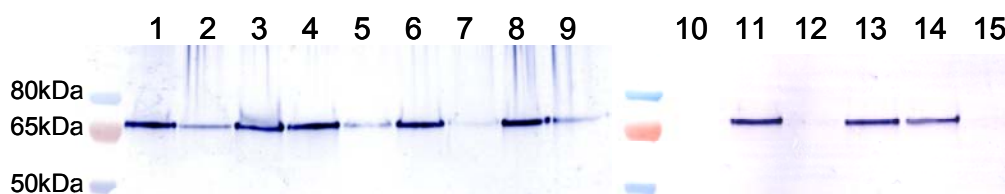


**Figure A2-1** Analysis of glucoside-3-dehydrogenase (G3DH) enzyme produced in *E. coli* cultures that express the gene from *A. tumefaciens* (E) or *S. maltophilia* (G). Expression was induced by adding IPTG (0.3 mM) for 2 h at room temperature. Bands at the correct molecular weight are circled. Some soluble protein was observed following recloning of the G3DH genes into an expression vector



containing a periplasmic export signal, however large amounts were present in the insoluble fraction.

In order to obtain larger amounts of soluble protein, inclusion bodies were purified and refolding conditions were tested using a kit from Athena Enzyme Systems (QuickFold™). The system provides 15 combinations of refolding reagents in a factorial matrix design to identify key buffer components resulting in soluble protein. The FAD cofactor (20 μM) was also included in all buffers. The amount of soluble protein recovered from each buffer combination was analysed by SDS-PAGE, transferred to PDVF membrane followed by detection with the Ni-conjugate. The results showed that several of the buffers allowed the protein to re-fold and remain in the soluble fraction (Figure A2-2).



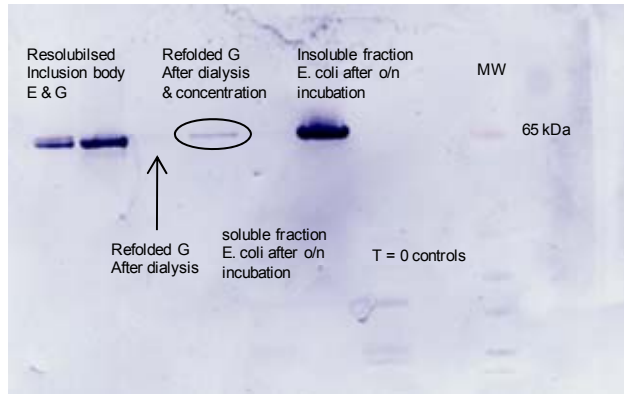
**Figure A2-2 Protein blot of soluble enzyme recovered by dialysis after refolding tests in 15 buffer combinations. In each lane, a blue band at 65 kDa molecular weight indicates that refolding was successful in this buffer.**

A further test was carried out on the refolded enzyme samples to test whether the enzymes retained dehydrogenase activity. This assay used an electron acceptor substrate (DCPIP) as an analogue for the native sugar substrate. Activity was detected as a decrease in absorbance at 595 nm, corresponding to electron transfer activity of the recombinant protein (Figure A2-3). This experiment demonstrated that our predictions of the enzyme action were correct and that the enzyme had been successfully cloned and expressed.

buffer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
$\Delta\text{Abs}_{(595)}$	0.10	0.17	0.21	0.17	0.21	0.20	0.06	0.02	0.00	0.00	0.03	0.06	0.04	0.02	0.00

**Figure A2-3 Results of an activity assay for enzyme recovered from each of 15 refolding buffer combinations. Dehydrogenase activity is expressed as change in absorbance at 595 nm. Buffers 2 to 6 produced soluble enzyme which retained dehydrogenase activity.**

Refolding using buffers #2 to #6 was then tested with a larger scale sample of protein. After dialysis and concentration of the refolded sample, the protein was successfully recovered from the soluble fraction (Figure A2-4). Electron acceptor assays confirmed that the protein retained the ability to transfer electrons. Although it was not possible to obtain a sample of ketosucrose as a standard, methods were available to assay for ketosucrose by HPLC, thin layer chromatography and colorimetric determination in the presence of NaOH. However, when the enzyme was incubated with sucrose under a variety of conditions, no ketosucrose could be detected in the reaction products. Since the native enzyme is found in the periplasm of *Agrobacterium*, it is possible that it requires additional proteins for activity against sucrose. Future work may be able to identify the accessory proteins and complete the synthesis of ketosucrose.



**Figure A2-4 Protein blot of soluble enzyme recovered after refolding**

## APPENDIX 4

### Fruit fly bioassay to distinguish ‘sweet’ sugar structures

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Keywords: carbohydrate, behavior, *Drosophila*, glucobiose, sucrose, glucose

#### **Abstract**

Palatable response to dietary sugars plays a significant role in influencing metabolic health. New structures are being explored with beneficial health properties, although consumer acceptance relies heavily on desirable sensory properties. Despite the importance of behavioral responses, the ability to elucidate structure-preference relationships of sugars is lacking. Using a wild population of *Drosophila melanogaster* as a model, we performed pair-wise comparisons across structural groups to characterize a fruit fly bioassay to determine sugar palatability. Preference was successfully described in structurally relevant terms, particularly through the ability to test sugars of related structures directly, in addition to standard sucrose comparisons. The fruit fly bioassay also provided the first report on the palatability of gentiobitol and in making reference to known human preferences also raises opportunities for greater understanding of behavioral response to sugars generally.

## **Introduction**

With a foundation dating back centuries, and once reserved for the privileged, sweet tasting carbohydrates (predominantly sucrose) are extensively added to many modern food products. As concerns grow about their health implications current efforts are focussed on developing alternative sugars with the proviso that suitable properties, including desirable taste, are maintained. Due to the complexities of performing human sensory trials, published data on structure-taste relationships is fragmentary, not well supported by experimental evidence or quoted in product information without reference. With a host of new carbohydrate products being developed and entering the market in recent years, an ability to assess define structures with favourable response is of interest.

The utility of the fruit fly as a model for research in chemosensory behavior is well accepted, with a degree of similarity between flies and mammals in the way palatable responses are invoked {Scott, 2005 #370}. A recent report has also demonstrated similarity in the response of *Drosophila melanogaster* (fruit fly) to high intensity sweeteners recognised by humans {Gordesky-Gold, 2008 #195}, extending on earlier work using *Phormia regina* (blow fly){Ahamed, 2001 #206}. This is particularly intriguing considering the variation in response displayed for these compounds in more closely related mammals, such as new world monkeys {Glaser, 2002 #368; Laska, 1998 #343}. With this is mind, the current study characterized structural groups of sugars in a fruit fly bioassay by comparing preferences to known ‘sweet’ sugars. The format also proved useful in targeting linkages responsible for heightened palatability through multiple comparisons with di- and trisaccharides containing related glucosidic linkages.

## **Materials & Methods**

### **Materials**

The di- and trisaccharides, kojibiose, nigerose, isomaltose, sophorose, gentiobiose, leucrose, and panose were sourced from Carbosynth (Berkshire, UK), while erlose, maltotriose and maltotriitol were supplied by Hayashibara Biochemical Laboratories (Okayama, Japan). Laminaribiose was purchased from Megazyme (Co. Wicklow, Ireland) and all remaining carbohydrates were purchased from Sigma-Aldrich (St. Louis, MO). A 200 mM stock solution of each carbohydrate was prepared in distilled water (dH<sub>2</sub>O) and stored in aliquots at -20 °C.

### **Synthesis of gentiobiitol**

Synthesis of gentiobiitol via the reduction of gentiobiose was performed by Epichem Ltd (Murdoch, Australia) and was based on a modification to the method of Abdel-Akher et al. {Abdel-Akher, 1951 #367} Briefly, 4.8 g of gentiobiose was dissolved in 50 mL of dH<sub>2</sub>O and combined with 1.0 g of sodium borohydride in 25 mL dH<sub>2</sub>O. The reaction was allowed to proceed at room temperature for 4 h and quenched with acetic acid after confirming the reaction to be complete by thin layer chromatography. The solution was then concentrated and the product precipitated using methanol. Further purification, deacetylation and concentration were carried out to yield a final solution of 0.70 M gentiobiitol in dH<sub>2</sub>O (4.05 g, 99 % purity).

### **Collection and maintenance of *Drosophila melanogaster* populations**

A new population of wild-type flies was established from 10 female *Drosophila melanogaster* captured on the University of Queensland campus between 27<sup>th</sup> February 2009 and 6<sup>th</sup> March 2009. Traps consisted of empty 1 mL pipette tip boxes baited with mashed banana and sprinkled with live yeast {Loeschcke, 2007 #358}. Standard 1 mL pipette tips

with the ends cut off were inserted to create a one-way entrance to the trap. Traps were deployed in the field for 24 h surrounded by Tanglefoot<sup>®</sup> insect barrier (The Tanglefoot Co. Grand Rapids, MI) to prevent ants and other crawling insects from entering. Traps were inspected for flies and females were transferred to separate vials containing standard corn meal nutrient medium. The individual females were monitored until their offspring eclosed and their sons could be identified. Identification of *Drosophila melanogaster* males was carried out by examining the sex combs, as *D. melanogaster* show distinct differences to other commonly found *Drosophila* species. Laboratory stocks of *Oregon-R* were used in pilot experiments. All flies were reared in 250 mL bottles at 25 °C on a 12:12 h light:dark regime on standard corn meal medium.

### **Two-choice behavioral assay**

Assays were carried out using a 96-well plate with a layer of Parafilm<sup>®</sup> stretched over it to keep the food and flies on the surface and out of the wells. Test sugars were mixed with either brilliant blue (25 mg mL<sup>-1</sup>) or erythrosine (90 mg mL<sup>-1</sup>) (New Directions; Marrickville, Australia) in 0.5 % agarose. Initially, plates were replicated with the colours inverted for each sugar to test for colour bias, but after 12 trials this approach was not continued as it was determined that colour had no effect on preference as had been reported previously {Thorne, 2004 #359}.

A minimum of 50, < 5 day-old flies were starved for 24 h on filter paper soaked in dH<sub>2</sub>O for each experiment {Thorne, 2004 #359}{Al-Anzi, 2006 #208}{Dahanukar, 2001 #360}{Dahanukar, 2007 #283}{Gordesky-Gold, 2008 #195}{Ueno, 2008 #361}. Both males and females were used as it has been reported that sex does not affect feeding preference {Dahanukar, 2001 #360}. All feeding experiments were carried out in the morning as circadian rhythm can affect feeding behavior {Meunier, 2007 #362}{Xu, 2008

#363}. Flies were allowed to feed for 2 h in the dark before being frozen for 48 h. Scoring of abdomen color was carried out visually with a dissecting microscope (Olympus SZ51, Center Valley, PA/ Zeiss Stemi 2000, Thornwood, NY) and the flies were grouped into the following categories: red (R), blue (B), purple (P) and none. Red and blue flies were distinct whereas the purple flies varied in shade depending on the quantity of sugar eaten. Preference Index (PI) was calculated as the number of red or blue flies + ½ number of purple flies divided by the total number of flies that fed;  $PI = R \text{ or } B + \frac{1}{2} P / (R + B + P)$ . Tests were only included in PI calculations if more than 20 % of the flies were feeding {Dahanukar, 2001 #360}{Gordesky-Gold, 2008 #195}. When using the wild-type population, feeding rate < 20% were not observed and rates were commonly > 50 %. Each paired comparison was replicated at least 3 times with separate plates and flies.

## **Results & Discussion**

*D. melanogaster* were exposed to traditional sugars, including glucose and sucrose, and a range of potential or current alternative sweeteners. Fruit fly sugar preferences were determined using two-choice behavioral assays based on the method of Tanimura et al. {Tanimura, 1982 #344} Initial optimization was performed to confirm that food dyes did not influence preference and to establish reproducible conditions for fruit fly behavior. In addition to confirming earlier studies, including the importance of ageing {Nestel, 1985 #375; Nigg, 1995 #374}, we found that a consistent time of day for starving and feeding improved reproducibility of assays. We suggest this improvement is likely due to the effect of circadian rhythms on feeding behaviour {Meunier, 2007 #362}{Xu, 2008 #363}.

A fruit fly line newly established from a wild population was employed for data on carbohydrate structures as it was generally observed to be more consistent in its behavioural response, displaying higher feeding rates throughout the experiments than inbred laboratory

lines. Preference index (PI) was calculated to measure the palatability of one carbohydrate in relation to another, determined from the feeding experiments following scoring of abdomen colour. PI for each carbohydrate choice was a proportion of 1.0, with a value of 0.50 equating to an equal preference of the two sugars being tested. This approach enabled gustatory responses to sugars to be reported in a defined and sensitive manner. The carbohydrate structures used in the study (**Table 1**) were selected to represent broad structural groups and target specific monosaccharide linkages through selection of related di- and trisaccharides. Tests were performed with equimolar (4 mM) solutions, including standard comparisons paired with either sucrose or glucose.

The first comparisons focussed on defining the preferences of the fruit fly, relative to commonly held views of human carbohydrate preference. A comparison of four  $\alpha$ -glucobioses along with their corresponding  $\beta$ -glucobiose were initially all tested against equimolar sucrose (**Figure 1a**), confirming the general preference for alpha structures over beta known in humans{Pangborn, 1966 #345}. While the  $\alpha$ -glucobiose samples were more preferred than their corresponding  $\beta$ -glucobiose in each case, significant consumption of the beta sugars did occur. This was particularly unexpected with the  $\beta$ -1,6 glucobiose (gentiobiose) which has been reported to have a 'bitter' taste in humans{Birch, 1970 #347}{Cote, 2009 #348}{Pangborn, 1961 #346}. A similar comparison was therefore conducted by directly pairing the alpha and beta carbohydrates as the two opposing choices (**Figure 1b**). Differences were more pronounced using a direct approach, with the preference for  $\alpha$ - over  $\beta$ -glucobioses confirmed and all statistically significant. The greatest difference in PI occurred with the  $\alpha$ -1,4 and  $\beta$ -1,4 samples (maltose and cellobiose respectively), suggesting a possible strong palatable response by *Drosophila* towards  $\alpha$ -1,4 carbohydrates in general. Interestingly, the difference between the  $\alpha$ -1,6 (isomaltose) and  $\beta$ -1,6 (gentiobiose) sugars remained small. This limited difference between gentiobiose and the

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related  $\alpha$ -linked structure may suggest a somewhat elevated preference for gentiobiose relative to other  $\beta$ -linked structures. The plausibility of this may be strengthened by the presence of  $\beta$ -1,6 glucans in yeast cell walls, an extract of which is included in the artificial diet of the fruit fly and found as part of their natural diet. Similarly, with such a pairwise test this may also indicate a reduced preference for isomaltose, or more specifically  $\alpha$ -1,6 linked glucose.

To characterise our model system further, we calculated preferences for a second structural group of sugars, the sucrose isomers. Sucrose and three isomers were compared to either sucrose or glucose (again equimolar). In these assays (**Figure 1c**) turanose was a more strongly preferred isomer than leucrose or palatinose. A previous study detecting responses of sugar-sensing neurons in fruit fly has reported similar findings, with sucrose and turanose displaying greater responses over leucrose and palatinose {Dahanukar, 2007 #283}. These preferences correlate with those suggested for humans {Godshall, 2007 #356}{Shibuya, 2004 #357}, although turanose is poorly studied in humans and suggested to be approximately half as 'sweet' as sucrose. *D. melanogaster* could be seen to have an elevated response to turanose, which may be a consequence of the greater natural abundance over the two other isomers tested, since turanose is present at higher levels in nectar and honey{Burgin, 1997 #349}{de la Fuente, 2007 #350}. In broad terms, these experiments reinforced the utility of the fruit fly behavioral assay relative to human preferences, as glucose comparisons resulted in consistently greater PI for the opposing carbohydrate than sucrose for all samples. Similarly glucose is generally accepted as being approximately 75 % as 'sweet' as sucrose in humans. Furthermore, the control comparing sucrose to itself in **Figure 1C** resulted in a PI close to 0.5 as expected.

Having characterized a range of linkages with generally accepted information on palatability, we then applied the assay to a compound with no previous data. The ability to increase preference for  $\alpha$ -linked disaccharides following conversion to a sugar alcohol is known; with disaccharide alcohols such as maltitol now commonly used as alternative sweeteners with desirable dental health properties. The effect of reduction on  $\beta$ -linked structures is less studied. Conversion of the  $\beta$ -glucobiose, cellobiose, into a sugar alcohol has been shown to decrease 'sweetness' in one study with human subjects {Kearsley, 1980 #351}. In the present study gentiobiitol, the reduced product of the  $\beta$ -1,6 linked glucobiose (gentiobiose), was assessed to determine any changes to preference. Gentiobiitol has recently been identified in transgenic sugarcane plants engineered to produce sorbitol {Chong, #365} and the potential applications of this novel sugar as an alternative sweetener is of interest. A comparison to equimolar sucrose was first performed (**Figure 2a**), and gentiobiitol was shown to be somewhat palatable with a PI of approximately 0.30, though not significantly more than gentiobiose compared to sucrose in the previous data (**Figure 1a**). Further comparisons were conducted with glucose and maltitol, which revealed preferences close to 0.50 or similar palatability to these known sweeteners. Glucose and maltitol have similar levels of 'sweet' taste in humans as seen with the fruit fly data and further testing of the  $\beta$ -linked sugar alcohol gentiobiitol is of interest. The comparison of gentiobiitol with gentiobiose was also conducted as it has been shown that comparing similar structures directly, resolves preferences between structures more clearly than indirect comparisons of both structures to sucrose for example. In this instance, although not statistically significant (at  $p < 0.05$ ), the final comparison in **Figure 2a** does suggest preference for the sugar alcohol over the related  $\beta$ -glucobiose. A similar comparison of the sugar alcohol sweetener maltitol was conducted along with the gentiobiitol data (**Figure 2b**), pairing sucrose, glucose, maltotriitol or maltose in feeding assays. Relative to sucrose, maltitol was reported to have a PI of  $0.44 (\pm 0.02)$ , and with glucose a PI of  $(0.62 \pm 0.03)$ . So while these comparisons are somewhat higher relative

to those of gentiobitol and may seem to contradict the result between maltitol and gentiobitol directly in **Figure 2a**, we are attempting to differentiate changes in PI of  $\sim 0.1$  which does seem to be at the limits of the bioassay. We do again see that results between the sucrose and glucose comparisons are greater with the less 'sweet' compound glucose, further indicative of consistency between assays.

Final behavioral experiments in **Figure 3** were performed firstly to investigate the effect of chain length through analysis of trisaccharide structures. Trisaccharides displayed elevated preferences when compared to sucrose, which is where variation to the accepted carbohydrate preferences of humans can be seen. Stronger preference towards erlose and melezitose (**Figure 3a**) over sucrose would not be expected in humans, as it is generally accepted that increased chain length decreases palatability, although further opportunity exists here for more detailed human studies also. For the fruit fly, however, this is not unexpected because of the greater likelihood of being encountered in their natural diet. Melezitose and erlose are present in honeydew for example, and have been described as a food source for other fruit fly species {Christenson, 1960 #364} {Hendrichs, 1993 #373} {Hogervorst, 2007 #352}. Additionally, the strong preference for these trisaccharides has been reported in other insects {Tinti, 2001 #353} {Glaser, 2002 #368}. In **Figure 3b** maltotriose exhibited a discerning preference over maltose, with increased chain length of an additional  $\alpha$ -1,4 glucose invoking an elevated response in *D. melanogaster*. This provides a further example of the strength of the bioassay in comparing related structures to define preferences structurally, as separate comparisons of maltotriose or maltose to sucrose (**Figure 3b** and **1a** respectively) showing minimal difference.

Preference for starch related linkages i.e. those containing  $\alpha$ -1,4 or  $\alpha$ -1,6 glucose, were able to be explored in the trisaccharide comparisons also, and highlighted because of their dietary

significance. Panose, which contains both an  $\alpha$ -1,4 and  $\alpha$ -1,6 linkage, showed higher preference over glucose although a markedly low PI when compared to maltose (**Figure 3a**). This was consistent with the suggestion of a reduced preference for isomaltose ( $\alpha$ -1,6 glucobiose) mentioned earlier. A separate assessment of maltotriose preference revealed a high PI ( $0.90 \pm 0.02$ ) over panose (**Figure 3b**), and further evidence for the preference of  $\alpha$ -1,4 glucose over  $\alpha$ -1,6 by *D. melanogaster*. This preference for  $\alpha$ -1,4 glucose also dominates response towards sugar alcohols, with maltotriose showing high PI over both maltitol and maltotriitol (**Figure 3b**). Similarly, in an earlier comparison maltitol demonstrated a significantly lower PI when paired with maltose (**Figure 2a**). So while this deviates from the increased response of humans to maltitol over maltose, it is consistent with the strong response invoked by  $\alpha$ -1,4 glucose with fruit fly. In terms of human preference for starch-related structures, we are not aware of any extensive studies to define the relative preference of  $\alpha$ -1,4 glucose over  $\alpha$ -1,6, though it has been reported that soluble starch can enhance sucrose ‘sweetness’ in humans {Kanemaru, 2002 #355}. So whilst human taste may be influenced by starch related carbohydrates, they directly invoke strong appetitive behavior in *Drosophila* with an ability to discriminate  $\alpha$ -1,4 over  $\alpha$ -1,6 glucose.

In summary, we have demonstrated the strength of a behavioral assay using the model organism *Drosophila melanogaster* that while seemingly simple in its execution, is reproducible, sensitive and can reveal specific structure-preference relationships of carbohydrates not easily obtained with humans. We extend work on the similarities between the fruit fly and human responses (summarised in **Table 2**) and note the strength in the similarity between species for disaccharides, with deviation reported in the response towards trisaccharides (particularly those containing  $\alpha$ -1,4 glucose). Opportunities for improved understanding of the response of specific carbohydrate structures in humans have been highlighted, including the sucrose isomer turanose, and starch related glucosidic linkages.

The latter may be particularly of interest because of the health benefits of altered starch structures for example, and sensory evaluation is being performed after incorporation into common food products {Baixauli, 2008 #371; Sanz, 2009 #372}. Finally, results suggest a sugar alcohol produced from a  $\beta$ -glucobiose may prove palatable and consider it of interest to examine the human response to gentiobiitol, particularly alongside gentiobiose.

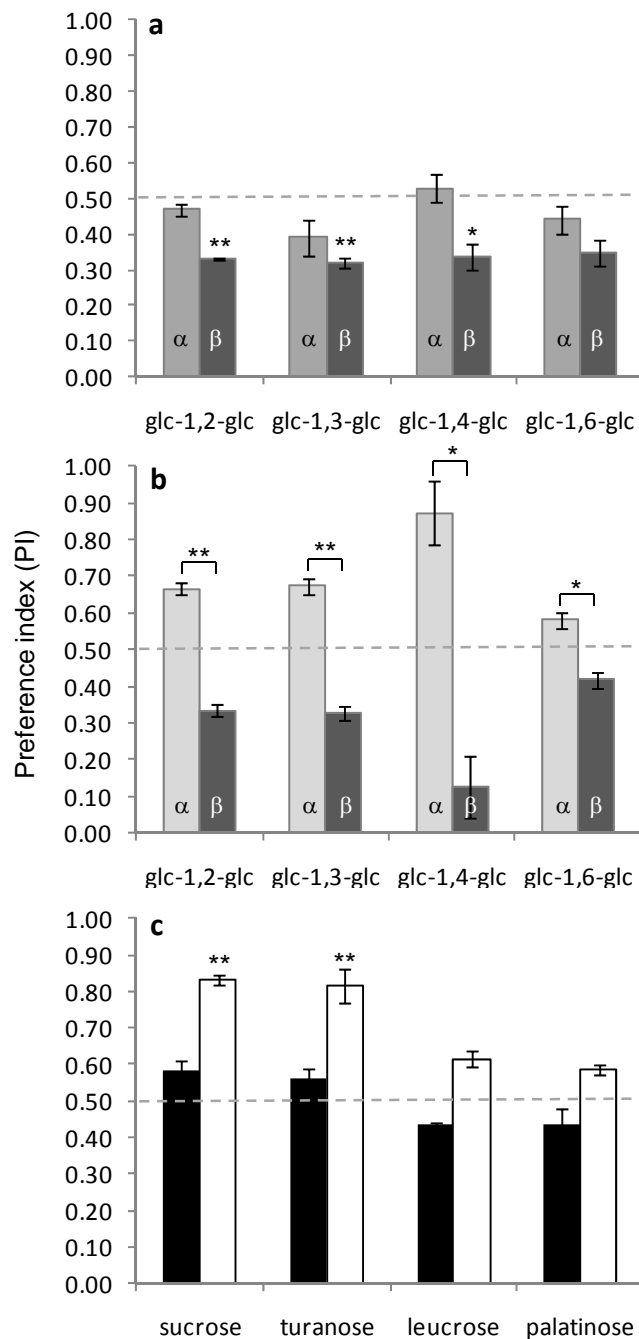
### **Acknowledgements**

We acknowledge the support of the Cooperative Research Centre for Sugar Industry Innovation through Biotechnology for project funding and thank Dr Rosanne Casu and Dr Graham Bonnett for their comments on the manuscript.

### **References**

**Table 1 : Structural groups of carbohydrates used in preference assays.** Rationalization of carbohydrate groups aided structure-preference analyses. All structures are alpha-linked except the group of beta-glucobioses as shown, and the reduced structure derived from the  $\beta$ -1,6 member of this group known as gentiobiitol. Monosaccharides are abbreviated to glc = glucose, and fru = fructose.

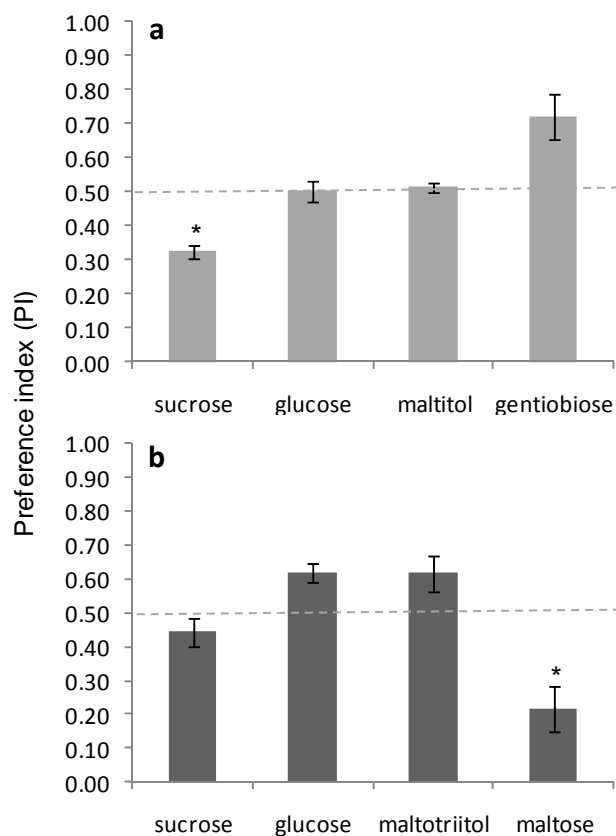
<b><math>\alpha</math>-glucobioses</b>	
kojibiose	glc-1,2-glc
nigerose	glc-1,3-glc
maltose	glc-1,4-glc
isomaltose	glc-1,6-glc
<b><math>\beta</math>-glucobioses</b>	
sophorose	glc-1,2-glc
laminaribiose	glc-1,3-glc
cellobiose	glc-1,4-glc
gentiobiose	glc-1,6-glc
<b>sucrose isomers</b>	
sucrose	glc-1,2-fru
turanose	glc-1,3-fru
leucrose	glc-1,5-fru
palatinose	glc-1,6-fru
<b>sugar alcohols</b>	
maltitol	reduced maltose
gentiobiitol	reduced gentiobiose
maltotriitol	reduced maltotriose
<b>trisaccharides</b>	
melezitose	glc-1,2-fru-1,3-glc
erlose	glc-1,4-glc-1,2-fru
panose	glc-1,6-glc-1,4-glc
maltotriose	glc-1,4-glc-1,4-glc



**Figure 1: Characterizing sugar preferences of *Drosophila melanogaster*.** Pairwise preference assays were performed with < 5 day old fruit flies by introducing them to a 96-well plate containing two equimolar sugar samples with either a red or blue food dye. After feeding, scoring of the abdomen color enabled calculation of the preference index (PI). A PI > 0.5 indicates elevated preference towards the indicated test sugar. Preference of  $\alpha$ - over  $\beta$ -glucobioses was examined by comparing all 8 samples to sucrose (**a**) as well as by directly comparing the alpha and beta structures to one another (**b**). The plots in **b**, which show PI for

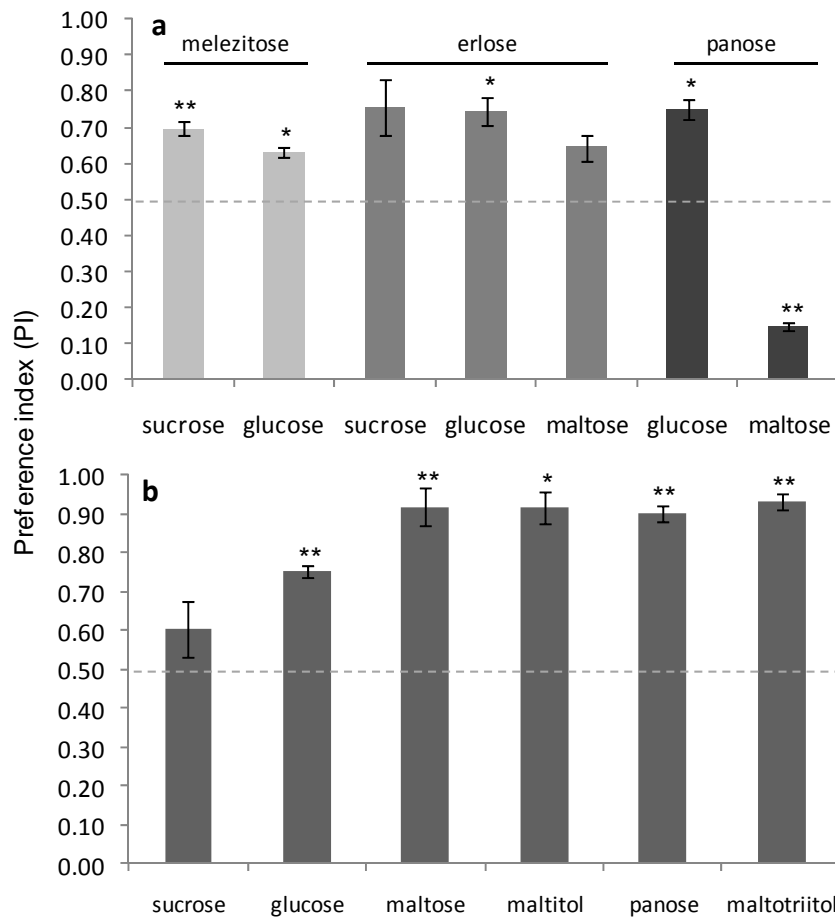
both sugars, more clearly reveals the preference for  $\alpha$ -glucobiose carbohydrates than when separately compared to sucrose. Further characterization of preference for a structural group was performed by examining sucrose isomers (c). PI was calculated relative to both sucrose (black bars) and glucose (white bars). A similar response towards the isomer turanose as sucrose was seen, while leucrose and palatinose were less preferred. The general observation of an increased response to all test sugars when compared to glucose than sucrose, confirms the elevated response of *D. melanogaster* towards sucrose over glucose that is consistent with humans. The control of sucrose with itself shows a PI close to 0.5 as expected also. Errors shown are  $\pm$  SEM, (n = 3 – 6). Paired t-test performed with \* corresponding to  $p < 0.05$  and \*\*  $p < 0.01$ .





**Figure 2 : Preference of novel compound using fruit fly bioassay. (a)** Preference of the sugar alcohol gentiobitol was determined relative to known ‘sweet’ compounds, including sucrose, glucose and maltitol, as well as gentiobiose from which it was prepared. While less preferred than sucrose, a similar PI to equimolar glucose and maltitol was observed. An increased preference relative to gentiobiose is suggestive of an improved palatable response to  $\alpha$ -linked structures through conversion to a sugar alcohol, though further testing is required. **(b)** Further assays with the known sugar alcohol maltitol enabled further, albeit indirect, comparisons for gentiobitol. The sucrose and glucose comparisons revealed only slightly elevated PI for maltitol than the same result for gentiobitol, though discerning a difference in PI of approximately 0.1 seems at the limits of the assay and may be seen to confirm the similarity of gentiobitol and maltitol. Unlike gentiobitol, maltitol is less preferred than the structure from which it is derived, with maltitol expected to be more ‘sweet’ in humans. This can be seen as consistent for the fruit fly, however, because of their high palatable response towards  $\alpha$ -1,4 glucose than seen in humans. Interestingly, the effect

of an additional -1,4 glucose seems minimal with the presence of a sugar alcohol, though, as PI for maltitol did not vary significantly when compared to maltotriitol. Errors shown are  $\pm$  SEM, (n = 3 – 5). Paired t-test with \* corresponding to  $p < 0.05$  and \*\*  $p < 0.01$ .



**Figure 3 : Preference trisaccharides with an emphasis on starch-related structures. (a)**

The PI was determined for the trisaccharide indicated at the top of the bars, compared to glucose as well as sucrose and / or maltose. In general trisaccharides showed higher preference over glucose and constituent disaccharides which is consistent with other insect studies, though increased chain length is suggested to reduce human response. A distinct reduction in PI with panose when paired with maltose provides further evidence of lower palatability towards  $\alpha$ -1,6 over  $\alpha$ -1,4 glucose structures. **(b)** Maltotriose shows elevated PI when paired with a variety of structures, including sugar alcohols. Again the strong response to  $\alpha$ -1,4 over  $\alpha$ -1,6 glucose when assessed against panose is observed. Additionally, increased chain length is favourable in the case of the high PI for maltotriose when compared to maltose. Indirect comparisons looking across comparisons with sucrose is unable to discern such a clear difference as this direct comparison. Errors shown are  $\pm$  SEM (n = 3 - 4). Paired t-test with \* corresponding to  $p < 0.05$  and \*\*  $p < 0.01$ .

**Table 2 : Summary of carbohydrate preferences by fruit fly relative to human responses.** Results from two-choice behavioral assays performed with *Drosophila melanogaster* are summarised with respect to structure-preference relationships. Comparisons to human preferences avoid the complexities associated with quoting relative values, rather providing a summary of accepted views and highlighting gaps in knowledge.

<b>Fruit fly</b>	<b>Human</b>
$\alpha$ -glucobiose > $\beta$ -glucobiose	$\alpha$ -glucobiose > $\beta$ -glucobiose
sucrose > glucose	sucrose > glucose
sucrose, turanose > leucrose, palatinose	sucrose > turanose, leucrose, palatinose
maltose > maltitol	maltitol > maltose
gentiobiitol $\cong$ maltitol $\cong$ glucose	maltitol $\cong$ glucose, gentiobiitol unknown
$\alpha$ -1,4 glucose > $\alpha$ -1,6 glucose	any difference unknown

## APPENDIX 5

# Oral and intestinal digestion of carbohydrates in structurally relevant terms

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**Keywords:** Sugar, glucoside, glucobiose, Streptococcus,  $\alpha$ -glucosidase

### Abstract

The impacts of dietary carbohydrates on human health are well recognized, representing both the need to minimize adverse effects (through high caloric and cariogenic sugars) and opportunity for improvements in wellbeing (with prebiotics for example). With the increasing market presence of alternative carbohydrate products aiming to address a range of modern health issues, a comparative study of oral and intestinal digestion across structural groups was conducted. Use of *in vitro* oral and intestinal  $\alpha$ -glucosidase assays provided comparison of various glucosidic linkages and chain length. Fermentation of carbohydrates by *Streptococcus mutans* highlighted the diversity of structures utilized by the oral bacterium, though  $\alpha$ -1,2,  $\alpha$ -1,3 and  $\beta$ -1,6 glucobioses were additional structures

to sugar alcohols and sucrose isomers found not to promote formation of organic acids. A mammalian glucosidase assay defined the relative digestibility of sucrose isomers, and the effect of variable linkages among  $\alpha$ -glucobiose substrates, with  $\alpha$ -1,4 >  $\alpha$ -1,3 >  $\alpha$ -1,2 >  $\alpha$ -1,6. Investigation of starch-related trisaccharides by anion-exchange chromatography revealed the reduction in glucose release observed for maltotriose, erlose and maltotriitol resulted from feedback inhibition of digestion products. Further comparisons of biological interactions among varying carbohydrate structures are likely to be informative in the design of functional food products.

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### QUOTATION N°. 081030-CSIRO

<i>Item</i>	<i>Structure</i>	<i>Quantity</i>	<i>Time</i>	<i>Cost (ex GST)</i>
1		All product derived from 1g Gentiobiose  All product derived from 5g Gentiobiose	1-2 weeks	\$4,000  \$6,000

**Notes:**

1. The product to be supplied >95% purity.
2. Gentiobiose starting material supplied by client.

Robert Gauci  
 Head of Laboratory (WA)

30 October 2008

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Fax: (07) 3214 2920  
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### Item 2—the Material

CRC SIBB Material:  
Gene sequence in a commercially available strain of *E. coli*

#### University Material:

Item 2A: 1L large-scale production of recombinant protein

Item 2B: Purification of recombinant protein from 1L expression  
using affinity chromatography.

### Item 3—Transfer Fee

\$2150 excluding GST as follows:

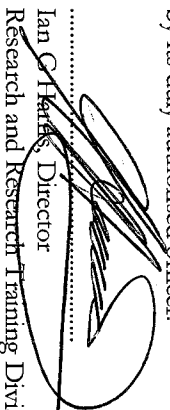
Item 3A: \$680

Item 3B: \$1470

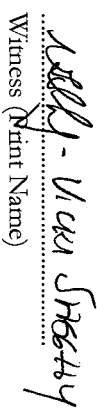
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Ian G Hanks, Director  
Research and Research Training Division

in the presence of:


  
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Witness (Print Name)

Date: 14/9/09 .....

SIGNED for and on behalf of C-SIIB MANAGEMENT PTY LTD  
by its duly authorised officer:

  
.....  
(Print Name)

in the presence of:

  
.....  
Witness (Print Name) GIDEON GOOSTEN

Date: 31 Aug '09 .....

.....  
Title )

## MATERIAL TRANSFER AGREEMENT

BETWEEN: **THE UNIVERSITY OF QUEENSLAND**, a body corporate constituted by *The University of Queensland Act 1998 (Qld)*, St Lucia, Queensland, Australia  
("the University")

AND: The entity/ies set out at item 1 of the Schedule ("CRC SIB")

### Background

- A. CRC SIBB is the owner of the CRC SIBB Material.
- B. The University is the licensee of expression and purification services under which the University will treat the CRC SIBB Material.
- C. The process of obtaining the University Material has unique value to the University, and may be the basis of applications for patents which may be jeopardised any unauthorised use.
- D. CRC SIBB wishes to be provided with a sample of the University Material for research purposes.

### The Parties Agree as Follows

1. **Meanings**
- 1.1 "Confidential Information" means all information relating to the Material including inventions; discoveries, facts, data, ideas, manner, method or process of manufacture, method or principle of construction, chemical composition or formulation, techniques, products, prototypes, processes, names, know how, routines, specifications, drawings, trade secrets, technology methods, computer programs, works in respect to which copyright subsists and other knowledge.
- 1.2 "Intellectual Property" means such of the following as relates to the Material and the Confidential Information:
- (a) an invention or discovery, manner, method or process of manufacture, method or principle of construction, chemical composition or formulation, biological material, computer program, integrated circuit, circuit layout or
- 1.3 "Material" means the "CRC SIBB Material" and the "University Material", as defined at item 3 of the Schedule.
- 1.4 "Purpose" means:
- (a) in respect of the CRC SIBB Material, the purpose of generating and providing the University Material in accordance with clause 2.2, and in respect of the University Material, the internal research purposes for which the University Material will be used by CRC SIBB, and expressly excludes any profit-making or commercial supply of the University Material to a commercial entity as well as any distribution of the University Material outside Australia.
- 1.5 "Recipient" means:
- (a) in respect of the CRC SIBB Material, the University, and
- (b) in respect of the University Material, CRC SIBB.

- 1.5 “Transfer Fee” has the meaning at item 4 of the Schedule.
- 2. Supply of Material**
- 2.1 CRC SIBB will supply to the University the CRC SIBB Material and any Confidential Information vested therein (collectively referred to as “the CRC SIBB Material” in this clause) for the Purpose.
- 2.2 The University will produce and supply to CRC SIBB the University Material and any Confidential Information and Intellectual Property vested therein (collectively referred to as “the University Material” in this clause) for the Purpose.
- 2.3 CRC SIBB will pay the University the Transfer Fee upon submission of a tax invoice by the University after supply of the University Material, as reimbursement of the University’s operating costs in generating and providing the University Material.
- 2.4 Intellectual Property in the University Material when used for the Purpose by CRC SIBB will be owned by the CRC SIBB. Intellectual Property in the protein expression and/or purification processes used by the University to generate the University Material will be either owned by or granted by licence rights to the University (as applicable). Intellectual Property in the CRC SIBB Material will remain owned by CRC SIBB.
- 2.5 Title to the physical sample of the Material provided to CRC SIBB will be vested in CRC SIBB upon payment of the Transfer Fee under clause 2.3.
- 3. Use of Material**
- 3.1 The Material is to be used by the Recipient for the Purpose, and is not to be given to a third party.
- 3.2 The Recipient acknowledges and agrees that if it wishes to use the Material for any activity outside the Purpose, then the Recipient may be required to obtain its own commercial licence from the relevant licensor of the expression and/or purification process.
- 4. No Warranty**
- 4.1 The Material being provided may have inherent defects or deficiencies, and is provided without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied.
- 4.2 To the full extent permitted by the laws of Australia or of any state or territory of Australia having jurisdiction, any conditions or warranties imposed under legislation are excluded.
- 4.3 On receiving the Material the Recipient should evaluate it prior to use to ensure it is appropriate for the Purpose. All appropriate guidelines for handling biohazards should be followed.
- 4.4 Neither party makes any representation that the use of the Material provided will not infringe the rights of any third party, including Intellectual Property rights.
- 5. Confidentiality**
- 5.1 The Recipient must keep the Material secret and confidential.
- 5.2 The Recipient must not disclose to any person or make known in any manner any part of the Material.
- 5.3 The Recipient may disclose the Material to such of its employees who need access to the Material for the Purpose and who are bound to the Recipient by obligations of confidentiality at least to the extent imposed on the Recipient by this Deed.
- 6. Publications**
- 6.1 CRC SIBB is free to publish any results derived from its use of the Material, provided:
- (a) any publications acknowledge the contribution of the University and its staff involved in the development of the Material; and

(b) a copy of the proposed publication is provided to the University for the purpose of determining whether it contains Confidential Information or Intellectual Property owned by the University.

6.2 The University may require amendment of the proposed publication if a University delay in publication would be prejudiced by publication. The parties will negotiate in good faith the extent of any amendment or period of delay.

**7. Commercial Use**

(a) If CRC SIIB wishes to use the University Material for profit making or commercial purposes, prior to such use the Parties will negotiate in good faith to establish the terms of a commercial license of any relevant Intellectual Property owned or licensed by the

University, if such rights exist. It is acknowledged by CRC SIIB that the University has no obligation to grant such a license to CRC SIIB, and may grant exclusive or non-exclusive licenses to others. For the avoidance of doubt, the commercialisation of patent rights enabled through the use of the University Material, but which do not claim the University Material as a composition of matter, is not use of the University Material for profit making or commercial purposes.

**8. Indemnity**

The Recipient agrees to indemnify the University for the Recipient's use or misuse of the Material except to the extent that the University is negligent, including for all damages, liabilities, and expenses including lawyer's fees.



## SCHEDULE

### Item 1—the Organisation

C-SIIB Management Pty Ltd  
(ABN 80 106 042 862)

#### Contact

Dr Peter Twine  
Level 5, John Hines Building, The University of Queensland  
St Lucia, QLD 4067  
Tel: +61 (0) 7 3365 7502  
Fax: +61 (0) 7 3365 4773  
[peter.twine@crsugar.com](mailto:peter.twine@crsugar.com)

### Item 2 – Delivery Address

Dr Anne L. Rae  
Care of CSIRO Plant Industry  
306 Carmody Road  
St Lucia, QLD 4067  
Tel: (07) 3214 2379  
Fax: (07) 3214 2920  
[Anne.rae@csiro.au](mailto:Anne.rae@csiro.au)

### Item 2—the Material

CRC SIIB Material:  
Gene sequence in a commercially available strain of *E. coli*

University Material:

Item 2A: 1L large-scale production of recombinant protein

Item 2B: Purification of recombinant protein from 1L expression  
using affinity chromatography.

### Item 3—Transfer Fee

\$2150 excluding GST as follows:

Item 3A: \$680

Item 3B: \$1470

EXECUTED AS AN AGREEMENT

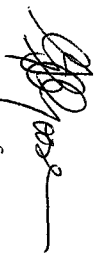
SIGNED for and on behalf of THE UNIVERSITY OF QUEENSLAND by its duly authorised officer:

in the presence of:

.....  
Ian G Harris, Director  
Research and Research Training Division

.....  
Witness (Print Name)  
.....  
Date:

SIGNED for and on behalf of C-SIIB MANAGEMENT PTY LTD by its duly authorised officer:

.....  
in the presence of:  
  
.....

.....  
(Print Name) *PT Harris*  
.....  
*CEO*  
.....  
Title )

.....  
Witness (Print Name) *GIDEON GOOSEN*  
.....  
Date: *28/8/09*  
.....



## Services Order

The New Zealand Institute for Plant and Food Research Limited a Crown Research Institute established under the Crown Research Institutes Act 1992 ("PFR")

CSIRO Plant Industry  
("Client")

<b>PFR Contact:</b>	Doug Rosendale	Phone: +64 6 355 6158
<b>Physical Address:</b>	Plant and Food Research Ltd, Food Industry Science Centre, Batchelar Rd, Palmerston North 4410	Fax: +64 6 351 7050
<b>Postal Address:</b>	Private Bag 11600, Palmerston North 4442	Email: douglas.rosendale@plantandfood.co.nz

<b>Client Contact:</b>	Jason Hodoniczky	Phone: +61 7 3214 2361
<b>Physical Address:</b>	CSIRO Plant Industry Level 4, Queensland Bioscience Precinct 306 Carmody Rd, St Lucia QLD 4067 AUSTRALIA	Fax: +61 7 3214 2920
<b>Postal Address:</b>	CSIRO Plant Industry Level 4, Queensland Bioscience Precinct 306 Carmody Rd, St Lucia QLD 4067 AUSTRALIA	Email: Jason.Hodoniczky@csiro.au

The Client agrees that PFR will carry out the Services and PFR agrees to carry out the Services, in accordance with the attached Terms and Conditions.

### Project Title: Prebiotic Potential of Alternative Sugars

**Services:**

The ability of alternative sugars to increase the growth of a panel of selected probiotic gut bacteria (Bifidobacteria, Lactobacilli) in broth culture, by measuring increases in turbidity (Optical Density, OD) of the culture. The trial will take the form of broth cultures in a 96-well microplate format inoculated with pure strains of bacteria (Bifidobacterium lactis HN019 (DR10™), Lactobacillus rhamnosus HN001 (DR20™)), appropriate growth media, and the modified sugar substrate, with growth monitored by OD.

**Commencement Date: 30 April 2010**

**Estimated Completion Date: 31 May 2010**

### Milestones and Fees:

Date	Milestone	Fee (excluding GST)
31 May 2010	Provision of culture raw data	\$5950.00
<b>TOTAL FEE (excl. GST)</b>		<b>NZ\$5950.00</b>

Executed on the later of the two dates written below.

<p>Signed by <b>The New Zealand Institute for Plant and Food Research Limited</b>  Name: Megan Woods Position: Business Manager Date: 31 March 2010</p>	<p>Signed by <b>CSIRO Plant Industry</b> _____ Name: Position: Date:</p>
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**Performance**

1. PFR will use all reasonable endeavours to undertake the Services in accordance with reasonable standards of scientific endeavour.
2. PFR may engage or contract a third party to provide goods or services to PFR to enable PFR to perform the services, and may supply confidential information to the third party provided that PFR requires the third party to comply with the confidentiality obligations of this agreement.
3. PFR does not predict or warrant any particular result or outcome of the Services, or the use or application of any result or information arising out of or in relation to the Services.
4. PFR is not liable for any delay or non-performance, directly or indirectly arising from, or attributable to, any fire, flood, hail, explosion, lightning, windstorm, earthquake, subsidence of soil, disease, genetic variation, failure of machinery or equipment or supply of material, discontinuity in the supply of power, court order, riot, war, strikes, labour disturbances, or by any other circumstances beyond its reasonable control.

**The Client's Obligations**

5. The Client shall pay the Fee, and any additional charges, disbursements and fees payable under this agreement, plus GST, and without deduction or set off, by the 20th of the month following the month of PFR's invoice(s). Interest is payable on overdue amounts at the rate of 18% p.a. calculated daily from the due date until PFR receives payment.
6. The Client will promptly provide all information, materials and assistance that PFR reasonably requires to undertake the Services.
7. If PFR incurs any additional costs by reason of the Client failing to supply any information, materials, and assistance that is to be provided by the Client, such costs shall be added to the Fee and shall be payable by the Client.

**Limitation of Liability**

8. To the extent allowed by law, PFR is liable to the Client, under or in relation to this agreement, only for actual loss suffered by the Client as a direct result of PFR's wilful default, and in no circumstances shall PFR's liability, and the liability of its officers and employees, exceed in aggregate an amount equal to the money that the Client has paid to PFR under this agreement.
9. The Client warrants that the Client is acquiring the Services for business purposes and acknowledges that the provisions of the Consumer Guarantees Act 1993 do not apply to the Services or this agreement.

**Indemnity**

10. The Client indemnifies PFR and its officers and employees against all losses, damages and costs (including reasonable legal costs on a solicitor/client basis) incurred (a) from claims made by third parties against PFR and/or its officers or employees, resulting from the application or the use of the results of the Services by the Client or any third parties; or (b) in relation to PFR's use of information or materials supplied by the Client.

**No Representations**

11. The Client acknowledges that the Client enters this agreement solely in reliance on the Client's own judgement and that PFR did not induce the Client to enter into this agreement by any statements, representations, assurances or warranties.

**Provision of Similar Services**

12. PFR may perform services similar, or identical, to the Services provided under this agreement, for other persons. The Client acknowledges that if PFR does so, it will not constitute a conflict of interest, provided that PFR complies with the confidentiality provisions of this agreement.

**Confidentiality**

13. The parties will keep confidential, and will not use nor permit the use of, any information concerning the affairs

of the other except as permitted by this agreement and except to the extent that:

- (a) the information lawfully enters the public domain; or
  - (b) the information is received in good faith from a third party, where no obligation of confidentiality is owed to the third party; or
  - (c) the party independently develops the information without reference to any information received from the other party; or
  - (d) the party to whom the information relates consents in writing to its disclosure; or
  - (e) disclosure is required by law, in which case the receiving party shall promptly notify the disclosing party and take all reasonable steps so as to allow the disclosing party the full opportunity to oppose disclosure.
14. Each party will ensure that its agents and employees who receive, or may receive, information which this agreement requires to be kept confidential, are aware of, and are bound by the confidentiality provisions of this agreement.
  15. Each party will, if required by the other party, obtain a written undertaking from any person to whom information which this agreement requires to be kept confidential is likely to be disclosed, which confirms that they have read and understood the confidentiality provisions of this agreement, and will comply with them.

**Reports**

16. All reports that PFR supplies to the Client under this agreement will become the Client's sole property provided that the Client pays all fees as they fall due and does not otherwise breach this agreement.

**Intellectual Property**

17. All intellectual property rights arising from this agreement or developed by PFR in the course of undertaking the Services will vest or remain with PFR as its sole property. Intellectual property rights includes, but is not restricted to, any right under the Patents Act 1953, the Designs Act 1953, the Trade Marks Act 2002, the Copyright Act 1994, the Plant Variety Rights Act 1987, and any like right under law in any country, and includes trade secrets, trade marks, trade names, data, computer software, pending rights, and any other right capable of being described as an intellectual property right.
18. PFR, or any person authorised by PFR, may publish any information arising out of or in relation to the Services, unless:
  - (a) the Client proves to PFR that the information is commercially sensitive to the Client; and
  - (b) the Client reasonably requests that PFR not publish that information.

**Use of PFR's Name**

19. The Client will not use PFR's name or any matter arising under this agreement to advertise or promote the Client in any manner.

**Assignment**

20. This agreement is personal to the Client and the Client may not assign any of the Client's rights or obligations under this agreement. Any material change in the Client's shareholding is an assignment for the purposes of this clause.

**General**

21. This agreement represents the entire agreement between the parties. Variations, waivers and modifications shall be valid only if in writing and signed by the parties.
22. Any failure of a party to enforce any provision of this agreement shall not constitute a waiver of any rights to future enforcement.
23. Should any part or provision of this agreement be held unenforceable or in conflict with the applicable laws or regulations of any jurisdiction, the invalid or unenforceable part or provision shall be replaced with a provision which accomplishes, to the extent possible, the original business purpose of such part or provision in a valid and enforceable manner, and the remainder of the agreement will remain binding upon the parties.

## TERMS AND CONDITIONS

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24. The Client acknowledges and agrees that if PFR carries out other services for the Client, then in the absence of a written agreement covering those services, those services will be subject to these Terms and Conditions.
25. This agreement is governed by New Zealand law. Both parties submit to the non-exclusive jurisdiction of the New Zealand courts, and neither party will object to the exercise of jurisdiction by those courts, either for forum non conveniens, or on any other basis.
26. The provisions of this agreement relating to limitation of liability, indemnity, provision of similar services, confidentiality, intellectual property, reports, use of PFR's name, further services, reconstruction and governing law shall not expire when this agreement ends.