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ORIGINAL ARTICLE

Protective effect of hop β-acids on microbial degradation of thick juice during storage

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Abstract

Aims: This study assessed the value of a commercial alkaline solution of hop β-acids (HBA) for prevention of microbial degradation of thick juice, a concentrated intermediate product in the production of beet sugar.

Methods and Results: The antimicrobial effect of different concentrations of HBA against microbial degradation of thick juice was tested in a pilot-scale storage experiment. Chemical, biochemical and microbial parameters were monitored during thick juice storage. Thick juice degradation, indicated as a decrease in pH, was generally accompanied by an increase in the count of fastidious bacteria (FB) on Columbia Agar with Sheep Blood (CAwSB), which were mainly identified as *Tetragenococcus halophilus*. Addition of HBA delayed juice acidification and the development of FB in a concentration-dependent manner. The susceptibility of FB to HBA was determined by plating degraded thick juice (FB $> 10^5$ CFU ml^{-1}) on CAwSB plates with different concentrations of HBA (0–160 ppm). None of the HBA concentrations tested reduced the number of FB colonies formed, but increasing HBA concentrations extended the lag time of colony formation.

Conclusions: HBA produce no measurable bactericidal effect, but retard the development of FB in thick juice. Moreover, HBA do not prevent the thick juice from deteriorating, but significantly delay its degradation.

Significance and Impact of the Study: These results indicate that adding a commercially available HBA formulation can prolong the storage life of thick juice in the sugar industry, although degradation cannot be eliminated. Future research will focus on the detailed characterization of FB consistently isolated from degraded thick juice and on determining their role in thick juice degradation.

Introduction

Storing thick juice beyond extraction and refining has been commonly practiced by many sugar companies worldwide since it was first introduced in 1960 in the USA. Earlier research and industrial practice (Asadi 2006) have demonstrated that thick juice stability is best managed by controlling parameters such as degrees Brix (°Bx; % soluble dry substance in a liquid), pH and temperature, since these parameters directly influence microbial growth (Willems *et al.* 2003). However, even under good storage practices, thick juice degradation resulting from microbial contamination still occurs. The most pronounced symptoms of degradation are a reduction in pH from pH 9 to pH 5 to 6 and typically, an increase in reducing sugar (RS) content (Sargent *et al.* 1997; Willems *et al.* 2003), resulting in financial loss.

Although the use of formalin and dithiocarbamates as technical aids is allowed in the sugar refinery, most sugar companies voluntarily avoid these chemicals in favour of more natural, harmless alternatives. In keeping with the concept of a chemical-free refinery, natural biocides are increasingly being screened for their antimicrobial effects during thick juice storage (Hein *et al.* 2006). Products derived from the hop plant (*Humulus lupulus* L.) were first successfully used in the sugar industry in 1994 to combat bacteria in beet extraction (Pollach *et al.* 1996). These natural, lipophilic hop components are considered harmless to humans and mammals and are even important components of beer (Sakamoto and Konings 2003).

Hop acids affect Gram-positive bacteria, but have no effect on their endospores or on Gram-negative bacteria, though there are some exceptions (Hollaus *et al.* 1997). Hop acids are believed to affect bacteria by disrupting the proper functioning of the membrane (Teuber and Schmalrek 1973) and by reducing the intracellular pH (Simpson and Hammond 1991; Blanco *et al.* 2006). Since the typical bacteria linked to thick juice spoilage are mainly Gram-positive (Hein *et al.* 2002; Willems *et al.* 2003), hop acids have attracted attention as potential biopreservatives for this product.

Currently, a 10% aqueous alkaline solution of hop β-acids (HBA) is available commercially to sugar refineries under the label Betastab® 10A (Beddie *et al.* 2004). HBAs have emerged as highly effective agents against formation of NO_2 and anaerobic infections in tower extractors, which are often operated intentionally under lactic acid fermentation conditions. Under slightly alkaline conditions, HBAs occur more dissociated and thus, are presumably less effective (Simpson and Fernandez 1994; Larson *et al.* 1996). On the other hand, they are more soluble under these conditions. HBAs have already shown surprising suppressive effects against *Thermus* spp. that can cause increases in NO_2 levels in thin juice, the purified beet juice containing approximately 15% sucrose which is evaporated till thick juice (Pollach *et al.* 2002).

The protective effects of HBA in thin juice kept at slightly alkaline conditions prompted other studies aimed at determining whether similar effects could be induced by HBA during thick juice storage, where slightly alkaline conditions also prevail. Both Pollach *et al.* (1999) and Hein *et al.* (2002) observed that addition of HBA to thick juice slows down microbial activity. In these studies, treatments of 3 and 6 ppm HBA in 60°Bx thick juice considerably delayed invert sugar and acid formation. However, industrial thick juice is typically stored between 68 and 70°Bx. Therefore, the primary objective of the research reported herein was to investigate whether a commercially available HBA formulation also retards microbial growth in and subsequent degradation of thick juice under industrial conditions.

Materials and methods

Thick juice storage experiments and sampling

Long-term thick juice storage experiments were conducted to monitor degradation and microbial dynamics using pilot-scale storage tanks. Each of these tanks consisted of an upright, sealable acrylic central cylinder (dimensions: $Ø_i$: 20 cm, H_i: 200 cm, V_i: 62·8 l) surrounded by a temperature-controlled circulating water mantle of 27·7 l. The tanks were each equipped with five taps, equally positioned from the bottom to the top, allowing aseptic sampling at different levels of the cylinder. For all pilot-scale experiments, 69°Bx thick juice was collected in sterile 25 l polyethylene buckets directly from a commercial processing system (Raffinerie Tirlemontoise, Belgium; RT). Samples were taken immediately after the evaporation and cooling stage. Thereafter, the buckets were sealed and stored at 4°C until use. When necessary, °Bx and pH were adjusted in thick juice with sterile demineralized water and 1 N NaOH, respectively.

Preliminary experiment

In an initial experiment, the effects of the starting °Bx content and pH on thick juice storage quality were studied. Thick juice in all tanks were incubated for 168 days at 25°C, using a factorial design for 13 cylinders (Systat software, Inc., San Jose, CA, USA) (Table 1A).

Hop experiment

In a subsequent experiment, the effect of different HBA concentrations on thick juice incubation at three relevant, challenging temperatures of 20, 25 or 30°C was studied using a fractional factorial design for nine cylinders (Systat software, Inc.) (Table 1B). The 69°Bx thick juice had an initial pH of 8·7.

For the HBA experiment, untreated thick juice was placed into four pilot-scale tanks that were designated as untreated controls (i.e. C3/C4/C5/C6). A commercial HBA product (BetaStab® 10A, BetaTec Hop Products, Nürnberg, Germany) was mixed into several thick juice buckets to achieve homogeneous concentrations of 20 or 40 ppm HBA (Bhattacharya *et al.* 2003). The 20 ppm HBA-treated thick juice was placed in one storage tank (i.e. C9), while the 40 ppm HBA-treated thick juice was decanted into each of the four remaining storage tanks (i.e. C1/C2/C7/C8). All thick juice cylinders were covered with a lid, creating a headspace of about 5 l air allowing circulation in and out of each cylinder. Two control tanks and two 40 ppm HBA-amended thick juice

Table 1 Experimental design of the pilot scale thick juice storage experiments. (A) C1–C13 represent the 13 cylinders from the preliminary Brix experiment at 25°C with their corresponding initial Brix (°) and pH. (B) C1–C9 represent the nine cylinders from the hop experiment with their corresponding temperature (°C) and initial hop ß-acid concentration (ppm)

A

	Brix (°)		
pH	65	67	69
9·2	C7/C8	C10	C3/C4
9·0	C9	C13	C11
8·8	C5/C6	C12	C1/C2

B

	Hop β-acid concentration (ppm)		
Temperature (°C)	0	20	40
30	C5/C6		C7/C8
25		C9	
20	C3/C4		C1/C2

tanks each were incubated at 20°C (C3/C4 and C1/C2) and 30°C (C5/C6 and C7/C8), respectively. The single 20 ppm HBA-treated thick juice tank (C9) was incubated at 25°C. All thick juice cylinders were stored for 272 days. Weekly, 20 ml thick juice samples were drawn aseptically into sterile screw cap tubes from the central tap of each cylinder and analysed for pH, microflora and RS content. Previous experiments indicated that there were no significant differences between samples taken from different taps during these prior thick juice storage assays (Justé, unpublished).

Additional thick juice storage laboratory experiment with autoclaved thick juice

In an additional experiment, two 400 ml thick juice samples with 69 °Bx were autoclaved for 15 min at 121°C in 500 ml Scott flasks and stored aerobically at 30°C. The evolution of pH was followed to investigate possible non-microbiological causes for the drop in pH during storage. During 45 days of storage, 10 ml thick juice samples were drawn aseptically twice a week and analyzed for pH.

Microbiological analyses

Table 2 presents the microbiological parameters monitored during thick juice storage as well as the incubation conditions used. Samples were analysed using ISO standard microbiological methods (Willems *et al.* 2003). Media were prepared according the supplier's instructions, except for the tetracycline glucose yeast extract agar where chloramphenicol was used in place of oxytetracycline or gentamycin (100 mg l^{-1}) as prescribed by the ISO standard 7945 method. De Whalley agar (DWA) contained l^{-1} deionized water: 5·0 g yeast extract (Oxoid Limited, Basingstoke, England); 2·0 g casein peptone (Serva, Heidelberg, Germany); 1·0 g glycerol (Merck, Darmstadt, Germany); 20·0 g glucose (Merck); 400·0 g sucrose (Merck) and 16·0 g agar (Oxoid). The term 'fastidious bacteria' (FB) is defined here as a collective term for those bacteria that form colonies on Columbia Agar with Sheep Blood (CAwSB, Oxoid) after 3 to 6 days of incubation at 30°C.

Biochemical and chemical analyses

Hop β-acid concentrations were determined with the HPLC-based method described by Hein and Pollach (1997). Briefly, HBA from 10 ml thick juice samples were extracted twice with hexane. Subsequently, extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The residue of each sample as well as HBA standards were dissolved in methanol and subjected to HPLC analysis (RP-18, Nucleosil 100-5 C18 HOP, Machery-Nagel). The eluent consisted of methanol, water, *o*-phosphoric acid and 0·1 mmol l^{-1} EDTA at volumetric ratios of 1700 : 350 : 5 : 2, respectively. Detection was performed at 270 nm.

Levels of the RS glucose and fructose were determined using a commercial enzymatic assay (D-glucose/D-fructose assay, r-biopharm AG, Darmstadt, Germany).

Table 2 Microbiological parameters, growth media and aerobic incubation conditions for monitoring different groups of micro-organisms during long-term thick juice storage

Microbiological parameter	Growth media	Incubation temperature (°C)	Incubation time (days)
Aerobic colony count	Plate count agar (PCA)	30	3
Lactic acid bacteria	De Man Rogosa Sharpe agar (MRSA)	30	3
Yeasts and moulds	Oxytetracycline glucose yeast extract agar (OGYEA)	25	5
Osmophilic flora	De Whalley agar	25	5
Fastidious colony count	Columbia agar with 5–7% sheep blood (CAwSB)	30	6

Sequencing of bacterial 16S rRNA genes

Sequencing was performed on purified PCR products from pure colonies obtained with the universal bacterial primers 27f and 1492r (Lane 1991). Samples were analysed on an Applied Biosystems 373A Automated Sequencer. Sequences were compared to online databases by using the BLAST program located at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) (Altschul *et al.* 1997; Benson *et al.* 2004).

Susceptibility of fastidious bacteria to hop β-acids

Thick juice samples collected after 169 days of storage from pilot-scale tanks C3 (0 ppm, 30°C) and C9 (20 ppm, 25°C), which contained approximately 10^5 CFU FB ml^{-1}, were used in a dose–response sensitivity assay in search of the minimum concentration of HBA required to inhibit FB colony formation (minimum inhibitory concentration, MIC). Both treated and untreated thick juices were plated to test the difference in hop resistance between their FB. Appropriate dilutions of the samples were plated on CAwSB containing HBA in concentrations varying between 0 and 160 ppm. Plates were incubated for 18 days at 30°C and colonies were counted after 3, 4, 5, 6, 7, 10, 14 and 18 days of incubation.

Data analysis

Data from the thick juice storage experiments were analysed by standard linear regression using the Statistical Analysis Software System (SAS Institute, Inc., Cary, NC, USA). Three parameters during thick juice storage were analysed as response variables: the first day of FB detection, the start of thick juice degradation defined as the day the pH had decreased by more than 1 unit, and the highest concentration of FB detected during the experiment. Initial HBA concentration and incubation temperature were considered as explanatory variables. The Shapiro-Wilk test was applied to test for a normal distribution of the residues and results were considered significant at a 95% confidence level ($P < 0{\cdot}05$).

Results

Effect of total soluble solids (°Bx) on thick juice degradation

In the preliminary experiment, the storage of thick juice at 65, 67 and 69°Bx was compared by regularly monitoring the pH evolution in pilot-scale storage tanks. Sugar refineries typically consider pH one of the more reliable indicators of thick juice quality during storage. Figure 1 demonstrates a rapid decline from almost pH 9·0 to pH 5·0 in less than 50 days in the 65°Bx thick juice, and a more gradual and less pronounced pH decline to pH 7·0 occurred after more than 150 days in the 69°Bx thick juice. The pH reduction and rate of decline observed in the 67°Bx thick juice were intermediate compared to those in the 65 and 69°Bx thick juice storage. This experiment highlights the relevance of the amount of total soluble solids for the degradation of thick juice. Since storage of thick juice in industrial practice is common at 69°Bx, thick juice with this soluble solids value was used for the remainder of experiments in this study.

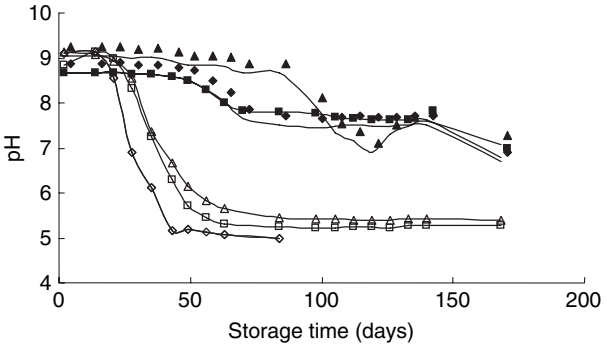

Figure 1 Temporal pH dynamics of 65° and 69°Bx thick juice during long-term storage in pilot-scale tanks at 25°C. ■ 65°Bx, pH_i = 9·2; ◆ 65°Bx, pH_i = 9·0; ▲ 65°Bx, pH_i = 8·8; ◇ 69°Bx, pH_i = 9·2; ○ 69°Bx, pH_i = 9·0; △ 69°Bx, pH_i = 8·8.

Stability of hop β-acids during thick juice storage

In order to detect potential breakdown of the HBA added to the thick juice, HBA concentrations were monitored throughout this pilot-scale storage experiment. The HBA concentration was relatively stable over the entire 260-day storage period studied, though a slightly declining trend could be observed overall. This small decline in HBA concentration was more pronounced with storage at 30°C than at 20°C, with residual concentrations of respectively 69 and 77% after 260 days of storage.

Effect of hop β-acids and temperature on thick juice degradation

In order to study the effects of HBA concentration and temperature on thick juice degradation, 69°Bx thick juice was subjected to multiple HBA concentration and incubation temperature treatments. Thick juice cylinders treated with HBA (C1/C2, C7/C8, C9) remained free of measurable thick juice degradation significantly longer ($P = 0{\cdot}02$)

Table 3 Summary of fastidious bacteria (FB) populations in relation with thick juice degradation

Concentration hop β-acid (ppm)	Temperature (°C)	Cylinder	Time to FB detection* (days)	Maximum level of FB (CFU ml^{-1})	Start thick juice degradation† (days)
0	20	C3	28	2×10^8 (day 76)	37
0	20	C4	23	3×10^8 (day 28)	34
0	30	C5	13	1×10^8 (day 28)	23
0	30	C6	13	1×10^9 (day 23)	23
20	25	C9	113	1×10^6 (day 134)	111
40	20	C1	190	6×10^2 (day 190)	272
40	20	C2	204	2×10^2 (day 204)	197
40	30	C7	198	1×10^3 (day 245)	61
40	30	C8	139	7×10^2 (day 139)	61

*Detection limit = 100 CFU ml^{-1} thick juice.

†'Start thick juice degradation' is defined as the day that the pH declined more than 1 unit below the initial pH.

than those cylinders lacking HBA treatment (Table 3). At 20°C, thick juice degradation started after 34 (C4) and 37 (C3) days in the untreated control treatments, whereas degradation occurred only after 197 (C2) and 272 (C1) days in the 40 ppm HBA treatments (Table 3). The pH of the thick juice in the untreated control columns stabilized at pH 5·0, while the pH in the HBA-treated columns had not yet stabilized within 272 days (Fig. 2). At 30°C, thick juice degradation started after 23 days in the untreated thick juice (C5/C6), and after 61 days in thick juice initially containing 40 ppm HBA (Table 3), indicating that thick juice was significantly less stable when stored at 30°C than at 20°C ($P = 0{\cdot}04$).

In general, the amount of RS increased during thick juice storage, demonstrating loss of sucrose (data not shown). However, the thick juice in cylinder C3 was heavily degraded but exhibited almost no increase in RS. Furthermore, neither pH nor any of the microbiological parameters measured during this experiment correlated with RS values.

Effect of hop β-acids on thick juice microflora

Counts on De Man Rogosa Sharpe (MRS) agar for lactic acid bacteria and OGYE/DWA counts for moulds were either zero or very low (< 100 CFU ml^{-1}) in all cylinders (data not shown). Levels of aerobic bacteria, yeasts and osmophilic bacteria were relatively low and varied from 10^3 to 10^4 CFU ml^{-1}. In addition, all these populations remained relatively stable during thick juice storage (data not shown), regardless of whether HBA was added or not. However, the maximum FB level was inversely related to the measured HBA concentration ($r = -0{\cdot}98$). HBA treatments also significantly prolonged the time to detect the first FB ($P < 0{\cdot}01$) and the time before the start of thick juice degradation ($P = 0{\cdot}02$), as illustrated in Table 3. In addition, all FB detected were confirmed to be Gram-positive cocci, and all micro-organisms recovered on DWA were yeasts.

The FB levels in untreated thick juice peaked at 10^8 to 10^9 CFU ml^{-1} within 30 days of storage at 30°C, and then declined thereafter (data not shown). This growth peak coincided with a considerable pH decline, indicating thick juice degradation. Growth of FB at 20°C was slower than that at 30°C, corresponding to a more moderate pH decline. In thick juice treated with 40 ppm HBA, FB were only detected after 139 (30°C) and 204 (20°C) days of storage, whereas addition of 20 ppm HBA delayed the growth of FB in 25°C stored thick juice until day 113 (Table 3). Moreover, significantly lower levels of FB ($P < 0{\cdot}01$) were detected with increasing HBA concentrations, suggesting a potential relation between FB level and thick juice degradation.

Nevertheless, as only few FB were detected in thick juice treated with 40 ppm HBA and since thick juice degradation already occurred at day 61 at 30°C storage temperature (Table 3), an additional or other reason than FB

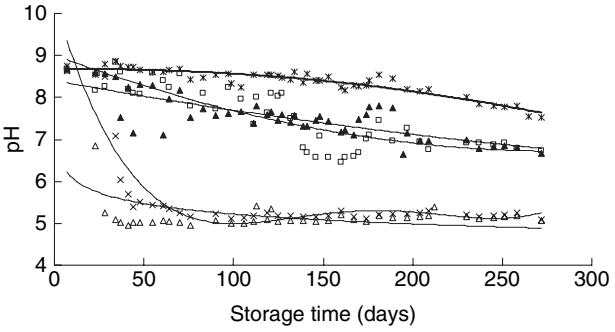

Figure 2 Comparison of the pH-decline in thick juice stored at various temperatures and treated with different concentrations of hop ß-acids, i.e. 0, 20 or 40 ppm. Duplicate cylinders are presented by their mean values. *40 ppm, 20°C; ▲ 40 ppm, 30°C; □ 20 ppm, 25°C; x 0 ppm 20°C; Δ 0 ppm, 30°C; — trend line.

level may be responsible for this pH decline. Since almost no micro-organisms were detected in this treated thick juice (C7/C8), except for a stable and relatively small yeast population, the hypothesis that this acidification could be caused by a nonmicrobial process was tested. Therefore, 400 ml fresh thick juice was sterilized in duplicate by autoclaving for 15 min at 121°C. Subsequent incubation at 30°C for 45 days showed an identical evolution of pH as measured both in cylinder C7 and C8 (40 ppm hop extract, 30°C; data not shown). No bacteria were detected in this thick juice upon plating, suggesting a nonmicrobial cause of the pH decline.

Preliminary identification of the fastidious bacteria (FB)

To have a first, quick identification of the dominant culturable microflora, 18 FB isolates from both hop-treated and untreated thick juice were subjected to sequence analysis of 16S rRNA genes. The 18 sequences thus obtained, have been deposited in the EMBL/Genbank/DDBJ Nucleotide Sequence Database under accession numbers EF595636–EF595649 and EF620529–EF620532. Comparison to this online database by using the BLAST program revealed a relatively limited bacterial population as presented in Table 4.

Sensitivity of fastidious bacteria to hop β-acids

Under the assumption that a causal correlation exists between FB level and pH decline, i.e. thick juice degradation, the effect of HBA on these FB and, in particular, the question whether FB would be able to adapt to hop extracts upon prolonged exposure, are of great interest. Therefore, the minimal concentration of HBA required to inhibit the growth of FB on CAwSB plates up to 18 days of incubation at 30°C was determined using FB populations from both hop-treated and untreated thick juice sampled from the previous experiment.

As presented in Table 5, the time to FB colony development increased with increasing HBA concentration up to 80 ppm. A further increase of the HBA concentration to 160 ppm had no additional inhibitory effect against FB, and none of the hop extract concentrations tested could suppress FB colony formation longer than 10 days. Furthermore, at all concentrations of HBA, the same level of FB was ultimately recovered on CAwSB after the maximum incubation time of 18 days. Remarkably, colony development of FB from HBA-treated thick juice was slower than that of FB from untreated thick juice.

Table 4 Preliminary identification of 18 'fastidious bacteria' (FB) based on 16S sequences after comparison to online databases by using the BLAST program (http://www.ncbi.nlm.nih.gov)

Cylinder	Presumptive ID	Genbank no.
C1	*Tetragenococcus halophilus*	EF595636
C1	*T. halophilus*	EF595637
C2	*T. halophilus*	EF595638
C2	*T. halophilus*	EF595639
C3	*T. halophilus*	EF595640
C3	*Staphylococcus* spp	EF595641
C4	*T. halophilus*	EF620529
C4	*T. halophilus*	EF595642
C5	*S. equorum*	EF595643
C5	*T. halophilus*	EF620531
C6	*T. halophilus*	EF595644
C6	*T. halophilus*	EF595645
C7	*T. halophilus*	EF595646
C7	*T. halophilus*	EF595647
C8	*T. halophilus*	EF595648
C8	*T. halophilus*	EF595649
C9	*Aerococcus viridans*	EF620532
C9	*T. halophilus*	EF635437

Table 5 Susceptibility of Fastidious Bacteria (FB) isolated from thick juice to Hop-β-acids. Thick juice from two cylinders (C3/0 ppm, 20°C and C9/20 ppm, 25°C) was plated on CAwSB with concentrations hop extract between 0 and 160 ppm. Plates were counted on incubation day 3, 4, 5, 6, 7, 10, 14 and day 18 (final FB level detected)

Hop β-acid concentration (ppm)	Time to FB detection* (day)		80% of inoculum detected (day)		Final FB level detected (day 18) (CFU ml^{-1})	
	C3	C9	C3	C9	C3	C9
0	3	5	5	6	4×10^5	1×10^5
20	4	6	6	10	3×10^5	1×10^5
40	6	10	10	14	3×10^5	1×10^5
80	7	10	10	14	3×10^5	1×10^5
120	7	10	10	14	3×10^5	1×10^5
160	6	10	10	14	2×10^6	1×10^6

*Detection limit = 100 CFU ml^{-1} thick juice.

Discussion

Storing sugar extracts as thick juice is common practice in the sugar refining industry. Even when good storage practice is followed, microbiologically induced thick juice degradation occasionally occurs (Sargent *et al.* 1997). In a preliminary experiment of this study, the importance of the initial solids content (Brix index) in thick juice storage is highlighted (Fig. 1), endorsing the industrial practice of storing at high Brix indices of about 69°Bx. The initial pH of the juice varied between pH 8·8 and 9·2 and showed less influence than the Brix index, although pH 9·0 and 9·2 slightly prolonged stable storage at 69°Bx, compared to pH 8·8. Pollach *et al.* (1999) and Hein *et al.*

(2002) showed a positive effect of HBA during 65 days of storage at 60°Bx, which is industrially irrelevant. Considering the different pH development in thick juice stored at 65 and at 69°Bx (Fig. 1), it is not unlikely that microfloras dominating at 60 and at 69 °Bx could be different during 2 months storage. Therefore, this research investigated the effect of hop extract on thick juice with an industrially relevant Brix index throughout 272 days of storage, covering the whole period of industrial thick juice storage. Willems *et al.* (2003) suggested that preservatives based on hydrogen peroxide, sulfur and formaldehyde are rapidly inactivated in bulk thick juice since their addition had only a limited effect on the stable storage life of thick juice. Formaldehyde for example, was completely degraded after 14 days of storage (Willems, unpublished data). In contrast, HBA proved very stable in 69 °Bx thick juice during the entire storage period of our experiments, and is in fact the first biocide shown to remain stable during thick juice storage.

Addition of hop extract significantly ($P < 0{\cdot}02$) delayed thick juice degradation as measured by the reduction in pH, and our results thus extend the initial observations of Pollach *et al.* (1999) and Hein *et al.* (2002) to thick juice at an industrially relevant Brix index of 69°Bx. Furthermore, although HBA are generally more effective in acidic foods (Larson *et al.* 1996), our study indicates that they can also work well in a slightly alkaline matrix such as thick juice.

Our results also confirm the crucial role of temperature during thick juice storage (Sargent *et al.* 1997; Willems *et al.* 2003) highlighting the increased risk of degradation above 20°C. Storage at 20°C clearly guarantees a longer stability of the thick juice compared to 25°C or 30°C ($P < 0{\cdot}05$).

During storage of untreated thick juice, a clear correlation ($r = 0{\cdot}99$) emerged between the drop in pH and the presence of FB. No relation was found with any other microbial parameter tested in our study. Generally, spoilage of sugar-rich foods is caused by osmophilic yeasts, particularly at an $a_w < 0{\cdot}85$ as in honey, syrups, jams, marmalades and dry fruits, since bacteria cannot grow at such low a_w values (Grant 2004). Some bacteria, including *Staphylococcus* spp., *Micrococcus* spp., *Pediococcus* spp, *Corynebacterium* spp., are able to grow at a_w values of 0·85–0·90, but these tend to be halophiles rather than osmophiles (Ventosa *et al.* 1998). The finding that the 69°Bx thick juice used in this study, with its a_w value of 0·87, favoured the development of a large bacterial flora during degradation was therefore unexpected. The FB in the degraded thick juice were Gram-positive, catalase-negative cocci as detected earlier in thick juice (Hein *et al.* 2002; Willems *et al.* 2003). Identification of 18 FB isolates revealed a rather limited diversity (Table 4). *Tetragenococcus halophilus* was most frequently isolated in both hop-treated and untreated thick juice, besides some *Staphylococcus* spp. and *Aerococcus viridans*. *Tetragenococcus halophilus* has been isolated primarily from salted food products such as fermented mustard (Chen *et al.* 2006), shoyu mash (Hanagata *et al.* 2003) and salted anchovies (Villar *et al.* 1985), but also once from thick juice, together with *Staphylococcus* spp. and *Aerococcus viridans* (Willems *et al.* 2003). Since *T. halophilus* is a homofermentative lactic acid bacterium, the pH decline during thick juice degradation may be explained by the production of lactic acid. It is not surprising however, that *T. halophilus* could not be detected on MRS plates, since it is known that for enumeration of *T. halophilus* on MRS, the pH should be adjusted to pH = 7·0 and 4 – 6% NaCl should be added (Holzapfel *et al.* 2006).

Yeasts were also consistently present in the thick juice, but their concentration remained constant in all cylinders at 10^3 to 10^4 CFU ml^{-1} throughout thick juice storage and degradation. Yeasts can thus be excluded as the cause of thick juice degradation, at least in the experiments conducted in this study.

The addition of hop extract to thick juice delayed both juice degradation (pH fall) and the development of FB. To our knowledge, nothing is known about the resistance of *T. halophilus* to hop constituents. The phylogenetically related genus *Pediococcus*, from which *T. halophilus* was separated to form a new genus (Collins *et al.* 1990) contains seven species and only one is known to be hop-resistant (Fernandez and Simpson 1993). However, hop resistance is not a species-specific property, but rather a property found in individual strains in different species (Behr *et al.* 2006).

The observed delay increased with increasing hop concentration and decreasing storage temperature. However, the link between the development of FB and the degradation of thick juice was not perfect. For example, thick juice treated with 40 ppm hop extract (C1 and C7, Table 3) decreased slowly in pH before any FB were detected. Since autoclaved thick juice, in which no viable bacteria could be detected, showed a similar evolution in pH as the 40 ppm hop-protected thick juice, the hypothesis is formulated that this slow decline of pH is caused by a nonmicrobiological process. The nature of this process is not yet clear, but it is known, for example, that in extremely alkaline environments, sucrose is transformed into lactic acid by a purely chemical reaction (Manley-Harris *et al.* 1980). Therefore, this slow decrease in pH might be inherent to the conditions prevailing within the thick juice during storage. On the other hand, cylinder C9 (20 ppm, 25°C) contained 10^5 to 10^6 CFU ml^{-1} FB, but pH declined only slightly (data not shown). Since the microflora in the different cylinders were not identified in

detail, it cannot be excluded that the FB in the hop-treated cylinder C9 could be different from those in the untreated thick juice.

However, a more plausible explanation is that acidification was less pronounced in cylinder C9 because the maximal level of FB reached in this cylinder was still 100 to 1000 times less than that in the untreated thick juice (C3 and C6) (Table 3). Taken together, these results show that HBA can be used to suppress thick juice degradation under conditions similar to industrial storage. Moreover, as β-acids are not active against yeasts and moulds (Pollach *et al.* 1999), these results strongly implicate the FB as the cause of degradation. This is the first report that clearly relates thick juice degradation with a specific microbial population.

The susceptibility of the FB flora, mainly represented by *T. halophilus*, to hop β-acids was further determined. Up to 160 ppm of β-acids, the FB were not inactivated, but their colony formation on CAwSB was delayed. Remarkably, samples of FB from column C9 (25°C, 20 ppm hop extract) showed slower colony development on the hop-containing CAwSB plates than those taken from column C3 (30°C, untreated control). This indicates that extended exposure to hop components in thick juice did not lead to adaptive tolerance in the FB, but rather to the opposite, i.e. increased sensitivity. The FB community in thick juice did not change upon addition of hop components, as was confirmed by preliminary identification of 18 isolates, but may have become stressed by exposure to the hop extract which may explain the delayed colony formation upon plating (Lambert and van der Ouderaa 1999). Although selection for resistance to hop components has been reported (Haas and Barsoumian 1994; Larson *et al.* 1996), it apparently did not occur in our experiments.

Notably, untreated thick juice showed a more pronounced decline of pH than in previous thick juice storage experiments published by Sargent *et al.* (1997) or Willems *et al.* (2003), where typical end points between pH = 7·5 and 6 were recorded, compared to pH = 5 in our experiment. This seemingly inexplicable variation may be related to a difference in the causal microflora since the thick juices have been obtained from different locations and different harvest years. On the other hand, this low pH may explain why the FB in our experiments declined or even disappeared after they reached their peak density. Still, another possibility is that a metabolite produced by other organisms inhibits these FB.

Although most of the thick juice cylinders showed a considerable rise of RS during storage, no clear relation could be found with the drop in pH in our experiment since at least one cylinder showed a steep pH decrease without liberating RS. Perhaps this discrepancy may be due to different metabolic capacities of the FB selected in the columns. Following up the sucrose concentration and a full and detailed characterization of the group of FB might clarify the experimental data.

Further work to identify and characterize the nature of the FB flora in detail is ongoing and will contribute to the elucidation of their role in thick juice degradation. Identification of the causal microflora will be pivotal to early prediction and control of thick juice degradation in the sugar industry.

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