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Optimizing red sorghum malt quality when *Bacillus subtilis* is used during steeping to control mould growth

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Previous work has shown that *Bacillus subtilis*-S499-based biocontrol treatments applied without aeration at the steeping stage of red sorghum malting offer good mould reduction, but yield malts with low levels of key hydrolytic enzymes. Thus we attempted to raise these levels by aerating the steeping liquor, varying the steeping time (from 8 to 40 h) and temperature (from 25 to 35 °C), and combining a biocontrol treatment with prior steeping in 0.2% NaOH. Aeration proved particularly important whenever *B. subtilis* cells were present in the steep liquor. The optimal temperatures for α- and β-amylase were 30 and 25 °C, respectively. By increasing the steeping time, it was possible to improve the α-amylase activity, but the β-amylase activity peaked sharply between 16 and 20 h, depending on the steeping medium. A good compromise was steeping in a biocontrol medium for 14–16 h at 30 °C. Combination steeping treatments (0.2% NaOH for 8 h followed by biocontrol for 8 h) yielded malts of a quality approaching that afforded by dilute alkaline treatment. Copyright © 2012 The Institute of Brewing & Distilling

Supporting information can be found in the online version of this article.

Keywords: red sorghum; biocontrol; mould reduction; malt quality; amylases; *Bacillus subtilis*

Introduction

In brewing, mould contamination of the grains used to produce malt can lead to the introduction of mycotoxins into the brewing process. Mycotoxins constitute a health hazard for consumers *(1–3)*. They can also affect the brewing yield *(4)*, the physiological state of the yeast *(4)* and beer quality *(5)*. Moulds and their mycotoxins are thus an expensive problem for the malting and brewing industries *(6)*. This concern exists in the case of barley malting [see Dodd *(6)*, for instance], but it is particularly acute in the case of sorghum malting. One reason is that sorghum grains have no protective husks and are therefore more vulnerable than barley to mould contamination owing to grain damage. A second reason is that, in many parts of Africa and Asia, traditional outdoor floor malting under uncontrolled conditions is the rule. Thirdly, whether traditional or industrial, the steeping and germination steps of sorghum malting require both high humidity and a relatively high temperature [ideally, about 30 °C *(7)* vs 12–18 °C for barley], and are thus highly favourable to microbial development.

As an alternative to the use of toxic chemicals such as formaldehyde, steeping in a dilute alkaline solution (0.1–0.5% NaOH or $CaOH_2$) has been found to reduce the level of mould contamination without adversely affecting the sorghum grain *(8,9)*. In addition, it can improve the quality of the malt, notably by inactivating polyphenols/condensed tannins and increasing both the diastatic power (DP) and the free amino nitrogen (FAN) *(7,10–12)*. Biocontrol strategies are also envisaged, that is, the use of harmless microbes to control the fungi that produce mycotoxins and gushing-promoting agents *(13–15)*. Lefyedi and Taylor *(13)* have tested the effect of steeping in the presence of a culture of the lactic acid bacterium (*Pediococcus pentosaceus* or *Lactobacillus plantarum*) or the yeast *Saccharomyces cerevisiae*. In their study, they achieved a good reduction in mould and bacteria levels, but in contrast to steeping in dilute alkaline solution, no increase in DP.

Our laboratory has been investigating the potential of *Bacillus subtilis* S499 as a biocontrol agent *(15,16)* in sorghum malting. The reasons for our interest are multiple: (1) as different biocontrol agents are active against different spectra of organisms *(13)*, it is good to have a choice; (2) it might not be ideal to use a lactic acid bacterium (LAB) or a yeast for biocontrol in brewing, given the need to distinguish biocontrol LABs from ones responsible for beer spoilage *(17–19)* and to avoid the presence of unwanted LABs or yeast during fermentation; (3) *B. subtilis* is known to synthesize lipopeptides having antifungal properties *(20)*; (4) *B. subtilis* is used to control mould growth in the field *(21)* and *in vitro (22)*; (5) Reddy *et al*. *(23)* have found treatment of sorghum grains with *B. subtilis* culture filtrate to reduce *Aspergillus flavus* contamination by 72%; and (6) *B. subtilis* is non-pathogenic, is not listed as a contaminant of beer *(19)* and is readily destroyed in the presence of hop *(24)*.

In a recent study *(15)* we showed that steeping for 16 h in the presence of *B. subtilis* S499 cells, culture medium or both could reduce the fungal count after kilning to 1.6–1.9 log cfu g^{-1}, as compared with 5.5 log cfu g^{-1} when the grains were steeped in water. This treatment proved somewhat more effective

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against moulds than treatment with 0.2% NaOH, but yielded considerably lower levels of several key enzymes (α- and β-amylases, limit dextrinase) and significantly higher levels of total phenolics and condensed tannins. Our current aim thus was to see if we could adjust this biocontrol treatment so as to maintain its mould-reducing power while improving the quality of the malt obtained, especially in regard to the levels of starch-hydrolysing enzymes.

Dewar *et al. (7)* attributed the improved DP and FAN content that they observed after dilute NaOH treatment to increased grain water uptake during steeping and they described the steep-out grain moisture as a good predictor of malt quality. Yet we found steeping in 0.1% $Ca(OH)_2$ to yield higher enzyme levels than soaking in 0.2% NaOH, although the former led to a lower steep-out moisture content *(15)*. Other phenomena that might limit enzyme levels when *B. subtilis* cells and/or conditioned medium are present include adverse effects of biosurfactants on some enzymes *(25)*, the antioxidant power of some biosurfactants *(26)* and competition for oxygen between a large microbial population and the sorghum grains during steeping *(14,27)*, which can notably reduce the percentage and vigour of germination *(27)*. In our previous study *(15)*, good amylase levels were obtained when steeping was carried out without aeration (as is common practice in traditional outdoor floor malting) in dilute alkaline solution. Yet in the case of steeping treatment with *B. subtilis* cells at high concentration, aeration, reported to improve malt quality *(7)*, could be critical.

In the present work we have thus investigated the possibility of improving the quality of malts obtained after *B. subtilis*-S499-based biocontrol treatment by adjusting the steeping conditions. Using steeping in 0.2% NaOH as a reference, we have examined in the context of our biocontrol treatments the effects of aeration, steeping temperature and steeping time on various malt characteristics. We have also tested the effect of combining two successive steeping treatments: soaking in 0.2% NaOH followed by a biocontrol treatment. Furthermore, we have looked at lipopeptide levels and total mesophilic aerobe counts in relation to mould control with *B. subtilis*.

Material and methods

Bacillus subtilis S499 cultures and preparation of biocontrol treatment fractions

Bacillus subtilis S499 strain was obtained from the Walloon Centre of Industrial Biology and grown on Luria Broth agar at 37 °C for 24 h. An inoculating loopful was transferred to 100 mL Landy broth optimized for *B. subtilis* S499 lipopeptide production and incubated for 16 h. Finally, 10 mL was transferred to 350 mL optimized Landy broth and incubated at 30 °C (under rotary shaking at 130 rpm) for 72 h. After cell counting under a microscope in a Burker cell, the culture was diluted with distilled water to 10^8 cells mL^{-1}. This suspension (henceforth called 'C + B' for 'cells plus broth') was either used as such or centrifuged at 10,000 × g to yield two fractions: a supernatant, which was centrifuged two more times at 10,000 ***g*** to eliminate as many cells as possible (the resulting fraction is henceforth called 'B'), and a cell pellet, which was subjected to two rounds of rinsing in distilled water and centrifuging and finally resuspended in distilled water so as to obtain a concentration of 10^8 cells mL^{-1}.

Total fungal and total mesophilic aerobe counts

Fungal populations and the total mesophilic aerobe count were evaluated after kilning as in Badau *(29)*.

Malting

Red sorghum was obtained from the Democratic Republic of Congo. After sorting and cleaning, 150 g sorghum grains was placed in a perforated stainless steel cylinder. The cylinder was immersed to about three-quarters of its height in a Julabo HP35 water bath containing 18 L steeping liquor (the amount of liquid in the cylinder was about 300 mL). When aeration was required, compressed air (0.7 bar) was pumped continuously into the bath from below (at about 15 L min^{-1}) and the steeping liquor was recirculated through the cylinder from above. After steeping, the grains were rinsed in distilled water and allowed to germinate at 30 °C for 72 h in a plastic mini-greenhouse, with the grains in the upper portion and water underneath to saturate the air. The green malts obtained were kilned at 40 °C for 48 h and rootlets were hand removed by gentle brushing.

Steeping treatments. All biocontrol treatments were applied at the steeping step. The grains were steeped at 25, 30 or 35 °C in either C + B, C or B for a time ranging from 0 to 40 h. Two controls were included: soaking in distilled water and soaking in 0.2% NaOH. Combination treatments were also tested. These involved steeping in 0.2% NaOH for 2–14 h, rinsing the grains and then subjecting them for 14–2 h to a biocontrol treatment (total steeping time 16 h).

Sorghum malt characteristics

The percentage of chitted grains after steeping was calculated as follows: 20 g grain was taken at random after steeping and divided into lots of approximately 5 g. The chitted grains of three randomly selected lots were counted, and the percentage of chitted grains was calculated as the average of the three measurements. All enzymatic assays were performed on extracts of malt flour obtained by grinding 20 g kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm). The α- and β-amylase activities were extracted and assayed using Megazyme methods (Ceralpha method: K-CERA 08/05; and Betamyl-3 Method: K-BETA 10/10, respectively). The β-glucanase extraction was carried out for 15 min at 30 °C in a centrifugation tube containing 0.5 g malt flour and 8 mL extraction buffer (50 mM Na-acetate, pH 4.8), with vigorous vortexing every 5 min. The mixture was then centrifuged at 1000 ***g*** for 10 min and the supernatant collected. The assay was performed at 40 °C in a reaction mixture containing 0.5 mL extract and 0.5 mL of 2% carboxymethyl cellulose as substrate. The incubation time was 5 min and the reaction was stopped by immersing the tubes in boiling water. The amount of glucose released was then determined by the method of Nelson-Somogyi *(30)* and the β-glucanase activity was expressed in μmoles of glucose released per min per kg of kilned malt.

Total phenolics were assayed using the method optimized by Georgé *et al. (31)* without eliminating water-soluble compounds, and results were expressed in milligrams gallic acid equivalents per gram of dried malt (mg GAE g^{-1}). Condensed tannins were estimated by the modified vanillin–HCl method of Price *et al. (32)* and results were expressed in percentage catechin equivalents. European Brewing Convention (EBC) methods were used for the following characteristics: EBC method 3.2. for raw sorghum moisture

content; EBC method 3.4, 2004 for the thousand corn weight; EBC method 3.5.2 for germination capacity; and EBC method 3.6.3 for germination energy (33). Soluble nitrogen was determined after mashing and filtration as described in EBC method 4.5.1 *(33)*. The nitrogen content was determined in the wort before and after 2 h of boiling and the soluble nitrogen/total nitrogen ratio was calculated.

Rehydration curves

After sorting and cleaning, the sorghum grains were soaked in 0.2% NaOH or supernatant B for 40 h. After 0.5, 1, 2, 4, 6, 8, 12, 14, 16, 20, 24, 36 and 40 h, the wet weight (w_w) was measured in triplicate. The dry weight (w_d) was determined by heating the grains in an oven at 105 °C until the weight remained unchanged. The dry-basis (d.b.) steep-out moisture content was calculated by subtracting w_d from w_w and dividing by w_d. Peleg's model *(34)* was used to draw the rehydration curves. This model can be written as: $1/M_t - M_0 = k_2 + k_1 t^{-1}$, where t is the rehydration time, M_0 is the initial moisture content (d.b.) and M_t is the moisture content (d.b.) at time t. The parameters k_1 (Peleg's rate constant) and k_2 (Peleg's capacity constant) are determined by plotting $1/M_t - M_0$ vs t^{-1} and fitting a straight line to the data. From these parameter values it is possible to deduce an initial sorption rate ($\mu_s = 1/k_1$) and a final (equilibrium) moisture content ($\omega_0 = \mu_0 + 1/k_2$).

Lipopeptide assays

Surfactins, iturins and fengycins were assayed by reversed-phase high-performance liquid chromatography hyphenated to diode array detection electrospray ionization mass spectrometry [RP-HPLC/(ESI$^+$)DAD-MS], as described by Nihorimbere *et al*. *(16)*.

Statistical analyses

XLSTAT 2011.4.01 software was used for statistical analyses. The Student–Neuman–Keuls test was used to determine the significance of differences between results obtained under different conditions. Three-way ANOVA was applied to the α- and β-amylase data obtained with different steeping treatments after different steeping times and at different temperatures. The goodness of fit of Peleg's model to the rehydration data was checked by estimating the mean relative percentage deviation modulus (E). Minitab 15 software was used to produce graphs and to perform the three-way ANOVA.

Results

Red sorghum characteristics

The characteristics of the red sorghum used here were as follows. Moisture content (wet basis, w.b.), 11.14 ± 0.19%; thousand grain weight, 26.20 ± 0.12 g; germination capacity, 96.33 ± 0.29%; germinative energy, 95.53 ± 0.50%; initial fungal plate count, 4.85 ± 0.14 log cfu g^{-1}.

Effect of aeration

As our previous study had been conducted without aeration *(15)*, we first compared the effects of aeration on our three biocontrol treatments (C + B, C and B) and our two controls (H_2O, NaOH). As shown in Table 1, aeration was found to have a slight, albeit positive effect on the w.b. steep-out moisture. The presence of the culture broth also seemed to increase, very slightly, the steep-out moisture (C + B and B vs C). On the other hand, aeration clearly stimulated germination, as indicated by increases in the number of chitted grains at the end of the steeping step. The most spectacular increases occurred in the presence of *B. subtilis* cells: 28- and 78-fold for treatments C and C + B, respectively, as compared with about 2-fold in distilled water and 5-fold in dilute alkaline solution. Also noteworthy was the high percentage of chitted grains observed after all three biocontrol treatments, as compared with either H_2O or NaOH. This last treatment would appear to have a germination-delaying effect (NaOH vs H_2O, not aerated and aerated).

Table 1. Effect of aeration on steep-out moisture and percentage of chitted grains

Treatment	Steep-out moisture content (%, w.b.)		Percentage of chitted grains	
	NA	A	NA	A
H_2O	30.7 ± 0.2^{f}	32.6 ± 0.3^{e}	25.5 ± 0.5^{f}	59.7 ± 3.8^{d}
NaOH	42.1 ± 0.1^{b}	43.8 ± 0.5^{a}	9.3 ± 1.5^{h}	45.7 ± 3.5^{e}
C + B	36.5 ± 0.1^{d}	37.9 ± 0.2^{c}	1.0 ± 1.0^{i}	78.0 ± 1.0^{b}
C	35.6 ± 0.1^{d}	36.8 ± 0.2^{d}	3.3 ± 1.2^{i}	85.0 ± 1.5^{a}
B	36.6 ± 0.0^{d}	38.6 ± 0.1^{c}	16.0 ± 2.0^{f}	74.3 ± 4.2^{c}

For each characteristic measured, data having a letter in common are not statistically different according to the Student–Neuman–Keuls test ($p < 0.05$). NA, Not aerated during steeping; A, aerated during steeping; w.b., wet basis.

Table 2 shows the effect of aeration on the post-kilning fungal count. It appeared to enhance, slightly, the antifungal efficacy of all three biocontrol treatments (C + B, B, and C, aerated vs not aerated) while reducing that of 0.2% NaOH. The biocontrol treatments were always more effective than dilute alkaline treatment. In terms of their fungus-reducing efficacy, the biocontrol treatments (with or without aeration) ranked as follows: B + C > B > C.

Table 2 also shows the total malting loss, total phenolics and condensed tannins after the various treatments. In the absence of aeration, the total malting loss was 46–48% lower after biocontrol treatment than after NaOH treatment. Aeration increased the total malting loss recorded after the biocontrol treatments (by 21, 11 and 24% for C + B, C and B, respectively) without affecting the loss after NaOH treatment, but the loss remained 34–39% lower after biocontrol treatment than after NaOH treatment. Aeration had no effect on total phenolics or condensed tannins, whose levels were lower after steeping in NaOH than after any biocontrol treatment.

Table 3 shows the effect of aeration on three key hydrolytic enzymes. In the absence of aeration, as observed previously (15), the α- and β-amylase levels achieved were lower after biocontrol treatment than after NaOH treatment, but higher than after steeping in water alone. Aeration improved these levels whatever the steeping conditions, but in all cases but one (β-amylase, treatment B), it had a greater effect when the grains were soaked in water (+64% for α-amylase, +67% for β-amylase) or subjected to a biocontrol treatment (α-amylase: +95, +82 and +42%, for C + B, C and B respectively; β-amylase:

Table 2. Effect of aeration on total fungal count, total malting loss, total phenolics and condensed tannins

Treatment	Total fungal count (log cfu g^{-1})		Total malting loss (%)		Total phenolics (mg GAE g^{-1})		Condensed tannins (%CE)	
	NA	A	NA	A	NA	A	NA	A
H_2O	4.81 ± 0.02^{a}	4.76 ± 0.04^{a}	21.1 ± 1.1^{b}	25.0 ± 0.5^{a}	7.9 ± 0.2^{ab}	8.0 ± 0.3^{a}	0.15 ± 0.01^{a}	0.16 ± 0.01^{a}
NaOH	2.87 ± 0.07^{c}	3.16 ± 0.03^{b}	26.0 ± 0.5^{a}	25.7 ± 0.6^{a}	5.9 ± 0.1^{e}	6.0 ± 0.1^{e}	0.06 ± 0.01^{c}	0.06 ± 0.01^{c}
C + B	1.63 ± 0.06^{f}	1.40 ± 0.07^{g}	13.5 ± 1.2^{e}	16.3 ± 0.6^{cd}	7.5 ± 0.1^{c}	7.4 ± 0.2^{cd}	0.12 ± 0.02^{a}	0.12 ± 0.02^{a}
C	2.11 ± 0.08^{d}	1.87 ± 0.04^{e}	14.1 ± 1.1^{de}	15.7 ± 1.1^{cde}	7.2 ± 0.0^{d}	7.2 ± 0.1^{d}	0.09 ± 0.01^{b}	0.09 ± 0.01^{b}
B	1.81 ± 0.03^{e}	1.61 ± 0.06^{f}	13.7 ± 2.1^{e}	17.0 ± 1.0^{c}	7.7 ± 0.1^{bc}	7.5 ± 0.1^{c}	0.12 ± 0.02^{a}	0.12 ± 0.02^{a}

For each characteristic measured, data having a letter in common are not statistically different according to the Student–Neuman–Keuls test ($p < 0.05$). GAE, gallic acid equivalents; NA, not aerated during steeping; A, aerated during steeping; %CE, percentage catechin equivalents.

Table 3. Effect of aeration on enzyme activities

Treatment	α-Amylase (U g^{-1} malt)		β-Amylase (U g^{-1} malt)		β-Glucanase (U kg^{-1} malt)	
	NA	A	NA	A	NA	A
H_2O	45.0 ± 0.7^{i}	73.6 ± 2.6^{g}	22.9 ± 1.1^{h}	38.3 ± 1.5^{f}	0.6 ± 0.0^{g}	0.9 ± 0.0^{f}
NaOH	262.5 ± 1.8^{b}	291.8 ± 2.7^{a}	50.6 ± 2.8^{b}	59.5 ± 1.7^{a}	1.8 ± 0.2^{e}	3.1 ± 0.2^{d}
C + B	69.6 ± 1.0^{h}	135.8 ± 1.9^{d}	32.0 ± 2.2^{g}	44.1 ± 1.2^{d}	3.9 ± 0.1^{c}	4.6 ± 0.1^{b}
C	80.5 ± 3.8^{f}	147.3 ± 1.7^{c}	40.4 ± 1.7^{ef}	49.6 ± 0.7^{c}	4.8 ± 0.2^{b}	5.8 ± 0.1^{a}
B	67.4 ± 0.7^{h}	96.1 ± 2.0^{e}	38.9 ± 1.7^{ef}	42.2 ± 0.6^{de}	1.1 ± 0.1^{f}	1.6 ± 0.1^{e}

For each characteristic measured, data having a letter in common are not statistically different according to the Student–Neuman–Keuls test ($p < 0.05$). NA, Not aerated during steeping; A, aerated during steeping.

+38 and +23% for C + B and C, respectively) than when they were steeped in 0.2% NaOH (α-amylase: +11%; β-amylase: +17%). Aeration also enhanced the level of β-glucanase activity under all steeping conditions. Interestingly, the highest levels of this enzyme activity were reached in the presence of *B. subtilis* cells (C + B and C, aerated and not aerated).

Given the considerable boosting effect of aeration on these enzyme activities, especially during biocontrol treatment, all subsequent experiments were performed with aeration.

Effects of steeping time and temperature on α- and β-amylase activity

We next examined whether the diastatic power of malts obtained after biocontrol treatment might be improved by extending the steeping time or changing the steeping temperature. Figures 1 and 2 show how these factors affected the α- and β-amylase activities. Three-way ANOVA applied to these data showed significant effects of steeping treatment, time and temperature, and their interactions (Tables 4 and 5). At each temperature and for each amylase, the peak activity recorded was highest after steeping in 0.2% NaOH, but at 35 °C, the β-amylase activity declined faster after peaking in the NaOH-treated samples than in the broth-containing biocontrol samples (C + B and B). In all treatment media the optimal temperature for α-amylase activity was 30 °C (Fig. 1). When treated at this temperature, samples steeped in NaOH showed a sharp rise in α-amylase between 8 and 16 h, followed by a broad peak (highest value recorded was 286.3 ± 1.0 U g^{-1}, at 24 h) and a final decline. Interestingly, the biocontrol treatments containing *B. subtilis* cells at high concentration led to higher α-amylase activities than steeping in conditioned broth alone (Fig. 1, C + B and C vs B). In the case of these cell-containing treatments, the α-amylase activity rose quickly for 24 h and then more slowly until the end of the experiment. The increase beyond 16 h (time corresponding to Tables 1–3) was 31% for treatment C + B, 27% for treatment C, and 11% for treatment B. Extending the steeping time is thus one way to increase the α-amylase activity of a red sorghum malt obtained after biocontrol treatment at 30 °C.

A different picture was obtained for β-amylase activity, which peaked between 12 and 20 h, and declined, often sharply, thereafter. The peak level obtained after NaOH treatment was about the same at 25 and 30 °C (respectively 56.3 and 55.3 U g^{-1}), but markedly lower at 35 °C (41.5 U g^{-1}). The peak levels recorded after biocontrol treatment were less sensitive to temperature, although 35 °C appeared generally less favourable. At 30 °C (the optimum for α-amylase), the β-amylase peak was higher after steeping in cell-containing medium (41.6 U g^{-1} for C + B and 42.0 U g^{-1} for C) than after treatment in the conditioned broth alone (26 U g^{-1}).

Enzyme levels in relation to grain hydration

Dewar *et al.* *(7)* attributed the increased DP observed after dilute NaOH treatment to increased water uptake by the grains during steeping, probably owing to the opening-up of the pericarp cell walls by the dilute alkaline solution. To investigate this matter,

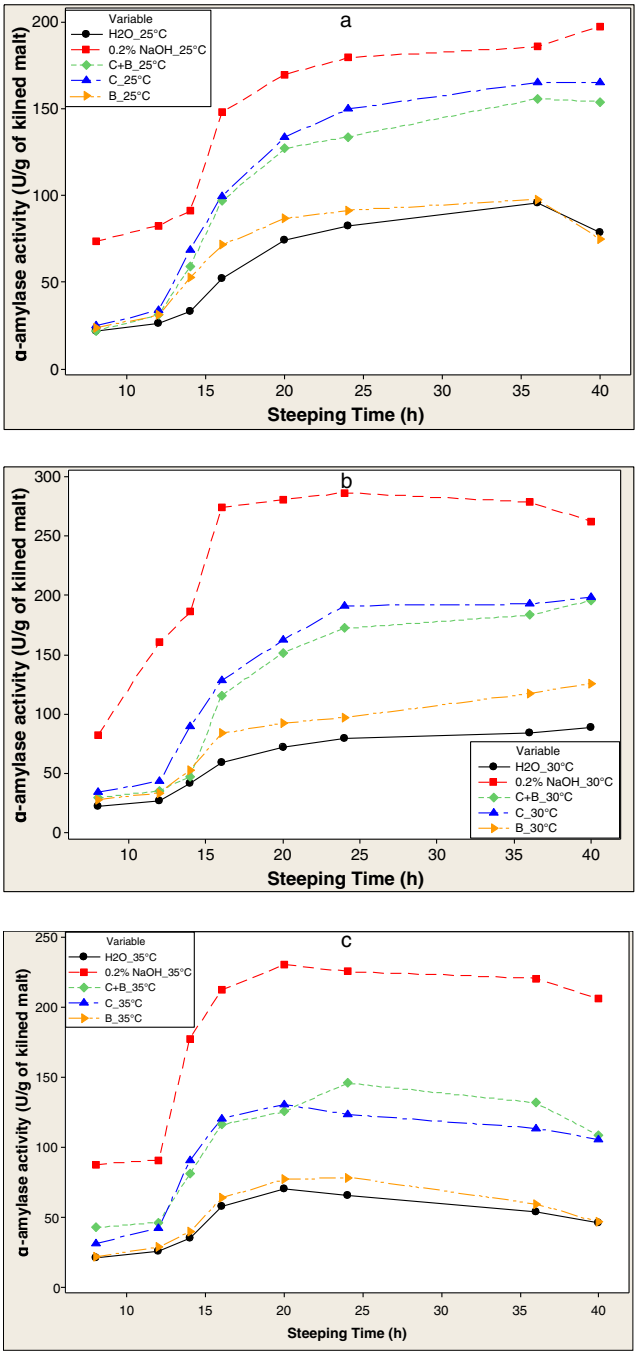

Figure 1. Effects of steeping time, temperature, and steeping liquor composition on α-amylase activities in kilned malts (A1–A3, α-amylase activities respectively for 25, 30 and 35 °C).

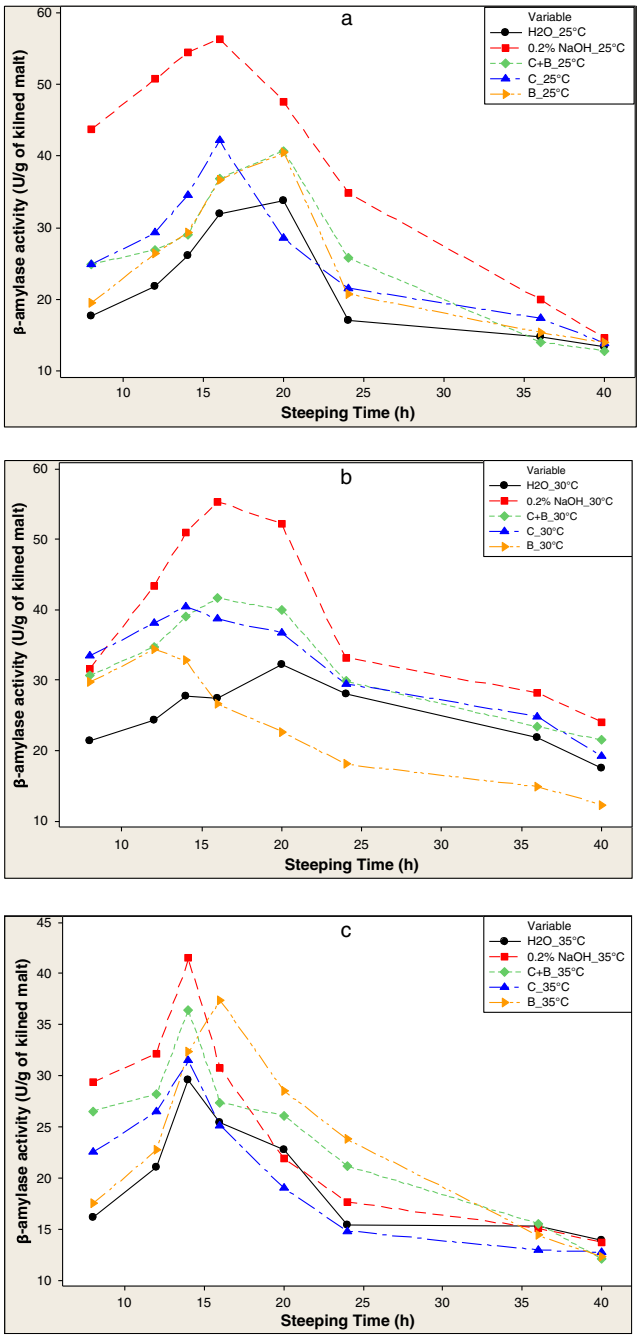

Figure 2. Effects of steeping time, temperature, and steeping liquor composition on β-amylase activities in kilned malts (B1–B3, β-amylase activities respectively for 25, 30 and 35 °C).

we measured the moisture content of the sorghum grains over a 40 h period of steeping under various conditions. Under all conditions tested, the dry-basis moisture content was found to increase over the entire period, being higher in the NaOH-treated samples than in the biocontrol samples. To obtain a really good fit with Peleg's model *(34)*, it was necessary to limit the time period considered to 16 h (data and model curves: Fig. S2; parameter values: Table 6). We attribute this to the changes accompanying grain germination, as attested by the appearance of chitted grains around this time. As shown by others *(35–40)*, the initial sorption rate μ_s, as calculated from the model fitted to the 16 h data, was found to increase with the temperature according to the Arrhenius equation (not shown). This was not true of the value obtained after fitting to the 40 h data (not shown). The model fitted to the 16 h data markedly underestimated the moisture at 40 h, by 4.1–8.3% when the grains were steeped in 0.2% NaOH and by 5.4–13.7% when they were steeped in *B. subtilis* culture broth. This suggests that the equilibrium moisture values (ω_o) calculated on the basis of this model should be considered with caution.

There appeared no absolute correlation between moisture content and the α- and β-amylase activities presented in the previous

Table 4. Results of three-way ANOVA applied to the data of Fig. 1 (for α-amylase vs steeping treatment, steeping temperature and steeping time) using the general linear model

Source	DF	SS	MS	*F*	*p*
Steeping treatments (STT)	4	732,130	183,033	5,490.35	0.0000
Steeping temperature (ST) (°C)	2	62,491	31,246	937.26	0.0000
Steeping time (SD) (h)	7	580,027	82,861	2,485.55	0.0000
STT*ST	8	56,745	7,093	212.77	0.0000
STT*SD	28	78,634	2,808	84.24	0.0000
ST*SD	14	42,028	3,002	90.05	0.0000
STT*ST*SD	56	26,116	466	13.99	0.0000
Error	240	8,001	33		
Total	359	1,586,173			
$S = 5.77383$		$R^2 = 99.50\%$		$R^2(\text{adj}) = 99.25\%$	

Table 5. Results of three-way ANOVA applied to the data of Fig. 2 (for β-amylase vs steeping treatment, steeping temperature and steeping time) using the general linear model

Source	DF	SS	MS	*F*	*p*
Steeping treatments (STT)	4	6,947.21	1,736.80	745.42	0.0000
Steeping temperature (ST) (°C)	2	4,021.97	2,010.98	863.09	0.0000
Steeping time (SD) (h)	7	19,896.86	2,842.41	1,219.93	0.0000
STT*ST	8	2,391.97	299.00	128.33	0.0000
STT*SD	28	2,200.84	78.60	33.73	0.0000
ST*SD	14	1,558.82	111.34	47.79	0.0000
STT*ST*SD	56	2,589.96	46.25	19.85	0.0000
Error	240	559.19	2.33		
Total	359	40,166.82			
$S = 1.52642$		$R^2 = 98.61\%$		$R^2(\text{adj}) = 97.92\%$	

Table 6. Red sorghum rehydration – kinetic parameters (Peleg's model)

Treatment	Temperature (°C)	k_1	k_2	μ_s	ω_0	*E*
16 h steeping in 0.2% NaOH	25 °C	2.92	1.59	0.34	0.75	0.55
	30 °C	2.53	1.31	0.40	0.88	0.43
	35 °C	2.07	1.17	0.48	0.97	0.32
16 h steeping in B	25 °C	8.14	1.89	0.12	0.65	1.10
	30 °C	7.17	1.73	0.14	0.70	1.42
	35 °C	5.89	1.66	0.17	0.72	0.83

section. On the one hand, it is true that both the moisture content and the amylase activities were highest after steeping in 0.2% NaOH. It is also true that the α-amylase activity rose concomitantly with the moisture content over the first 20 h. Yet both enzyme activities were lower at 35 than at 30 °C, and β-amylase reached its highest levels at 25 °C (both steeping liquors).

Combining steeping in 0.2% NaOH with a biocontrol treatment

We then examined the possibility of combining NaOH treatment with a biocontrol treatment, so as to possibly accumulate the advantages of both. In an experiment where the total steeping time was set at 16 h, steeping in 0.2% NaOH for 2–14 h was followed by 14–2 h of resteeping under conditions C + B or C. Figure 3 shows that, as the time spent in the dilute alkaline solution increased, the fungal count was found to rise, the percentage of chitted grains to decrease and the moisture content to rise before stabilizing at 8 h. To maximize the moisture content while minimizing the mould count increase and germination lag, 8 h in NaOH followed by 8 h biocontrol treatment (C + B or C) was chosen for the next experiment. As shown in Table 7, the characteristics of the malts obtained were encouraging. As compared with the corresponding 16 h biocontrol treatments (see Tables 2 and 3, tests with aeration), both combination treatments offered higher α-amylase activities and lower levels of total phenolics and tannins. The β-amylase activity was improved only by the combination treatment with C + B. These improvements were associated with an increased steep-out moisture (to a level only 3% lower than for the 16 h NaOH treatment). Although the combination treatments gave rise to somewhat higher malting losses and total fungal counts than the corresponding 16 h biocontrol treatments, these characteristics remained more favourable than after the 16 h NaOH treatment. There was no significant difference between treatments in regard to the soluble-to-total nitrogen content after 2 h of boiling. Both tested combination treatments thus offer slightly better mould control than a 16 h NaOH treatment, while yielding malts of approaching quality.

Lipopeptides and total mesophilic aerobes

We next examined whether lipopeptide production by *B. subtilis* S499 and/or competition between moulds and bacteria might be responsible for the biocontrol effects observed here. Table 8

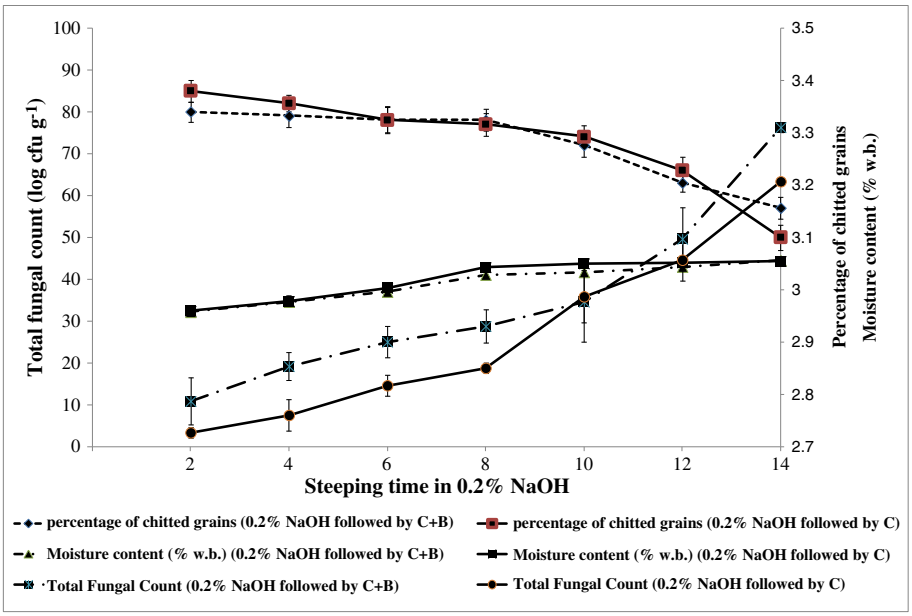

Figure 3. Steep-out moisture, percentage of chitted grains, and total fungal count as affected by residence time in 0.2% NaOH prior to rinsing and transfer to C + B or C (total steeping time: 16 h).

shows the total mesophilic aerobe counts and lipopeptide (surfactin, iturin and fengycin) levels observed after 16 h of C + B, C, or B treatment, and Figs S3 and S4 show their variations in the course of each treatment. As expected of extracellular compounds in a culture medium optimized for their production, all three lipopeptide families were found at high levels in the presence of conditioned broth (C + B and B). Although these levels decreased somewhat in the course of the experiment, they remained high at the end of the 16 h period. In contrast, all three families were undetectable for the first 8 h of treatment with rinsed cells, reaching low levels by the end of the experiment. As expected, the total mesophilic aerobe count was highest in the cell-based treatments. It was lower in the case of treatment B (centrifuged broth) than after NaOH treatment.

Discussion

We have shown here that, during sorghum malting, the use of steeping treatments based on *B. subtilis* S499 can greatly reduce the post-kilning level of fungal contamination. All of the biocontrol treatments tested (with and without aeration, with and without cells or conditioned broth, combined or not with NaOH treatment) reduced the total fungal count from an initial 4.85 log cfu g^{-1} to below the limit of 3 log cfu g^{-1} recommended by the Association Française de Normalisation *(41)*. The results of Lefyedi and Taylor *(13)* offer a comparison: these authors reduced the total fungal count in malt from an initial 2.6×10^3 to 1.3×10^2 cfu g^{-1} with a half-and-half mixture of *Lactobacillus plantarum* and *S. cerevisiae* at 10^7 cells mL^{-1}. All of the biocontrol treatments tested here appeared to limit the post-kilning mould count more effectively than NaOH treatment.

The mechanism(s) underlying mould control by *B. subtilis*-based steeping treatments are as yet unclear. On the one hand, lipopeptides produced by *B. subtilis* are known to inhibit the growth of plant pathogens such as *Fusarium oxysporum* and *Rhizoctonia solani (16,28)*. Another possible mechanism is competition between moulds and the biocontrol agent during steeping. Yet our results cannot readily be explained by either of these mechanisms, as strong biocontrol effects were observed after both B treatment (low mesophilic aerobe count) and C treatment (low lipopeptide levels after 16 h of steeping). Perhaps bacterial compounds other than lipopeptides exert antifungal effects. Or perhaps both mechanisms intervene, since the strongest effects were observed with cells and broth together. This matter deserves further investigation.

We confirm here, as shown by others (see Introduction), the DP-enhancing power of steeping in 0.2% NaOH during sorghum malting. With biocontrol treatments alone, we failed to approach the α- and β-amylase levels recorded for dilute alkaline treatment. Interestingly, however, the recorded β-glucanase activity was between 1.5 and 2.7 times as high after steeping with *B. subtilis* cells (alone or with their broth) as after dilute alkaline treatment. The reason for this enhanced activity is unclear. The effect was not observed with conditioned culture broth alone, which seems to rule out penetration of a secreted *B. subtilis* enzyme into the grains. A high β-glucanase activity could be an advantage in situations where sorghum malt is used for brewing *(42)*. Cell wall β-glucan hydrolysis and solubilization during malting and brewing are two important steps of the process, because they affect the viscosity of the mash and the stability of the beer. Yet preliminary results (unpublished) fail to show any significant change in β-glucan levels according to the steeping treatment.

Several measures taken here to raise the enzyme activities achieved after biocontrol treatment were successful. Aeration is clearly of the essence, since all measured enzyme levels were found to increase with aeration, whatever the steeping liquor. The effect was particularly pronounced for α-amylase, when *B. subtilis* cells were added to the steeping mixture. This suggests that the bacterial cells compete strongly with the grains for the available oxygen during steeping without aeration. This is further suggested by the marked increases in the percentage of chitted grains observed upon aeration, most pronounced when *B. subtilis* cells are present. Alternatively or additionally,

Table 7. Characteristics of malts obtained with combined steeping treatments

Treatment	Steep-out moisture (%, w.b.)	Total fungal count (log cfu g^{-1})	Total malting loss (%)	α-Amylase (U g^{-1})	β-Amylase (U g^{-1})	β-Glucanase (U kg^{-1})	Total phenolics (GAE g^{-1})	Condensed tannins (%CE)	Soluble nitrogen/total nitrogen (%)	Soluble nitrogen/total nitrogen after 2 h boiling (%)
NaOH, C + B	*40.8 ± 0.9^{b}	1.65 ± 0.04^{c}	20.1 ± 0.9^{b}	*224.9 ± 4.0^{c}	*46.5 ± 0.7^{c}	*4.3 ± 0.1^{b}	*6.6 ± 0.3^{b}	*0.08 ± 0.01^{b}	35.9 ± 1.0^{b}	32.7 ± 0.7^{a}
NaOH, C	*40.9 ± 0.9^{b}	2.05 ± 0.03^{b}	21.1 ± 0.4^{b}	*254.5 ± 4.4^{b}	*49.2 ± 1.6^{b}	*6.0 ± 0.0^{a}	*6.0 ± 0.2^{a}	0.08 ± 0.01^{b}	38.5 ± 0.9^{b}	33.8 ± 1.0^{a}
NaOH	43.8 ± 0.5^{a}	3.16 ± 0.03^{a}	25.7 ± 0.6^{a}	291.8 ± 4.9^{a}	59.5 ± 1.7^{a}	3.1 ± 0.2^{c}	6.0 ± 0.1^{a}	0.06 ± 0.01^{a}	41.4 ± 0.2^{a}	33.2 ± 1.5^{a}

For each characteristic measured, data having a letter in common are not statistically different according to the Student–Neuman–Keuls test ($p < 0.05$).
* Results for the 8 + 8 h treatments that differ significantly from the data for the corresponding 16 h biocontrol treatments. GAE, gallic acid equivalents.

Table 8. Lipopeptides and mesophilic aerobes in steeping liquor

Treatment	Surfactins (μg mL^{-1})	Iturins (μg mL^{-1})	Fengycins (μg mL^{-1})	Total mesophilic aerobe count (log cfu mL^{-1})
C + B	332.9	100.0	249.8	10.02
C	26.6	11.2	18.8	10.64
B	368.4	89.6	176.7	4.01
0.2% NaOH	—	—	—	6.0

the mixing and bubbling that accompany aeration might favour evacuation of CO_2, known to inhibit the synthesis of certain enzymes, especially α-amylase *(43)*. It would be interesting to test different types and levels of aeration to see if enzyme levels after biocontrol treatment might be improved further.

Regarding the steeping time, Dewar *et al. (7)* report a significant increase in DP with the steeping time, whereas Novéllie *(44)* reports no effect. Our results suggest that increasing the steeping time is a two-edged sword. On the one hand, at 25 or 30 °C, the α-amylase level can be increased somewhat by extending the steeping time beyond 16 h. On the other hand, the β-amylase level in the kilned malt shows a rather sharp peak, the timing of which shifts according to the steeping conditions. One must thus be careful not to extend the steeping time beyond the peak. In the case of treatment C + B or C carried out at 30 °C, this means limiting the treatment to about 16 h.

Changing the temperature affects the α- and β-amylase activities differently. The former was highest at 30 °C and the latter at 25 °C. Overall, the cell-containing biocontrol treatments yielded higher activities than treatment with supernatant alone, with the exception of β-amylase at 35 °C. A good compromise would appear to be treatment C + B or C at 30 °C with a 14- to 16-h steeping time (see Figs 1 and 2).

The relationship between water uptake and individual enzyme levels is unclear. A higher moisture content might explain the superiority of NaOH treatment (in terms of α- and β-amylase activities) over biocontrol or H_2O treatment, but it cannot explain either the temperature-related variations in enzyme levels presented here or the superiority of 0.1% $Ca(OH)_2$ treatment over 0.2% NaOH treatment observed by Bwanganga *et al. (15)*. If necessary, the moisture content can be corrected by spraying the grains during germination, but limitations to endosperm modification caused by suboptimal moisture are hard to correct later in the germination process *(45)*.

We highlight here a limitation of the use of Peleg's equation to model grain hydration. This model appears to offer a good description up to the time the grains show signs of germination, but less so thereafter. This is not really surprising, as Peleg's model is intended for modelling diffusion into a homogeneous medium. It takes into account neither the grains' internal structure (water may have successive barriers to pass, as shown by Holmbero and Hámáláinen *(46)*) nor the physiological changes that occur when a plantlet emerges and begins to take up water and nutrients actively.

By combining dilute alkaline treatment with a biocontrol treatment, we have obtained malts whose quality approaches that of malts produced from NaOH-steeped grains, the most

noteworthy improvement being at the level of the recorded enzyme activities. Disappointingly, we observed no synergy between the mould-reducing effects of NaOH and biocontrol treatment. In fact, the total fungal count was not quite so effectively reduced with the combined treatment as with the 16 h biocontrol treatments, but it remained at an acceptably low level.

Our results raise several questions worth answering. It would certainly be important to identify any compounds that contribute to the antifungal effects of steeping treatments based on *B. subtilis* S499 (see above). Another question is: how do the tested biocontrol treatments induce grain chitting? Some *Bacillus* species such as *B. pumilus* and *B. licheniformis* can produce gibberellic acid *(47)*, which can induce barley germination and affect both α-amylase synthesis and the activation of preformed β-amylase in barley. It would also be useful to understand in depth the relationship between grain chitting during steeping and the development of enzyme activities. With such knowledge gained from biocontrol experiments, it might be possible to devise new biocontrol strategies or to produce bioadditives with which to modulate the effects of dilute alkaline treatment.

Conclusions

It is possible to limit mould development during red sorghum malting by means of *B. subtilis*-S499-based biocontrol treatments applied at the steeping stage. Aeration of the biocontrol steeping medium improves both the fungal-count reduction and the levels of hydrolytic enzymes in the kilned malts. Steeping in 0.2% NaOH for 8 h followed by an 8 h biocontrol treatment yields malts approaching the quality of those obtained after steeping for 16 h in dilute alkaline solution, with better mould control.

Supporting information

Supporting information can be found in the online version of this article.

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