Mannitol as a sensitive indicator of sugarcane deterioration and bacterial contamination in fuel alcohol production*

Mannit als sensitiver Indikator zur Bestimmung der Verschlechterung der Zuckerrohrqualität und der bakteriellen Kontamination bei der Kraftstoffethanolerzeugung

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Mannitol, formed mainly by *Leuconostoc mesenteroides* bacteria, is a very sensitive indicator of sugarcane deterioration that can predict processing problems. A rapid (4 to 7 min) enzymatic method has been developed to measure mannitol in juice pressed from consignments of sugarcane delivered to the factory. This method can be easily performed using existing equipment in sugarcane factories, with mannitol being measured spectrophotometrically using mannitol dehydrogenase (MDH) as the enzyme catalyst. The stability of the reagents, limited cane juice preparation, linearity, accuracy, and precision are described. The method is highly specific for mannitol and was not affected by the presence of sucrose, glucose, fructose, or dextran. The current cost is only ~60 U.S. cents per analysis. Mannitol has also been proved to be an advantageous indicator of bacterial contamination. Compared to other indicators, mannitol is not produced by yeast cells but only by some contaminating bacteria (mostly *Lactobacillus* strains) during fermentation. Its presence can account for unexpected yield drops, and it can be measured easily. A strong correlation existed between mannitol formation and bacteria counts in sugarcane juice and molasses fermentations with induced mannitol producing bacterial contaminations.

Key words: mannitol, sugarcane deterioration, mannitol dehydrogenase, fuel alcohol production, mannitol enzymatic method, bacterial contamination

Mannit, das hauptsächlich von *Leuconostoc-mesenteroides* gebildedt wird, ist ein sehr sensitiver Indikator für die Verschlechterung der Zuckerrohrqualität zur Voraussage von Verarbeitungsproblemen. Es wurde eine enzymatische Schnellanalyse (4 bis 7 min) entwickelt zur Messung des Mannitgehaltes im ausgepressten Saft, der aus den verschiedenen Zuckerrohranlieferungen stammt. Die Analyse ist einfach durchzuführen, in dem Mannit spektrophotometrisch bei Einsatz von Mannit-Dehydrogenase (MDH) als Enzymkatalysator erfasst wird. Die Stabilität der Reagenzien, eingeschränkte Aufbereitung des Rohrsaftes sowie die Linearität, Richtigkeit und Präzision der Analyse werden beschrieben. Die Methode ist hochspezifisch und wird durch die Anwesenheit von Saccharose, Glucose, Fructose oder Dextran nicht beeinflusst. Die gegenwärtigen Kosten liegen bei ca. 0,47 Euro pro Analyse.
Mannit kann bei der Fermentation von Bioethanol ebenfalls ein Indikator für eine bakterielle Kontamination sein. Verglichen mit anderen Indikatoren wird Mannit während der Fermentation nicht durch Hefen produziert, sondern nur durch einige kontaminierende Bakterien (*Lactobacillus*-Stämme). Es existiert eine starke Korrelation zwischen der Bildung von Mannit und Bakterienzahlen in Rohrsäften und Fermentation(brühen) mit durch bakterielle Kontamination hervorgerufener Manniterzeugung.

Stichwörter: Mannit, Rohrqualität, Mannit-Dehydrogenase, Kraftstoffethanolerzeugung, enzymatische Mannitbestimmung, bakterielle Kontamination

1 Introduction

The delivery of consignments of deteriorated sugarcane to factories can detrimentally affect multiple process units and even lead to a factory shut-down. Currently, there is no reliable, rapid, easy and inexpensive method to measure cane deterioration at the factory. This has meant that factory staff has not been able to screen individual consignments of cane and, thus, be unable to know which consignments would detrimentally affect processing in order to reject unsuitable consignments of cane.

The major (but not sole) contributor to cane deterioration in the United States is *Leuconostoc*, lactic acid bacterial infection. Dextran is considered the major deterioration product from *Leuconostoc* infection. Current methods to determine dextran suffer from being either too long and complicated, not specific enough, too expensive [1], or too difficult in the interpretation of results [2].

Numerous metabolic products other than dextran are formed by *Leuconostoc* which are of importance in sugar manufacture, including levan (a fructose polysaccharide) and mannitol. Mannitol is a sugar alcohol that does not degrade under processing conditions [3], can be contained in large amounts in factory syrups and massecuites processed from deteriorated cane, and directly affects processing by reducing sugar recovery [4] and evaporation rates [5]. Mannitol has been repeatedly proven to be a sensitive measure of sugarcane [3, 6–7] and sugarbeet deterioration [8–9]. Recently, *Eggleston* et al. [5] found that approximately ≥2500 mg/kg RDS of mannitol in sugarcane juice predicted processing problems at the sugarcane factory.

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Previously in the sugar industry [3, 8], mannitol has been measured with IC-IPAD (ion chromatography with integrated pulsed amperometric detection) – a sophisticated technique that cannot be used at the industrial site because of its expense, complication, and the level of expertise required of the operator. Consequently, an enzymatic method to measure mannitol in sugarcane juices at the factory was developed. An enzymatic assay was chosen for its high specificity and because a chemical method would be too complicated, require unsafe chemicals and temperatures, and be too long.

Mannitol is currently determined in hospitals as an indicator of intestinal permeability, with mannitol being measured enzymatically in urine [9] – a simpler matrix than sugarcane juice. Mannitol dehydrogenase (MDH) is used to convert mannitol to fructose in the presence of co-enzyme NAD^+. The NADH formed can be easily measured spectrophotometrically at 340 nm:

$$\text{Mannitol} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{Mannitol}} \text{Fructose} + NADH + H^+$$

By extrapolating *Eggleston*'s research on the use of mannitol to predict sugarcane deterioration [3] to the fuel alcohol industry, it was discovered that mannitol is an advantageous indicator of bacterial contamination in fuel alcohol production from sugarcane juice or molasses [10]. Commercial fuel ethanol in Brazil is currently produced by a fed-batch or continuous fermentation process of sugarcane juice and/or molasses by *Saccharomyces cerevisiae* culture yeast with cell recycle. Microbial contaminants (including bacteria and wild yeast) can also be recycled with the culture yeast that can cause many problems due to competition between bacteria and yeasts for the same substrate. Bacterial contamination of alcohol fermentations is regarded as a major technological problem that can cause (i) significant drops in fermentation yields, (ii) yeast (*Saccharomyces*) flocculation (not all contaminating bacteria cause floc formation), (iii) foaming problems (see Fig. 1), and (iv) production of by-products that detrimentally affect the quality of distillates. Bacterial contamination control in Brazil is currently undertaken in (1) factory crushing, by cleaning with hot water or the sometime addition of biocides (depends on the factory) or in (2) fermentation, by sulfuric acid washing of yeast cell suspensions [11] or the sometime addition of antibiotics (penicillin, virginiamicin, Kamoran HJ) [12]. *Oliveira* et al. [13, 14] developed a procedure to optimize the use of antibiotics by evaluating the sensitiveness of the bacterial population. However, bacterial contaminants are frequently adaptable to the products used for their control, particularly antibiotics, that makes industrial control difficult.

To effectively control bacterial contamination monitoring and measurement are essential. Typical monitoring methods are either direct or indirect. Direct methods include the counting of live bacteria by light microscopy (15 min), or plating of bacteria on rich medium (very time consuming). Indirect methods include the acidification of the culture medium, or the measurement of lactic acid. However, some methods are not specific enough for bacterial contamination, while others could be tedious, complex, or expensive.

In this paper it is described the development of a rapid, enzymatic method using MDH that can be undertaken at the factory to measure mannitol in pressed cane juices, which represent individual sugarcane consignments. The use and advantages of using mannitol as an indicator of bacterial contamination in fuel alcohol production are also described.

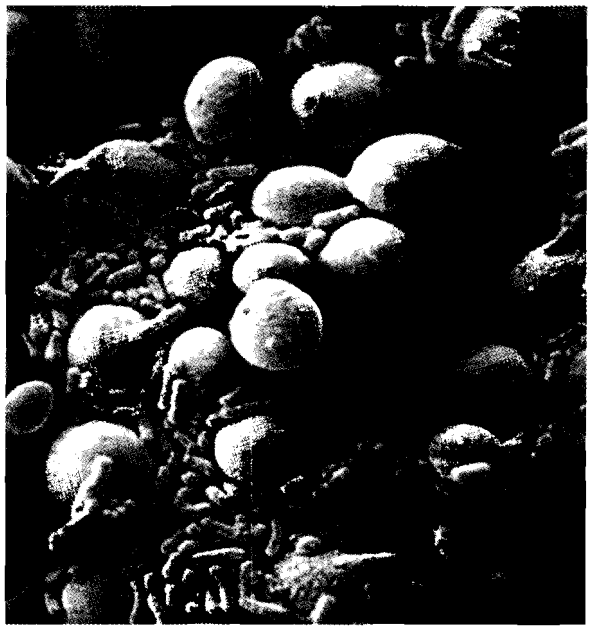

Fig. 1: Bacterial contamination of fuel alcohol producing yeast. Photograph reproduced with permission from Fermentec Ltda, Brazil.

2 Materials and methods

2.1 Development of enzymatic method to measure mannitol

2.1.1 Enzyme, chemicals, sugarcane juices and buffers

Mannitol dehydrogenase (EC 1.1.1.67) was purchased as a freeze-dried powder (8.45 Units/mg dry mass) from Biocatalysts Ltd, Cardiff, Wales. All chemicals were analytical grade. Dextran T2000™ (molecular mass 2,000,000 Da) was from G.E. Healthcare (U.S.). Sugarcane juices were obtained from Louisiana factories. All juices (120 mL) were stored with 5 drops of biocide (Bussan 881™, Buckman Labs.) in a –60 °C freezer. Glycine buffer (100 mmol/L; pH = 10.5) and phosphate buffer (25 mmol/L; pH = 6.0) with 30% glycerol were prepared [15].

2.1.2 NAD solution

NAD (0.22 g) was dissolved in 10 mL of de-ionized water and prepared *daily*.

2.1.3 Preparation of enzyme

A stock solution of enzyme was first prepared by dissolving 0.01 g freeze-dried MDH in 1 mL ice cold phosphate +30% glycerol buffer. For the assays, a further dilution was made by pipetting 100 µL of stock/10 mL phosphate +30% glycerol buffer (10,000-fold dilution). Both the stock and diluted enzyme solutions were stored in a –20 °C freezer. The stock solution can be stored for ~1 month, and the diluted enzyme for 1–2 weeks.

2.1.4 Mannitol dehydrogenase activity

See *Eggleston* and *Harper* [15] for method.

2.1.5 Factory mannitol enzymatic method

The method was first standardized using five mannitol standards (1, 10, 100, 500 and 1000 ppm) diluted in de-ionized water, to generate a linear standard curve and equation. A new standard curve must be generated for each batch of enzyme. Sugarcane juice was first diluted 1:1 (i.e., 2-fold) in glycine buffer and then filtered through a 0.45 µm then 0.22 µm pore-size PVDF filter. For difficult to filter samples, celite can be first added to the juice before filtering through the PVDF filters or the juice can first be filtered through Whatman™ 91 filter paper (185 mm; 10 µm). (Note: If PVDF filters are not available then add 0.5 g celite to 10 mL juice in a syringe body, mix, filter juice through a glass filter, discard first 2 mL of filtrate, and dilute 2-fold with glycine buffer). To two test-tubes the following are added:

Sample Test-Tube	**Blank Test-Tube**
1.4 mL glycine buffer	1.4 mL glycine buffer
0.2 mL diluted and filtered juice	0.2 mL diluted and filtered juice
0.2 mL NAD	0.2 mL NAD
0.2 mL MDH enzyme	0.2 mL water

The test-tube mixtures were vortex stirred and immediately added to a 1 cm cuvette then placed in a spectrophotometer. The change in absorbance at 340 nm is measured from 0 to 5 min. Final absorbance was [sample absorbance – blank absorbance]. Calculations were based on the equation of the standard curve and dilution factors. For deteriorated juices containing high amounts of mannitol which cause the mannitol absorbance to be higher than the upper limit of the standard curve, further dilutions of 1:3 (4-fold) or 1:7 (8-fold) in glycine buffer are required.

2.1.6 Effect of temperature on the factory enzymatic method

The factory mannitol method was followed except 0.2 mL of mannitol (1400 ppm) replaced the juice and, after the enzyme was added the two test-tubes were placed in a shaking (90 min^{-1}) waterbath (Julaba SW22) at different temperatures (23–48 °C) for 5 min, before the absorbance was measured.

2.1.7 Effect of added sugars on the factory enzymatic method

Model solutions were made to simulate levels of sugars in a typical sugarcane juice, and all the solutions had a final refractometric dry substance content (RDS) of 14.0%. All the solutions contained either 1000 or 2000 ppm mannitol. Glucose and fructose additions were on ~3% on solids basis, sucrose ~90% solids. Dextran was added at 1000 ppm level. The factory mannitol method was followed except the model sugar solution containing mannitol (1000 or 2000 ppm) replaced the juice.

2.1.8 Haze dextran in sugarcane juices

Haze dextran in juices was based on the modified method of *Eggleston* and *Monge* [16].

2.1.9 Mannitol determined by IC-IPAD

See *Eggleston* [6] for method. Dilutions varied, depending on the juice, from 1 g/50 mL to 1 g/500 mL.

2.1.10 Statistics

Single factor ANOVA was conducted using Microsoft Excel™, version 2002 with SP-2.

2.2 Development of mannitol as an indicator of bacterial contamination in fuel alcohol production

2.2.1 Growth of bacterial isolates

Bacteria, in freeze-dried form, were from the Fermentec (Piracicaba, Brazil) industrial collection. They were reactivated in MRS medium (24 h at 32 °C) and then grown for 48 h at 32 °C in cane juice medium (containing 2.5% reducing sugars) supplemented with 1% yeast extract. Bacterial counts were performed either by plating or optical microscopy. Mannitol concentrations in the growth mixtures were measured by IC-IPAD.

2.2.2 Yeasts

Two *Saccharomyces cerevisiae* strains (BG-1 and PE-2, from the Fermentec collection) commonly used in industrial fermentation were propagated under anaerobic conditions at 32 °C using molasses medium with 8% total sugar, doubling the medium volume each 24 h until there was sufficient biomass for the assay, which was collected by centrifugation (20 min, 800 · *g*).

2.2.3 Mannitol determined by IC-IPAD

Mannitol was measured on a Dionex DX-300 system.

2.2.4 Alcohol fermentation

Fermentation was performed at 33 °C in 150 mL centrifuge vials containing 8 g of yeast (fresh mass with 25% dry substance content) and 80 mL of substrate (a mixture of cane juice and molasses containing 18% total sugar, each source representing 50% of the sugar). Two different strains of *Saccharomyces cerevisiae* (BG-1 and PE-2) were used in separate experiments (in duplicate) with induced bacterial contamination (*Lactobacillus fermentum* inoculated during the first fermentation at different levels: $2 \cdot 10^6$, $1 \cdot 10^7$, and $5 \cdot 10^7$ cells/mL). After fermentation (8–10 h) the fermented medium was centrifuged (800 · *g*, 20 min) and the yeast and bacteria settled were fed again with substrate, performing two additional fermentations cycles. Bacterial counts (microscopy) and mannitol content were measured in the fermented media at the end of the last two fermentation cycles.

3 Results and discussion

3.1 Development of enzymatic method to measure mannitol

3.1.1 Stability of the enzyme

Initially there was a problem with the stability of the enzyme which had been diluted in a phosphate buffer without glycerol, even if the stock or diluted enzyme was stored in a –40 °C freezer. This is illustrated in Figure 2. After 8 days of storage in a –20 °C freezer the MDH activity had markedly decreased from 1.88 to 0.20 Units/mL. Stabilization studies were undertaken, and it was found that the addition of glycerol to the buffer stabilized the enzyme stored as both stock or as a further diluted solution. There was a slightly higher level of stability of the enzyme diluted from stock that had been stored in buffer containing 30% glycerol, compared to that from stock that contained no glycerol, and this increased stability was observed even in fresh, unstored preparations (day 1). As can be seen in Figure 3, both the preparation of the enzyme stock and diluted enzyme from the stock, need to be undertaken in phosphate buffer with glycerol. Glycerol at the 30% level was found to be optimum (Fig. 3). The freeze dried enzyme is stable in a –20 °C freezer for up to 6 months. The enzyme stock and diluted enzyme can be stored in conventional –20 °C freezers.

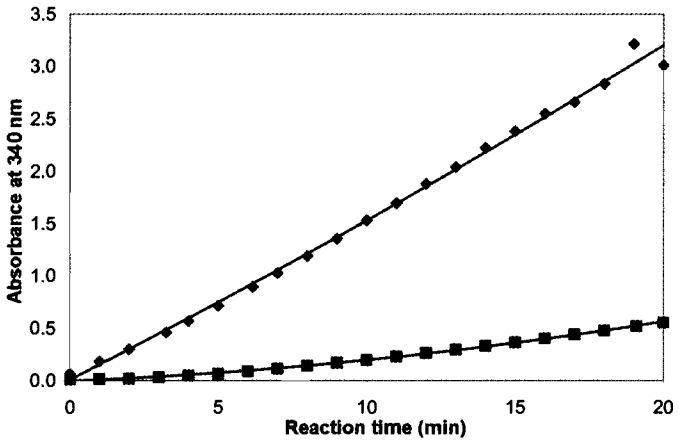

Fig. 2: Instability of MDH when the stock enzyme solution was prepared in phosphate buffer only with no added glycerol [15]. ◆ day 1; ■ day 8

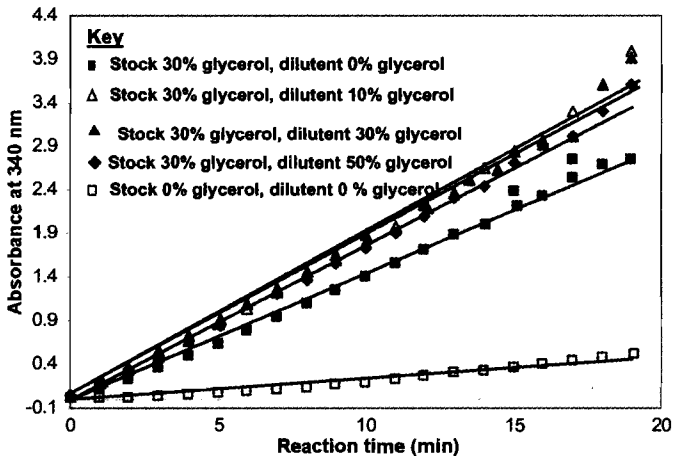

Fig. 3: Stabilization of MDH after 8 days when the stock solution was prepared in phosphate buffer, with added glycerol at different concentrations [15]

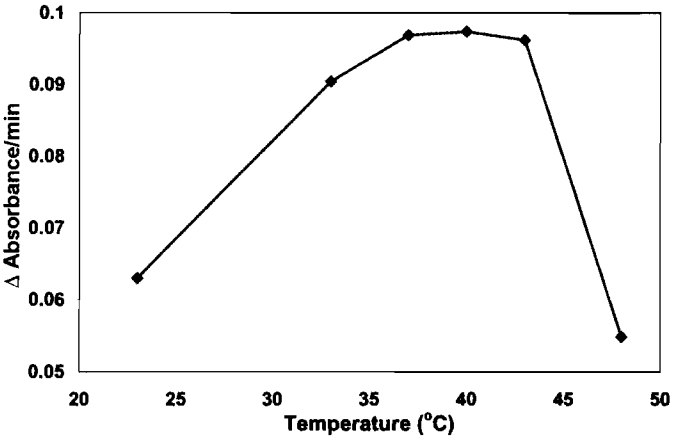

Fig. 4: Effect of temperature on the activity of mannitol dehydrogenase (MDH) [15]

3.1.2 Effect of temperature

In early efforts to develop a simple method to determine mannitol in sugarcane consignments, there was concern that the method was relatively rapid. For this reason, the effect of temperature on MDH activity was investigated – see Figure 4.

Although the activity of MDH is optimum between 37–43 °C (Fig. 4), incubating the enzyme/juice mixture in a waterbath at the factory would only add another level of complexity and cost. It was, therefore, decided to keep the reaction at room temperature (23 °C) for the final factory method. Nevertheless, if factory staff want an even faster method, they can incubate the enzyme/juice mixture in a waterbath at 40 °C for 2 min, instead of leaving it at room temperature for 5 min.

3.1.3 Linearity

The relationship between the mannitol concentration and the absorbance at 340 nm after 5 min was found to be only approximately linear up to 1000 ppm, which may be due to lack of substrate at very lower levels, and product inhibition ~1000 ppm level. Therefore, up to the 1000 ppm level, a quadratic fit would be better. A better linear fit was found from 1–500 ppm mannitol (Fig. 5). Quadratic fits can also be applied.

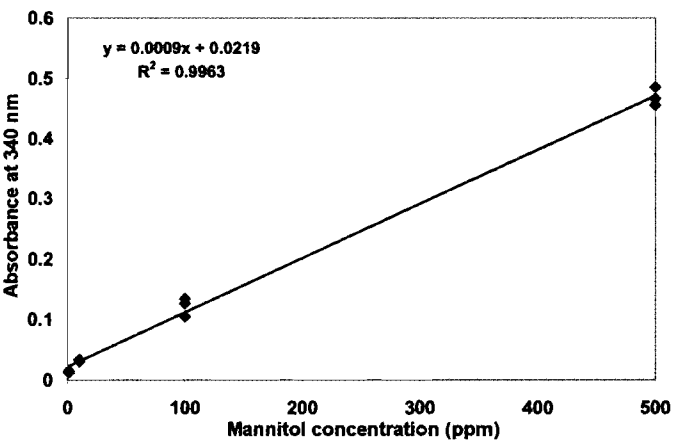

Fig. 5: The linear relationship between mannitol concentration and absorbance at 340 nm after 5 min incubation at room temperature [15]

Table 1: Precision of the enzymatic method for the determination of mannitol in sugarcane pressed juices expressed as the coefficient of variance (CV)

Cane juice sample	RDS (%)	N	Mean concentration of mannitol (ppm)	Mannitol variation CV (%)	Haze dextran (mg/kg RDS)
Deteriorated, pressed cane juice*	14.89	10	20,455	3.32	4,688
Crusher juice from Factory A	14.70	8	3,870	4.50	585
Crusher juice from Factory B	16.01	10	3,259	5.32	204
Crusher juice from Factory C	14.67	9	7,180	1.73	944

* Had to be diluted 8-fold

3.1.4 Precision of the enzymatic factory method to determine mannitol in cane juices

The method precision was very acceptable in four cane juices assayed (Table 1), with coefficients of variation ranging from 1.73–5.13%. The precision tended to become worse when relatively lower amounts of mannitol were present in slightly deteriorated and undeteriorated cane juices (Table 1).

3.1.5 Determination of mannitol in the presence of other cane sugars

Sugarcane juices have high levels of glucose, fructose, and particularly sucrose. Dextran will also be present when mannitol is present in cane juice because both are formed from *Leuconostoc* [6]. These short and long chain carbohydrate sugars could potentially interfere with the measurement of mannitol. Therefore, it was investigated the effect of different sugars as they approximately occur in sugarcane juices, using simulated, model juices and results are shown in Table 2. None of the sugars showed any statistical interference at the 95% probability level with the mannitol determination, which confirms the ability of the method to accurately measure mannitol in cane juices. The analytical recoveries were very acceptable between 99.1–104.2% (Table 2).

Table 2: Determination of mannitol in the presence of other sugarcane sugars in simulated juices with 14.0 % RDS

Simulated juice[a]	Mannitol conc. added (mg/kg RDS)	Mean % recovery of mannitol[b, c]	Total variation CV[c] (%)
Sucrose	2000	100.6	1.86
Sucrose + glucose	2000	102.5	3.16
Sucrose + glucose + fructose	2000	99.6	3.28
Sucrose	1000	103.6	2.97
Sucrose + glucose	1000	104.2	3.26
Sucrose + fructose	1000	103.6	1.36
Sucrose + glucose + fructose	1000	99.1	7.16
Sucrose + glucose + fructose + dextran (diluted 1:2; 7.0% RDS)	1000	100.5	1.63

[a] See Materials and methods section. [b] N = 3. [c] No statistical differences were found amongst the simulated juices with different sugars added using single factor ANOVA.

3.1.6 Accuracy

The accuracy of the developed enzymatic method to measure mannitol concentrations in cane juices was determined by comparing the results with an ion chromatography (IC-IPAD) method (Fig. 6).

An excellent correlation existed (R^2 = 0.99) between the two methods, that validates the accuracy of the enzymatic method. Furthermore, there were no significant differences at the 95% probability level, between the two methods for any of the juices analyzed. Precision was, however, worse for both methods in the juice containing <550 ppm mannitol (Fig. 6), which reflects the difficulty of detecting small amounts of mannitol, and confirms results listed in Table 1. As factory staff are more concerned with detecting mannitol concentrations >550 ppm in deteriorated pressed cane juice at the factory, this lower precision should have limited impact at the factory.

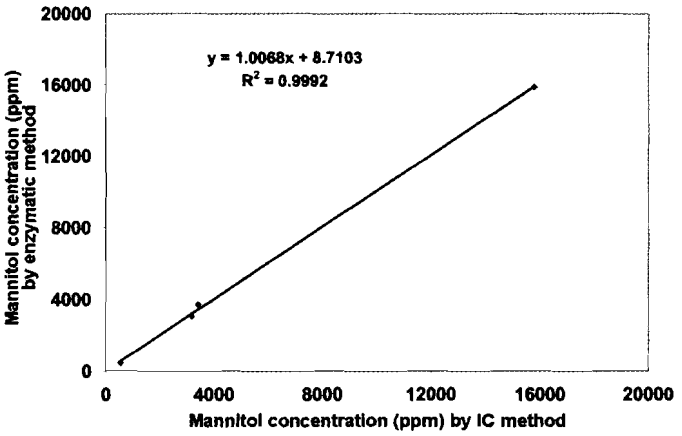

Fig. 6: Linear correlation between the enzymatic method for determining mannitol and cane juices and an ion chromatography (IC) method. No statistical differences between the two methods were found at the 95% probability level for any sugarcane juice studied [15].

3.1.7 Relationship of mannitol with dextran measured by the Haze method

As well as mannitol concentrations, Haze dextran concentrations in four cane juices are shown in Table 1, and the correlation between Haze dextran and mannitol was excellent at R^2 = 0.99 at the 99% probability level. This confirms previous observations from a laboratory cane deterioration study [6]. In more complex field studies of cane deterioration [3, 7], other strong correlations were found between mannitol measured by IC and dextran, but with slightly lower R^2 values of 0.84 which reflects the further complexity of the multiple field samples representing numerous cane varieties, and possibly other bacterial sources of sugarcane deterioration (see Table 3).

As seen in Table 1, mannitol concentrations were markedly higher than Haze dextran concentrations. Higher mannitol than dextran concentrations have been previously observed in deteriorated juices

from different cane varieties [3, 7] and in juices from *Rhizoctonia* affected beets that were susceptible to *Leuconostoc* deterioration in the late stages [17]. This not only highlights the usefulness and higher sensitivity of mannitol to better predict *Leuconostoc* and other bacterial cane deterioration (see Table 3) than dextran, but the underestimation by sugar industry personnel of the relatively large amounts of mannitol present in deteriorated cane that can affect processing.

3.2 Development of mannitol as an indicator of bacterial contamination in fuel alcohol production

3.2.1 Mannitol in contaminating bacteria

Numerous types of bacteria can contaminate yeast fuel ethanol fermentations. Lactic acid bacteria, *Leuconostoc* and *Lactobacillus*, are common contaminants and frequently associated with process problems. *Lactobacillus* is adapted to the alcoholic and nutritional conditions of the process [18], but *Leuconostoc* is more sensitive to alcohol and usually does not persist as long. The lactic acid formed by such bacteria can strongly inhibit yeast metabolism and decrease alcohol yield.

Known contamination bacteria were grown on a medium of sugarcane juice to evaluate their ability to produce mannitol, and results are listed in Table 3.

Table 3: Formation of mannitol by bacterial isolates from industrial processes

Bacteria	Mannitol (mg/kg RDS)	Plating counts (CFU · 10^7/mL)
Acetobacter pasteurianus	<50	71.0
Bacillus coagulans	<50	2.8
Bacillus megaterium	<50	1.0
Bacillus subtilis	<50	2.0
Bacillus stearothermophilus	<50	2.0
Lactobacillus buchneri	1070	15.0
Lactobacillus fermentum	5110	4.8
Lactobacillus fructosus	5970	3.4
Lactobacillus vaccinostercus	1875	16.0
Leuconostoc mesenteroides	7480	8.3

Mannitol production depended on the bacterial species and strain. The highest producer of mannitol was *Leuconostoc mesenteroides*, which supports the evidence that mannitol is a sensitive indicator of sugarcane [3, 6] and sugarbeet deterioration [9]. Furthermore, it was the common contaminant of fuel ethanol fermentations from *Lactobacillus fermentum* and *fructosus* that produced the most mannitol (Table 3). This suggested that mannitol would be a useful indicator of bacterial contamination. More importantly, it also explained why ethanol yields are sometimes lower than expected with respect to the acidification of the fermentation medium, because these bacteria competed with the yeast for substrate to produce mannitol rather than ethanol.

3.2.2 Mannitol as an indicator of bacterial fermentation in alcohol fermentation

To verify if mannitol could be an indicator of bacterial fermentation, an experiment was conducted to measure mannitol in fermentations performed with induced bacterial contaminants, and results

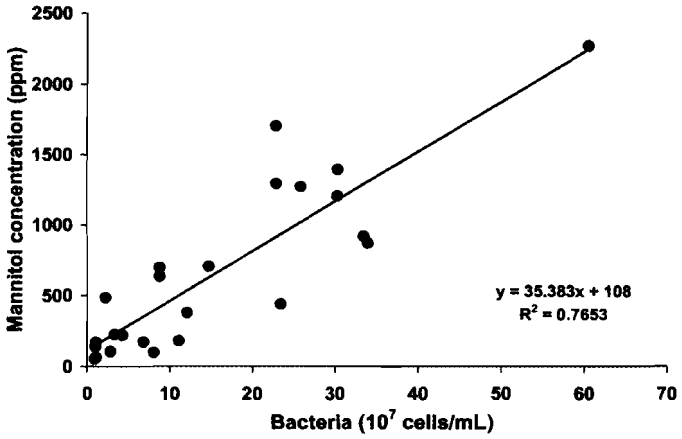

Fig. 7: Relationship between bacteria grown in alcoholic fermentation and mannitol formation. Fermentation was performed with induced bacterial contamination.

are illustrated in Figure 7. A good correlation existed between mannitol and the bacterial cell count (Fig. 7), proving that mannitol was a valid indicator.

3.2.3 Advantages of mannitol as an indicator of bacterial fermentation

Mannitol serves as a very important complementary method to other direct and indirect methods to monitor bacterial contamination in sugarcane processing to fuel alcohol production:

- Mannitol is not produced by the starter strain or by wild *Saccharomyces cerevisiae* yeast;
- Mannitol is produced (mannitol at the end of fermentation – mannitol in the imput must and yeast) by bacteria during fermentation and can account partly or wholly for decreased ethanol yields. Approximately 6000 ppm mannitol can cause an approximate 4% drop in ethanol yield;
- Mannitol is easily measured.

4 Conclusions

An enzymatic method has been developed to measure mannitol and, therefore, the extent of deterioration in juice pressed from consignments of sugarcane delivered to the factory. This enzymatic method has many advantages for the factory staff:

- Simplicity;
- Rapid;
- Uses existing equipment at most factories;
- Accurate;
- Precise;
- Not affected by the presence of other sugarcane sugars;
- Only ~60 U.S. cents per analysis (largest cost is the NAD at 45 cents; MDH cost is12.5 cents per analysis) that is much lower than the cost for rapid dextran analysis by monoclonal antibody technology [1];
- Mannitol is a more sensitive indicator of sugarcane deterioration than dextran.

Further research needs to be undertaken to verify if the method can be used to measure bacterial contamination in fuel alcohol production. A bright purple color forms when the enzymatic method is used to measure mannitol in deteriorated sugarbeet juice; research

is needed to ensure that this color does not interfere with the determination of mannitol in sugarbeet juice.

Mannitol has been shown to be an indicator of bacterial contamination in fuel alcohol fermentations that can complement other indicators and methods to monitor contamination:

- It is not produced by the starter or wild strains of *Saccharomyces cerevisiae* yeast;
- It is produced by bacteria during fermentation;
- It can account for unexpected alcohol yield drops;
- It is easily measured.

Not all contamination bacteria produce mannitol so other monitoring techniques are also needed for a full picture. Future research is planned to evaluate mannitol as a bacterial contamination indicator further using other bacteria isolated from industrial processes.

5 Acknowledgements

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Le mannitol comme indicateur sensible de la détérioration de la canne à sucre et de la contamination bactérienne dans la production d'alcool carburant (Résumé)

Le mannitol formé principalement par des *Leuconostoc mesenteroides* est un indicateur très sensible de la détérioration de la canne à sucre qui peut annoncer des problèmes de fabrication. Une méthode enzymatique rapide (de 4 à 7 min.) a été mise au point pour mesurer le mannitol dans le jus de pression des arrivages de cannes amenées à l'usine. Cette méthode peut être exécutée facilement en utilisant les équipements présents dans les sucreries par mesure spectrophotométrique du mannitol au moyen de mannitol déshydrogénase (MDH) comme catalyseur enzymatique. La stabilité des réactifs, la préparation réduite du jus de canne ainsi que la linéarité, l'exactitude et la précision sont décrites. La méthode est très spécifique pour le mannitol et n'est pas affectée par la présence de saccharose, de glucose, de fructose ou de dextrane. Le coût par analyse ne s'élève qu'à ~60 U.S. cents (0,47 Euro). Le mannitol s'est aussi avéré être un indicateur avantageux de contamination bactérienne. Comparé à d'autres indicateurs, le mannitol n'est pas produit par les cellules de levure, mais seulement par certaines bactéries au cours de la fermentation (le plus souvent par des souches de *Lactobacillus*). Sa présence peut expliquer des chutes de rendement imprévues et est facile à mesurer. Il existe une forte corrélation entre la formation de mannitol et la population bactérienne dans le jus de la canne à sucre et les fermentations de mélasse avec production de mannitol provoquées par contamination bactérienne.

Manitol como indicador sensitivo para la determinación del deterioro de la calidad de caña de azúcar y de la contaminación bacteriana en la producción de etanol para combustible (Resumen)

El manitol, sobre todo formado por *Leuconostoc mesenteroides*, es un indicador muy sensitivo para el deterioro de la calidad de la caña de azúcar y para el pronóstico de problemas durante los procesos de producción. Para la medida del contenido de manitol en jugo extraido de diferentes acarreos de caña de azúcar se desarrolló un análisis enzimático simple y rápido (4 a 7 min): se determina el manitol espectrofotométricamente empleando manitol dehidrogenasa (MDH) como catalizador enzimático. Se describen la estabilidad de los reactivos y la preparación limitada del jugo de caña como también la linearidad, exactitud y precisión del análisis. El método es altamente específico y no es influenciado por la presencia de sacarosa, glucosa, fructosa o dextrano. Los costes actuales suman unos 0,47 Euro por análisis. En la fermentación de bioetanol el manitol también puede ser un indicador para una contaminación bacteriana. Comparándolo con otros indicadores el manitol no es producido por levaduras durante la fermentación sino solamente por la contaminación con algunas bacterias (tipos de *Lactobacillus*). Existe una fuerte correlación entre la formación de manitol y el número de bacterias en jugos crudos y caldos de fermentación con una producción de manitol por contaminación bacteriana.

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Final Recommendations of the 25th Session of ICUMSA, 2006

GS1: Raw Sugar (Referee: Dr. Les A. Edye, Australia)
No Recommendations

GS2: White Sugar (Referee: Geoff Parkin, UK)
1. Method GS2/3-19, The Determination of Insoluble Matter in White Sugar by Membrane Filtration should be rewritten to incorporate the modifications tested in the collaborative study and should be Officially adopted for all levels of insoluble matter.
2. ICUMSA Draft Method No.7 (2006), The Determination of Arsenic, Cadmium, Copper and Iron in White Sugar by Atomic Absorption Spectroscopy should be collaboratively tested without delay. If the results of this study prove satisfactory then it should also be Officially adopted.
3. Every assistance should be given to the Referee for Subject 3 and the ISBT in validating their proposed method for the Determination of Turbidity in White Sugar.
This work has been done and will be reported during the S7 presentation.
4. The status of Method GS2/3-40 Part B, The ICUMSA 24 Hour Acid Beverage Floc Test for Beet White Sugar should be changed to Accepted. A rapid test for Acid Beverage Floc in Cane White Sugar is still required.
5. Method GS2/3-35, The Determination of Sulphite in Brown Sugars by an Enzymatic Method, should be collaboratively tested in conjunction with the Referee for GS3.

GS3: Specialty Sugars (Referee: Gloria Negbenebor, UK)
1. To develop an alternate method for determination of Starch (anticaking agent) in Icing Sugar based on a Starch-Iodine reaction. To obtain shorter time for analysis compared with time required for filtration and drying in Method GS3-21
2. Progress a collaborative trial to demonstrate precision of Method GS4-13 Determination of Refractometric Dry Substance of Molasses, for liquid sucrose solutions. With the view of proving and writing up method for GS3 subject group.
3. Following from a preliminary work done on Sulphite in Raw Sugars by Mr Maurice Daruty, Associate Referee GS1 & GS3. I would like to recommend a collaborative trial for determination of sulphite in Demerara sugars in conjunction with GS1 referee.

GS4: Molasses (Referee: Dr. Gillian Eggleston, USA)
1. Confirm (ratify) the Official Status of GS4/3-3 "The Determination of Total Reducing Sugars in Cane Molasses and Refined Syrups by the Lane & Eynon Constant Volume Procedure". Adopt the precision indicators obtained in the 2002 Collaborative Test, namely repeatability (r) 0.77 and reproducibility (R), 1.60. (Mean Horwitz Ratio, 1.50)
2. Confirm (ratify) the Official Status of GS4/3-7 "The Determination of Total Reducing Sugars in Molasses and Refined Syrups after Hydrolysis by the Lane & Eynon Constant Volume Procedure" for both cane and beet molasses. Adopt the precision indicators obtained in the 2002 Collaborative Test, namely repeatability (r) 1.51 and reproducibility (R), 2.47. (Mean Horwitz Ratio, 0.77)
3. Continue research studies on Draft Status Method "Determination of Apparent Total Sucrose from Sucrose, Glucose and Fructose in Molasses by an Enzymatic Method" (No Status). Review the text of the method in light of the results of the research study presented at ICUMSA 2006 Brazil session by Gillian Eggleston and M. Füsün Kavas, and comments of the participants. Improve the stability and shelf-life of the enzymes
4. Continue to establish a method for the determination of mineral hydrocarbons in molasses

GS5: Cane (Referee: Ashok Sitaram Patil, India)
1. To investigate and standardize the technology to find out polyphenols and amino acids in cane juice using GC-MS technology.

GS6: Beet (Referee: Dr. Dierk Martin, Germany)
1. Draft Method No. 5 (2005) "Determination of Potassium and Sodium in Sugar Beet by Flame Photometry" should be given Official status and be numbered GS6-7 (2006).
2. Draft Method No. 6 (2005) "Determination of α-Amino Nitrogen in Sugar Beet by the Copper Method ('Blue Number')" led to acceptable precision data in the collaborative study for the aluminium salt defecation and should therefore be given Official status. It should be numbered GS6-5 (2006).
3. Further collaborative studies for the determination of potassium, sodium and α-amino-nitrogen should be conducted in order to collect more precision data under improved conditions.

GS7: Cane Sugar Processing (Referee: M.B. Londhe, India)
1. The method "Use of non-lead clarificants for high-grade products clarification with NIR polarimeter proposed by Réunion" (Appendix 1) is to be accepted as Draft Method and collaboratively tested for syrups, intermediate molasses and high-grade massecuites.
2. The method proposed by Brazil for replacement of lead clarificants by aluminum chloride and calcium hydroxide is to be accepted as a Draft method for analysis of high grade products of cane sugar process streams.
3. For determination of MA & CV in massecuite samples, further method validation study of the VSI & CERF methods is recommended
4. Use of gamma ray irradiation technique for preservation of cane sugar processing products during international inter laboratory/ collaborative tests shall be explored, and if appropriate, be written as standard specification with official status.
5. It is recommended to extend the scope of Method GS7-21 for syrup and clarified syrup for determination of turbidity – Accepted status as proposed in Appendix 6 (amended).

GS8: Beet Sugar Processing (Referee: Jean-Pierre Lescure, France)
1. The ICUMSA Draft Method n°1 (2005) "The determination of Mannitol in Beet Brei by HPAEC" should be further studied and evaluated.
2. The ICUMSA Method GS1-15 (2005) "The Determination of Dextran in Raw Sugar by a Modified Alcohol Haze Method – Of-