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Evaluation of the risk of fungal spoilage when substituting sucrose with commercial purified Stevia glycosides in sweetened bakery products

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Abstract

The objectives of this study were to compare the addition of different Stevia-based sugar substitutes (S1-S3) with sucrose alone and sucrose + S1 on (a) humectant a_w properties, (b) relative colonisation rates of sponge cake slices at 0.90 a_w by strains of *Aspergillus flavus*, *Eurotium amstelodami*, *Fusarium graminearum* and *Penicillium verrucosum* at 20 and 25°C and (c) shelf-life periods in days prior to visible growth. This showed that sucrose, S1 and S1+sucrose in water solutions were able to reach a_w levels similar to those of glycerol and glucose mixtures. The S2 and S3 commercial sugar substitutes were unable to reduce a_w levels significantly. Colonisation of sponge cake slices by *E. amsteladami*, *A. flavus* and *P. verrucosum* occurred with all the treatments at 25°C. At 20°C, sucrose alone inhibited growth of *A. flavus*. *F. graminearum* growth only occurred in sponge cake slices containing S2 and S3 Stevia-based products at both temperatures. The longest shelf-life periods (30 days), without spoilage, was for *A. flavus* at 20°C with sucrose and S1 Stevia product and for *E. amstelodami* at the same temperature with sucrose. *F. graminearum* was completely inhibited, with no growth occurring at both temperatures and sucrose alone, S1 and sucrose + S1 treatments. This study suggests that, as part of a hurdle technology approach, replacing sucrose with low calorie sugar substitutes based on Stevia glycosides needs to be done with care as different products may have variable humectant properties and bulking agents which may shorten the shelf-life of intermediate moisture bakery products.

Introduction

Sucrose is currently one of the main ingredients in the food industry and is especially important in sweetened bakery products and confectionary for its preservation characteristics and as an important source of energy, providing 394 kcal/100 g of refined sugar. It preservation properties are to act as a humectant by reducing the water activity (a_w) of bakery products as part of a hurdle technology strategy. Intermediate bakery products have a relatively short shelf-life although this can be extended by addition of aliphatic acids and sometimes modified atmosphere packaging. However, these intermediate moisture products are prone to colonisation by xerophilic and xerotolerant fungi when not stored properly. The most important spoilage moulds in bakery products are species from *Eurotium*, *Aspergillus* and *Penicillium* (Abellana et al., 1997; Arroyo et al., 2005; Guynot et al., 2005; Spicher, 1980; Williams, 1990; 1999; 2001;).

There has been interest in the substitution of sucrose with alternative lower calorie sweetners in bakery products. The most common high-intensity sweeteners in the world market (e.g. saccharin, aspartame, sucralose) are made of synthetic compounds (Abdalbasit et al., 2014). There has thus been interest in other alternative plant-based products based on extracts from the plant *Stevia rebaudiana* (Bertoni). The compounds of interest are known as steviol glycosides (Boileau et al., 2012). Stevia products have redefined the category of intense sweeteners globally, because, for the first time, food manufacturers have access to an effective non-calorific sweetener that has a 'natural' image (Gibson-Moore, 2013). In 2011, the European Commission authorised the use of pure steviol glycosides (95%) in foods and beverages in the European Union.

Stevia products are being increasingly used (Chattopadhyay et al., 2014) by food companies which produce sweetened bakery products and recommended as a substitute for home baking. However, there have surprisingly been no studies on whether substituting sucrose with Stevia glycosides completely, or in combination

with sucrose, will result in the required target a_w levels of products such as cakes and provide similar shelf-life properties as sucrose in terms of preventing fungal spoilage from being initiated.

The objectives of this study were to (a) evaluate the water binding capabilities of different commercial Stevia-based sweetener formulations by comparing them with sucrose, glucose and glycerol, (b) to examine the effect of three Stevia substitute products (S1-S3) alone or with sucrose on fungal colonisation rates on sponge cake slices at 20 and 25^{o}C by different spoilage fungi (*Aspergillus flavus*, *Penicillium verrucosum*, *Eurotium amstelodami* and *Fusarium graminearum*), and (c) relative shelf-life of different formulated sponge cake slices in terms of time (days) before visible spoilage was initiated.

2. Materials and methods

2.1. Commercially purified Stevia products

Three different commercially available products were bought from UK retail shops and identified as S1, S2 and S3. The information on the product labels with regard to weight, composition, manufacturer dosage guidelines and energy per 100 g was recorded and are shown in Table 1.

2.2. Determination of the water activity (a_w) of solutions of different sugars and additives

RQ-Water solutions containing 5, 10, 20, 30, 40, 80 and 100 g/100ml of water were prepared using the different Stevia products. Because at the higher concentrations, Stevia products in water solutions were close to their maximum solubility, the temperature was increased when required to allow complete dissolution.

For comparison, glycerol-water, sucrose-water and glucose-water solutions were prepared, according to the literature in the range 0.995 and 0.80 water activity (a_w; Dallyn and Fox, 1980; Scott, 1957). The a_w of all solutions were then measured using

122 an Aqualab 3TE instrument (Decagon, Pullman, WA, USA). All measurements were
123 made with three replicates per treatment solution.
124
125 2.3. Preparation of the cake analogue
126
127 A sponge cake analogue similar to that used by Abellana et al. (1999) was prepared
128 for these studies. The recipe consisted of the following basic ingredients: 275 g self-
129 rising wheat flour, 250 ml of vegetable oil and 4 medium sized eggs. In this study 5
130 different treatments were included. These were (i) sucrose-based cakes (control), (ii)
131 100% substitution of sucrose by the manufacturers recommended amounts of Stevia
132 products for S1, S2 and S3 and (iii) 75 % substitution of sucrose by the Stevia product
133 S1 according to an online cake recipe.
134
135 The ingredients were mixed in a multifunctional kitchen mixer to make the cake
136 dough. The dough batches were placed in grease (vegetable oil) disposable aluminium
137 tins and baked in an oven at 160-170^{o}C for 40-45 min.
138
139 After baking, the tins were covered with sterilised cooking foil and transferred to a
140 laminar flow bench for further processing. The cooled cakes were cut into ≈4 mm thick
141 slices and placed in sterile 9 cm Petri plates. The cake slices were then exposed to
142 254nm UV light for 10 min in a Herolab CleneCab Plus (Herolab GmbH Laborgeräte,
143 Germany) to eliminate any surface contamination. The a_w of the cake slices were
144 checked using an Aqualab 3TE instrument (Decagon).
145
146 Subsequently, all the treatments were equilibrated at 0.90 a_w in order to carry out
147 fungal growth experiments. The cake slices in Petri plates were divided into 4 groups
148 and placed in plastic chambers together with two 500ml beakers containing a glycerol-
149 water solution with an equilibrium relative humidity value identical to the a_w
150 treatments (=0.90 a_w). Equilibration was achieved by incubating for 48 h. Appropriate
151 equilibration was confirmed using an Aqualab 3TE instrument and found to be within
152 ±0.02 of the target a_w level.
153

154 2.4. Strains used in these studies and inoculation method

155

156 Fungal strains *Aspergillus flavus* (NRRL3357), *Penicillium verrucosum* (OTA11),
157 *Eurotium amstelodami* (IMI229971) and *Fusarium graminearum* (FgB (L1-2/2D) were
158 grown on Malt Extract Agar (MEA) for 10 days, except for the *E. amstelodami* strain
159 which was grown on MEA modified to 0.95 a_w. From these cultures spores were
160 collected using 10 mL sterile saline solution containing 0.05% Tween 80 (Acros
161 Organics, USA) and rubbing the surface with a sterile glass rod in order to remove
162 conidia. The spore suspensions were counted using a haemocytometer (Fisher
163 Scientific, United Kingdom) and adjusted to $1x10^6$ spores/ml and directly used to
164 inoculate the cake slices. Each cake slice was centrally inoculated with 3 µl of the spore
165 suspension.

166

167 2.5. Incubation and measurement of growth and data analyses

168

169 Experiments were conducted at 20 and 25^{o}C. In all cases, observations were carried
170 out every two days or as necessary, and the diameter of the growing colonies
171 measured in two directions at right angles to each other. Growth was observed with
172 the aid of a binocular magnifier (Olympus SZ, Olympus, Japan). Measurements were
173 taken for a maximum of 1 month. All experiments were carried out with at least three
174 replicates per treatment.

175

176 The temporal colony diameters were measured and subjected to primary modelling
177 using the linear model. The maximum growth rate (μ_{max})(mm diameter/day) of each
178 fungal treatment on the different cake analogues was determined. Regression lines
179 were made using the time points which represented the linear phase of the growth
180 curves using Microsoft®Excel®:MAC 2011 (14.4.8) (Microsoft Corporation, Redmond,
181 USA). The slope of the linear equation with an associated correlation coefficient of not
182 < R^2 = 0.98 was considered the μ_{max}. Lag times (λ) were calculated by equalling the
183 regression lines to the size of the inoculum point.

184

185 2.6. Statistical analysis

The normality if the data sets was investigated using the Shapiro–Wilk test (α=0.05). This was followed by Levene's test (α=0.05) to determine variance homogeneity. Due to non-normality of the growth data, analysis was performed using non-parametric tests for testing whether distributions across factor levels were centered at the same location. Differences between independent groups at each temperature, using the different stevia formulations, sucrose only and the sucrose-S1 mixture as factors, were examined by the Kruskal–Wallis analysis of ranks. Nonparametric multiple comparison using the Wilcoxon each pair test were performed to identify differences within treatments. When growth occurred only under two conditions, homogeneity of variance was tested and the appropriate t-tests were used. When analysing the shelf-life prior to visible growth datasets values of 30 days were removed to avoid bias.
The statistical package JMP®12.1 Pro (SAS Institute Inc., 2015, Cary NC, USA) was used in the analysis.

3. Results

3.1. Comparison of the water activity of different solutions of sugars and humectants

The three Stevia products (S1-S3) were all able to reduce the a_w of the solutions, although the actual final levels differed significantly (Figure 1). The S1 Stevia product was much more efficient in reducing the a_w in water solutions when compared with S2 and S3. These two products showed a very similar behaviour pattern with regard to modifying a_w.

Figure 1 also shows that the solubility of S1 in water was higher than for the other Stevia treatments (S2 and S3). For this reason, there are more data points. For S1 the solubility limit was very close to 100g/100mL of water. We observed that under these conditions solubility was temperature-dependent and we had immediate precipitation when the solution was cooled. However, product S1 was able to reduce the a_w from 1.00 to 0.871 whilst products S2 and S3 were able to only slightly reduce a_w to 0.986 and 0.987 a_w, respectively.

In comparison to other common compounds used as ingredients in bakery products (sucrose, glucose and glycerol) the S2 and S3 commercial Stevia products were relatively ineffective in decreasing a_w. However, S1 was able to do so. In addition, on a weight/volume basis reductions were higher than those obtained with sucrose and glucose. Among all compounds tested, glycerol was the most effective humectant closely followed by S1 (see Figure 1).

3.2. Comparison between the a_w achieved in the sponge cakes modified with different sugars

The cakes were cooled down for 120 min and then cut into slices. The a_w of the cakes ranged from 0.852 (S1+Sucrose) to 0.971 a_w (S3 alone). This showed that cakes prepared with only sucrose (0.869 a_w), product S1 (0.893 a_w) or their mixture (Sucrose+S1) exhibited lower a_w values when compared with cake analogues baked using S2 (0.949 a_w) or S3 (0.971 a_w).

3.3. Effect of treatments on colonisation by spoilage fungi

Figure 2 shows the effect of treatments at 20 and 25^{o}C on the colonisation rates by the different spoilage fungi. This shows that *E. amstelodami* was particularly tolerant of all the treatments used with colonisation rates faster than for the other spoilage fungi, especially *A. flavus* and *P. verrucosum* at 25^{o}C (p=0.0238 and p<0.001 respectively). Sucrose alone or sucrose + S1 was also effective at reducing relative growth rates of this xerophilic species. *A. flavus* was also able to grow effectively, regardless of treatment at 25^{o}C (p=0.062). However, at 20^{o}C it was inhibited by sucrose alone, S1 and sucrose + S1 (p<0.0001). For *P. verrucosum*, while growth was relatively slower, colonisation was observed for all the treatments at both 20 and 25^{o}C. For both temperatures significant differences where observed between different treatments (20^{o}C p=0.0004, 25^{o}C p<0.0001) where they grew faster with products S2 and S3. *F. graminearum* was the most sensitive species tested and it was only able to grow in the presence of S2 and S3. Between them, growth with S2 was

248 significantly higher (p=0.0043). It was inhibited in the other treatments at both
249 temperatures.
250
251 3.4 Effect of sugar/Stevia treatments on shelf-life prior to visible growth
252
253 Table 2 shows the shelf-life in terms of number of days prior to any visible growth
254 being observed. For both *A. flavus* and *F. graminearum* at both 20 and 25^{o}C the
255 sucrose alone and S1 treatments inhibit growth for the maximum observation period
256 of 30 days. For the latter species, a mixture of sucrose + S1 also controlled growth for
257 30 days. However, the shelf-life in the S2 and S3 products were significantly shorter
258 (at 20^{o}C all p-values for comparisons between S2 and S3 and the other treatments
259 were <0.0275).
260
261 Sucrose was able to completely inhibit growth of *E. amstelodami* at 20^{o}C. For the other
262 treatments, at both temperatures, the shelf-life was <4 days. For *P. verrucosum* shelf-
263 life was very short for all treatments at both temperatures, being <5 days, except for
264 sucrose at 20^{o}C where this was 7 days.
265
266 **4. Discussion**
267
268 This study suggests that while Stevia glycosides may have many positive
269 characteristics as a low calorie sugar substitute, it may not be as effective as part of a
270 hurdle technology approach to control fungal spoilage unless used with other hurdles
271 in intermediate moisture bakery products. It is interesting to note that the a_w
272 reduction of different concentrations of Stevia products was variable. This may partly
273 be because of the formulation of the products themselves and their solubility. Thus
274 while S1 was effective at reducing the a_w in mixed water solutions down to levels
275 achieved with sucrose, glucose and glycerol. However, the S2 and S3 commercial
276 products did not reduce the a_w significantly.
277
278 S1 has only 1% of steviol glycosides, with the bulking agent being erythritol. Erythritol
279 is a 4-C sugar alcohol which acts as a compatible solute in fungi and is a very effective

humectant, almost as good as glycerol. It also occurs naturally in a number of horticultural products, is heat stable up to 160^{o}C, non-caloric and non-glycemic. Studies of a_w tolerance by mycotoxigenic spoilage fungi have shown high levels of biosynthesis of endogenous erythritol as a mechanism of adaptation to low a_w environments (Nesci et al, 2004; Ramirez et al, 2004). Conversely, in Stevia products S2 and S3, 2% of steviol glycosides were bulked with maltodextrin. Maltodextrins are starch-derived α (1→4)-linked glucose polymers up to 7 to 8 glucose units. They are common food additives used as a thickening or filling agent in a range of commercial foods and beverages. It is nearly tasteless but is often described as being slightly sweet. However, the density (g/volume) of both maltodextrin-bulked Stevia-based products was very low, and if the manufacturer recommendations are followed (1 teaspoon is equivalent to 1 teaspoon of sucrose) only 1/10 of the weight is consumed. This, coupled with the inability of maltodextrins to capture water are the reasons why very little reduction of a_w was observed when substituting sucrose with products S2 and S3 in the sponge cake studies. Overall, the lowest a_w was obtained in the cake where the mixture S1+Sucrose was used (0.852 a_w). This was just slightly lower than when comparing with the sucrose-baked sponge cake.

The effect of different sponge cake treatments in terms of fungal colonisation and shelf-life were subsequently standardised to maintain an
a_w of 0.90 to eliminate any differences with regard to the original a_w levels. This allowed us to make comparisons of colonisation rates by the four different fungi. It was clear that the use of Stevia products S2 and S3 supported the colonisation of the sponge cake slices by all the species at both 20 and 25^{o}C.

With these products the spoilage fungi also had very short lag times prior to visible growth suggesting rapid potential for contamination after baking of the product if contaminated with spores of these species. The ability of maltodextrin to enhance fungal growth on plants and potentially increase the amount of mycotoxins has been raised by EFSA when assessing maltodextrin use as an insecticide (EFSA, 2013). Although some reports have described the ability of extracts from *Stevia rebaudiana* (Bertoni) to control fungal growth and mycotoxins production (Garcia et al., 2012), we

have not observed this effect when using purified steviol glycosides. However, the proportion of steviol glycosides present in the products used in this study ranged from 1-2%, which might be far lower than concentrations used in the trials by Garcia et al. (2012) and this may explain their results. It may not be economic at higher concentrations in terms of commercial use. However, fungal spoilage issues may not have been considered when developing these products as substitutes for sucrose in bakery products.

In the reference cake containing 100% sucrose was an excellent hurdle to inhibit and control the initiation of growth of these spoilage fungi, especially *E. amstelodami*, *A. flavus* and *P. verrucosum*. At the lower temperature it was able to completely inhibit all the test species. However, S1 product was unable to stop growth of *E. amstelodami and P. verrucosum*. The partial substitution of sugar made the cake more susceptible to fungal spoilage. Only the *F. graminearum* strain used was completely inhibited by this product at both temperatures.

The shelf-life time prior to initiation of microscopic or visible moulding is critical for bakery products. Sucrose and the S1 product, which included eryhtritol as a bulking agent, were effective in controlling initiation of growth for up to 30 days. However, S2 and S3 were much less effective with very short shelf-lifes prior to visible spoilage becoming visible. This would suggest that unless combined with a range of other hurdles such as preservatives or modified atmosphere packaging, products using these two sugar substitutes would be prone to rapid fungal spoilage (Guenot et al., 2005).

Conclusions

This study suggests that commercial Stevia sugar substitute products alone may not be effective at controlling growth of spoilage fungi in cake-type bakery products. This needs to be taken into account despite the fact that Struck et al. (2014) obtained good results in terms of bakery product quality when substituting sucrose with Stevia-based products. This study suggests that modified bakery product formulations which

incorporate Stevia products to reduce the overall sugar content needs to take into account potential shorter shelf-life issues with regard to fungal spoilage in such intermediate moisture bakery products. This may increase the relative risk for shorter shelf-life of such products. Perhaps the use of such products in home baking requires some additional information for consumers in relation to storage conditions to minimise the risks of fungal spoilage in the domestic environment.

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419 Table 1: General specific information in the products label with regard to weight,
420 composition manufacturer, use guidelines and energy
421

Product	Weight of the package	Composition	Manufacturer dosage recommendations	Energy values per 100 g
S1	270 g	Bulking Agent: Erythritol Sweetener: Steviol Glycosides (1% Stevia Leaf Extract), Natural Flavourings	1/3 teaspoon sweetens like 1 teaspoon of sugar	0 kJ (0 kcal)
S2	75 g	Bulking Agent: Maltodextrin Sweetener: Steviol Glycosides (2%), Natural Flavourings	1 teaspoon (2 kcal) is equivalent in sweetness to one teaspoon of sugar (20 kcal)	1598 kJ (376 kcal)
S3	75g	Bulking Agent: Maltodextrin Sweetener: Steviol Glycosides (2%)	1 teaspoon is equivalent in sweetness to one teaspoon of sugar	1656kJ (390kcal)

422
423
424
425

426 **Figure Legends**
427

428 Figure 1. Comparison of the effect of different solutions (0-180 g solute/ 100 ml of
429 water) of sucrose, glucose, glycerol, and Stevia-based products S1, S2 and S3 on the
430 water activity levels.

431

432 Figure 2. Effect of temperature on the lag phases (days) of *A. flavus*, *F. graminearum*,
433 *E. amstelodami* and *P. verrucosum* on the cake analogue slices over a 1 month
434 incubation period. *Denotes conditions at which the lag time was longer than the
435 duration of the experiment.

436

437 Figure 3. Effect of temperature on the growth rate (mm diameter/day) of *A. flavus*, *F.*
438 *graminearum*, *E. amstelodami* and *P. verrucosum* on the cake analogue slices over a 1
439 month incubation period.

440

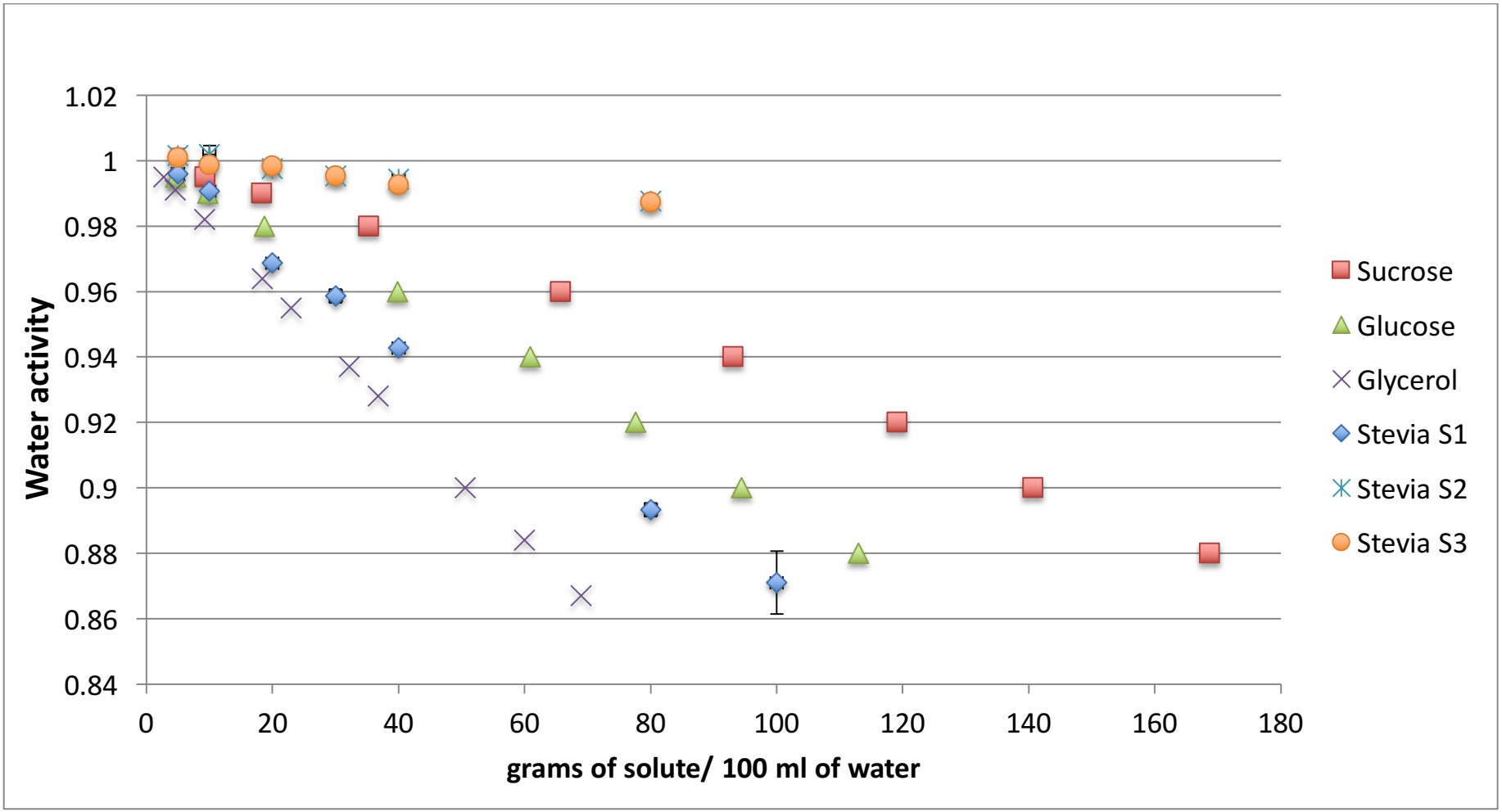
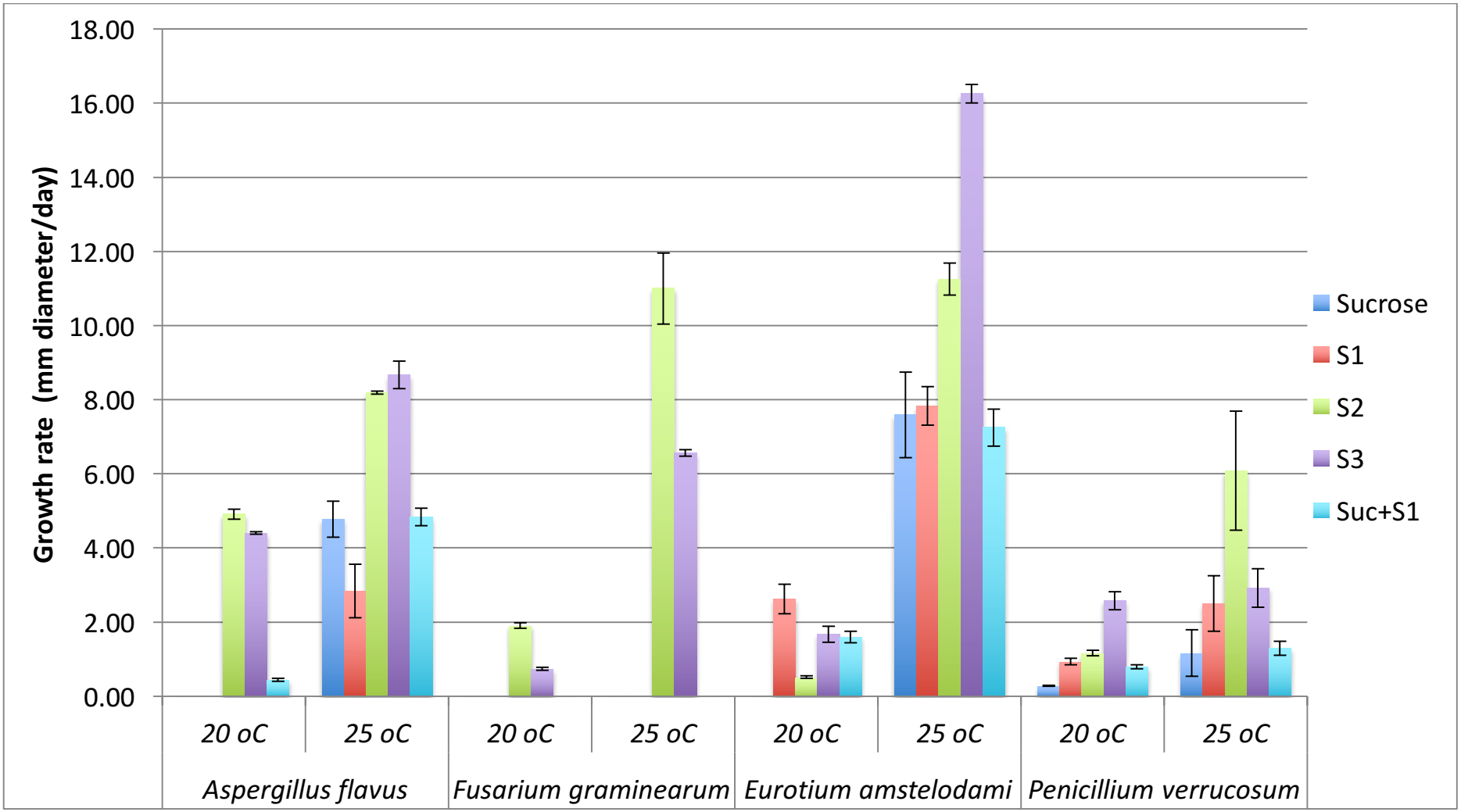
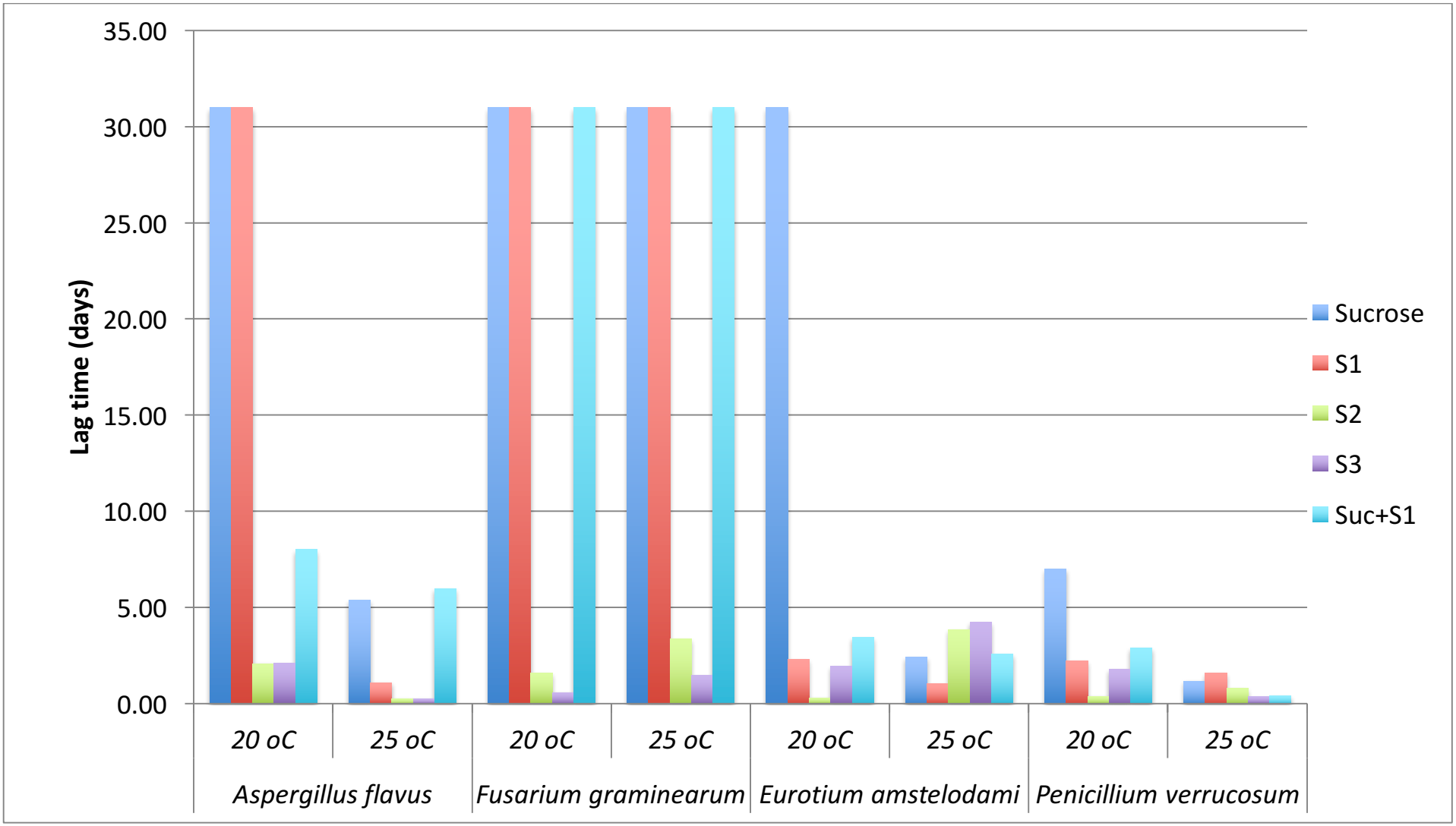


Figure 1 Rodriguez et al


Growth rate (mm diameter/day)
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Sucrose
S1
S2
S3
Suc+S1
20 oC
25 oC
20 oC
25 oC
20 oC
25 oC
20 oC
25 oC
Aspergillus flavus
Fusarium graminearum
Eurotium amstelodami
Penicillium verrucosum


35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Lag time (days)
Sucrose
S1
S2
S3
Suc+S1
20 oC
25 oC
20 oC
25 oC
20 oC
25 oC
20 oC
25 oC
Aspergillus flavus
Fusarium graminearum
Eurotium amstelodami
Penicillium verrucosum

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http://dspace.lib.cranfield.ac.uk/handle/1826/10055