Development of the *Bacillus subtilis*-S499 biocontrol of mould during red sorghum malting

Jean-Claude Bwanganga Tawaba, François Béra, Philippe Thonart

1Department of Food Technology. GemblouxAgroBioTech, University of Liege. Passage des Déportés 2, B-5030 Gembloux (Belgium). E-mail: jcbwanganga@alumni.uliege.be; jcbwangson@yahoo.fr
2Department of Bioindustries. GemblouxAgroBioTech, University of Liege. Passage des Déportés 2, B-5030 Gembloux (Belgium).
3University of Kinshasa. Agricultural Faculty. BP 14071 Kinshasa 1 (DRC).

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ABSTRACT

*Bacillus subtilis* exerts an inhibitory effect on moulds isolated from red sorghum raw grain. The total fungal count reduction time course, affected by the dilution of *B. subtilis* culture, follows a sigmoidal function type. Therefore, one can distinguish three zones: a first zone of high inhibition, where the dilution of *B. subtilis* culture does not greatly affect the steeping treatment on reducing mould growth (DF > minID); a second zone of dilution where the inhibitory effect of the steeping treatment is almost proportional to the dilution of the *B. subtilis* culture (MaxID < DF < minID); and a third zone of dilution where treatment is simply ineffective i.e., no significant reduction in total fungal count upon increasing the concentration of *B. subtilis* (DF < MaxID). Steeping in the biocontrol allows malt production with a low level of fungal contamination, relatively low malting losses and high β-glucanase levels. When compared with dilute alkaline steeping, the biocontrol treatments result in malts with low α- and β-amylase activities and a relatively high content of total phenolic compounds and condensed tannins. *Bacillus subtilis* dilution has been found to significantly affect kilned malt enzyme activities depending on the type of enzyme studied.

Keywords: *Bacillus-subtilis*-based biocontrol, mould growth inhibition, red sorghum malting

RESUME

*Bacillus subtilis* exerce un effet inhibiteur sur les moisissures isolées des grains de sorgho rouge. La flore fongique totale est décrite comme une fonction sigmoïdale du logarithme du facteur de dilution de la culture de *B. subtilis*. On peut ainsi distinguer trois zones: une première zone de haute inhibition où la dilution de la culture de *B. subtilis* n’affecte pas grandement la capacité de la solution de trempage à réduire la croissance des moisissures (DF > minID); une deuxième zone de dilution où l’effet inhibiteur de la solution de trempage est proportionnel à la dilution de la culture de *B. subtilis* (MaxID < DF < minID); et une troisième zone de dilution où le traitement est simplement inefficace, c’est-à-dire qu’il n’y a pas de réduction de la flore fongique totale lorsque la population de *B. subtilis* augmente (DF < MaxID). Le trempage dans le biocontrôle permet de produire un malt dont la flore fongique totale est faible, une freinte au maltage relativement faible et des niveaux élevés d’activités β-glucanases. Comparés aux malts obtenus après trempage dans la solution alcaline, les malts obtenus après trempage dans le biocontrôle ont donné des faibles activités α- et β-amylases et des teneurs relativement élevées en polyphénols totaux et en tannins condensés. La dilution de la culture de *B. subtilis* affecte significativement les activités enzymatiques des malts tourailles et, ce, de manière parfois différente en fonction du type d’enzyme en question.

Mots-clés : Biocontrôle-avec-*Bacillus-subtilis*, inhibition des moisissures, maltage de sorgho rouge

1. INTRODUCTION

Sorghum is a major crop in Africa, Asia and South America, and malted sorghum is used notably in making products for local consumption, such as beer, infant porridge and non-fermented beverages. During malting, the maltster has to create good grain germination conditions (moisture, aeration, temperature, etc.) in order to take advantage of the underlying transformations (enzyme production, grain modification, reducing the importance of certain substances such as tannins, reduction of undesirable microorganism development, removal of...
raw flavour, development of malty flavour and colour, etc. (Lewis and Bamforth, 2006). The importance of microbes during barley malting was elucidated (Laitila, 2007); as was the contribution of the microbial ecosystem on malt properties. Maltsters also recognize the influence of the natural barley grain microflora on grain physiology during malting (Camphnhoui et al., 1998). It is also known that the grain microflora interact with the malted grain both by their presence and metabolic activity (Noots et al., 1999). However, the presence of certain microorganisms during malting is disadvantageous, as they can exert an adverse effect on the quality of both malt and malted products such as beer. The negative role of mould is well known (mycotoxin production during malting and the effect on beer quality and consumer health) (Schapira et al., 1989) and procedures to prevent mould toxin production during malting are currently employed.

During sorghum malting, mould control is of paramount importance in the sense that sorghum malting – unlike that of barley – is conducted at relatively high temperatures (Dewar et al., 1997). In addition, grain sorghum is not dressed; hence the endosperm cell wall is attacked to a varying degree, allowing the microorganisms easy access to nutrients. Sorghum raw grain is recognized to be a susceptible material to invasion by potentially toxigenic fungi. Thus, several studies have been conducted on mould control and mycotoxin production during sorghum malting; the use of chemical treatments (diluted acids, dilute alkalines) and the use of biocontrols (lactic bacteria and yeasts) (Rabie and Lübben 1984; Lefyedi and Taylor, 2006, 2007; Agu and Palmer, 1997; – to name only a few).

Biological control of postharvest diseases in general (reviewed by Sharma et al., 2009) and mouldbiocontrol during malting (sorghum as well as barley), is an environmental and economic challenge, and several studies have already been performed in this direction (Lowe and Arendt, 2004; Lefyedi and Taylor, 2007; van Sinderen and Rouse, 2008).

Beer spoilage microorganisms and hop resistance were presented in a detailed and interesting review (Sakamoto and Konings, 2003), placing several species of lactic acid bacteria at the head of list. It has been shown that the presence of lactic acid bacteria in the brewing environment is not of interest, especially as counting methods and identification of lactic acid bacteria are very expensive (different auxotrophic profiles, several lactic acid bacteria grow poorly in mainstream media) (Simpson, 1993; Simpson and Fernandez, 1994).

Recently, (Reddy et al., 2010) showed the effect of B. subtilis on the inhibition of mould growth in the field (Makoto, 2000). Indeed, B. subtilis produced a series of lipopeptides with antifungal properties (Ongena and Jacques, 2007; Nihorimbere et al., 2011). Its presence in the brewing environment is not a concern as B. subtilis is a non-pathogenic bacterium; is not a potential beer spoilage bacterium (Sakamoto and Konings, 2003) and is easily destroyed in the presence of hops (Teuber and Schmalreck, 1973).

Thus in this work, we have focused specifically on the possibility of using a harmless microbe, B. subtilis, to control mould development during red sorghum malting. The inhibitory effect of this strain on various moulds isolated from raw sorghum grain was first evaluated in vitro, and – during red sorghum malting – the inhibitory effect of the B. subtilis biocontrol was evaluated after using this bacteria as a starter in the steeping liquor. The effect of the dilution of a B. subtilis culture on the grain total fungal count was modelled using a sigmoidal type function. Parallel to the monitoring of fungal development, other malt properties were also evaluated (enzymatic activities: α- and β-amylose, β-glucanases, polyphenol and condensed tannin contents, total malting loss).

2. METHODS

2.1. Sorghum grain characteristics

Red sorghum grain was obtained from the Democratic Republic of Congo (DRC) (vernacular cultivar Imbutongufi). The thousand grain weight (TGW), germination energy (GE), germination capacity (GC), and moisture content were determined as described in Analytica EBC (2004), EBC method 3.4, 2004, EBC method 3.6.3 and EBC method 3.5.2, respectively.

2.2. Malting

Sorghum grain was cleaned and then 150 g was steeped without aeration for 16 h and allowed to germinate at 30 °C for 72 h. Green malt was dried at 40 °C for 48 h and rootlets were hand removed by gentle brushing. Six steeping treatments were tested: H2O (16 h in distilled water), NaOH (16 h in 0.2% NaOH), Ca(OH)2 (16 h in 0.1% Ca(OH)2), C+B (16 h in a B. subtilis S499 culture diluted with distilled water to 105 cells/mL), C (16 h in distilled water containing B. subtilis S499 cells at 106 cells/mL), and B (16 h in the cell-free supernatant obtained after centrifuging a culture diluted as in C+B). For C+B, C, and B, B. subtilis S499 was first grown on Luria Broth agar at 37 °C for 24 h. An inoculating loopful was transferred to 100 mL Landy broth optimized for B. subtilislipopeptide production and incubated for 16 h. 10 mL was then finally 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 $10^6$ cells/mL. This diluted culture was used directly for C+B and centrifuged at 10,000×g for 15 min for C and B. The supernatant was used in B and the pellet was washed and resuspended in distilled water (so as to obtain $10^6$ cells/mL) for C. The RP-HPLC-DAD-MS method was used to assay lipopeptide families (Nihorimbere et al., 2011). The total fungal population was counted on potato dextrose agar supplemented with 0.005 % chloramphenicol (PDA+C).

### 2.3. Microbial growth inhibition tests

*Bacillus subtilis* S499 was grown under conditions optimized for lipopeptide production as in Bwanganga et al. (2012) (Fig. 1). The culture was diluted with water to $10^8$ cells/mL, centrifuged, and the supernatant collected. This cell-free supernatant was used undiluted and at various dilutions in the inhibition tests. 150μL samples were placed in wells of 5 mm diameter cut into PDA+C plates. Each sample was allowed to diffuse into the agar for 4 hours at room temperature before incubation at 30°C. Inhibition was graded as follows: (+) when the strain was inhibited by the undiluted *B. subtilis* culture, (+ +) when it was inhibited by dilution 1/10, (+ + +) when it was inhibited by dilution 1/100, and (++ +++) when it was inhibited by dilution 1/1000.

Three randomly sampled lots of 100 red sorghum grains were obtained and immersed for 2 min in 70% ethanol followed by 2 min in 0.4% chlorine and then rinsed with sterile distilled water (Pitt and Hocking, 2009). The grains were transferred to pre-sterilized Petri dish humidity chambers (25 grains/dish) under aseptic conditions and were incubated, for 5 days at room temperature with a 12 h light cycle, for observation (see Navi et al., 1999). Infected kernels were soaked in sterile distilled water at 4°C, diluted before inoculation on PDA+C and incubated at room temperature. Seven morphologically distinct isolated colonies were selected and grown on PDA+C until spore formation (in the dark). The spores were resuspended in distilled water (0.01 % Tween) and re-inoculated on PDA+C to produce isolated colonies. Individual colony forming units were placed on PDA+C for the inhibition test. Mould identification was performed as described in Pitt and Hocking (2009) and results were compared with that of the Fusarium interactive key (Agri. and Agri-Food Canada, 1996; Navi et al., 1999; Rafi and Sajjad-Ur-Rahman, 2002).

### 2.4. The effect of *B. subtilis* population on malt total fungal count

When a biocontrol is used it is important that the bacterial population is at the minimum necessary to obtain the expected response. Thus, before establishing a biocontrol strategy, we must first answer a crucial question: what is the minimum bacterial population required? To answer this question, sorghum grains (10 g) were soaked in 20 mL *B. subtilis* culture (C+B), washed cells (C) or cell-free supernatant (B) and in diluted (1/10, 1/20, etc.) treatments for 16 hours. Malting was conducted as described previously and the kilned mals total fungal count was evaluated in log CFU on PDA+C agar after incubation at 30°C for 48 h. The total fungal count has been described as a function of the steeping liquor dilution, and the time course of the grain total fungal count for steeping treatment C+B has been modelled as follows:

$$y = (αxβ)\exp(γx)$$

Eq. 1. where y and x represent the total fungal population (log CFU g⁻¹ kilned malt), and the logarithm of *B. subtilis* culture dilution. “α, β and γ”, represent the model kinetic parameters and have been calculated as follows:

1. γ was considered equal to 1, which helped to write the equation Eq. 1 as follows:

$$y = (αxβ)\exp(\chi)$$

Eq. 2. and calculating the approximate values of α and β by plotting the line:

$$y^1 = β^1  + (α^1 - β^1) \exp (\chi)$$

Eq. 3.

Eq. 3. is a straight line whose slope and x-intercept are respectively $(α^1 - β^1)$ and $[\beta/(α - β)]$.

2. The values of α and β obtained were used as start values of iterations and Minitab 16 was used to obtain the final model (confidence level for all intervals: 95 %, the Gauss-Newton algorithm and a convergence tolerance of $0.00001$). From Eq. 1, when $x→0$, $y→y_0=α$, and when $x→∞$, $y→y_c=β$.

The root mean square error (RMSE) $= (1/n)\sum (experimental \ data-predicted \ data)^2/n$ has been used to evaluate the goodness of fit.

### 2.5. Sorghum malt properties

All enzymatic assays were performed on extracts of malt flour obtained by grinding kilned malt in an IKA mill followed by sieving (mesh size: 0.5 mm). 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). β-(1-4)-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 1000xg 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 2 % carboxymethylcellulase as the substrate for β-(1-4)-glucanase. 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 (Primarini and Yoshibuki, 2000) and the β-(1-4)-glucanase activity was expressed in µmoles of glucose released per minute per kg of kilned malt. Megazyme assay procedure for cereal flours and malt
endo-(1,3)-β-glucanase activity and Megazyme S-ABGI003/11 method were used for β-(1,3)- and β-(1,4)-glucanase activities.

Total phenolics were assayed using the method optimized by Georgé et al. (2005) without eliminating water-soluble compounds and results are expressed in mg 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. (1978) and results are expressed in per cent catechin equivalents (% CE).

### 2.6. Statistical analysis

Tukey’s HSD tests, statistical computing and graphics were performed with Minitab 16 software.

### 3. RESULTS

The red sorghum used in this study contained: 11.1±0.2 (% wet weight) moisture content, 26.2 g ± 0.1 thousand grain weight, 96.3 % ± 0.3 germination energy and a germination capacity of 95.5 % ± 0.5. The raw grain total aerobic count was 6.4±0.1 log CFU/g and the initial fungal plate count was 4.8±0.2 log CFU/g. The protein content was 12.7±0.3 g/100 g and the total phenolic compounds and condensed tannins were 8.1±0.1 mg gallic acid equivalent and 0.18±0.0 % catechin equivalent, respectively.

Lipopeptide levels in the *B. subtilis* supernatant (RP-HPLC-DAD-MS) were: surfactins: 385.21 µg/mL; fengycins: 264.54 µg/mL. Lipopeptide levels in *B. subtilis* supernatant included: surfactins: 385.21 µg/mL; fengycins: 264.54 µg/mL. Lipopeptide levels in *B. subtilis* supernatant included: surfactins: 385.21 µg/mL; fengycins: 264.54 µg/mL. Lipopeptide levels in *B. subtilis* supernatant included: surfactins: 385.21 µg/mL; fengycins: 264.54 µg/mL.

### 3.1. Effect of *B. subtilis* on Malt properties

<table>
<thead>
<tr>
<th>Malting Loss (%)</th>
<th>Kilned malt</th>
<th>Total fungal count (log CFU)</th>
<th>Mixed media</th>
<th>H2O</th>
<th>NaOH</th>
<th>C+B</th>
<th>C</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.2±0.5 b</td>
<td>4.6±0.1 a</td>
<td>3.7±0.0 b</td>
<td>3.7±0.1 c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.9±0.2 b</td>
<td>2.8±0.0 c</td>
<td>3.4±0.1 c</td>
<td>3.4±0.1 c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.9±0.2 a</td>
<td>2.8±0.0 c</td>
<td>3.4±0.1 c</td>
<td>3.4±0.1 c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.7±0.1 c</td>
<td>1.7±0.0</td>
<td>2.3±0.0</td>
<td>2.3±0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17.9±0.0 c</td>
<td>1.4±0.0</td>
<td>2.0±0.0</td>
<td>2.0±0.0</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>14.1±0.1 d</td>
<td>1.1±0.0</td>
<td>2.5±0.0</td>
<td>2.5±0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

### 3.2. Effect of *B. subtilis* on Microbial Load

- *B. subtilis* cells/mL, C (16 h in distilled water containing C+B (16 h in a B. subtilis culture diluted with distilled water to 10³ cells/mL), C (16 h in distilled water containing B. subtilis cells at 10³ cells/mL), and B (16 h in the cell-free supernatant obtained after centrifuging a culture diluted as in C+B).

High percentages of inhibition were obtained with biocontrol treatments were compared to dilute alkalines and distilled water steeping (≈99.99% for C+B and B treatments). All seven have been inhibited by *B. subtilis* S499 to differing degrees, as follows: (a) *F. oxysporum* (+++), (b) *F. solani* (+), (c) *A. niger* (+++), (d) *A. versicolor* (+), (e) *Penicillium expansum* (++), (f) *Penicillium chrysogenum* (+++), (g) penicillium sp. (+). Values are mean±StDev. Treatments within a line having a letter in common are not statistically different according to Tukey’s HSD test (p<0.05).

### 3.3. Effect of *B. subtilis* on Enzyme Activities

Enzyme activities assayed were: α-amylase, β-amylase, β-(1,4)-, β-(1,3)-, β-(1,3)-(1,4)-

### Table 1. Red sorghum malts properties as affected by steeping treatment

<table>
<thead>
<tr>
<th>Malting property</th>
<th>H2O</th>
<th>NaOH</th>
<th>C+B</th>
<th>C</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilned malt Total fungal count (log CFU)</td>
<td>4.6±0.1 a</td>
<td>2.8±0.0 c</td>
<td>3.7±0.0 b</td>
<td>3.7±0.1 c</td>
<td>3.7±0.1 c</td>
</tr>
<tr>
<td>Total Malting Loss (%)</td>
<td>24.2±0.5 b</td>
<td>24.9±0.2 b</td>
<td>26.9±0.2 a</td>
<td>18.7±0.1 c</td>
<td>17.9±0.0 c</td>
</tr>
</tbody>
</table>

### Figure 1. A. Moulds isolation and inhibition by *Bacillus subtilis*; B. Malting steps

The effect of a *B. subtilis* population on malt enzyme activities is presented in Fig. 2.
Figure 2. Effect of *B. subtilis* dilution on kilned malt α-amylase, β-amylase and β-glucanase activities. Values are mean±StDev. Treatments having a letter in common are not statistically different according to Tukey's honestly significant difference test (p<0.05). C+B: steeping 16 h in a *B. subtilis* culture diluted with distilled water to $10^8$ cells/mL; Diluted C+B (4 log CFU/mL): steeping 16 h in a *B. subtilis* culture diluted with distilled water to $10^4$ cells/mL.

The effect of a *B. subtilis* population in the steeping liquor on mould development has been modelled as described in the materials and methods. The result of the first approximation is presented in Fig. 3 and according to the result obtained, Eq.3, can be written as follows:

$$y^{-1} = 0.2038 + 1.418 \exp(x)$$

Eq. 4

Figure 3. Regression line of 1/experimental values of total fungal count versus exp(log DF) for the first iteration ($\gamma=1$)

From Eq. 4., approximate values of $\alpha$ and $\beta$ have been calculated ($\alpha \approx 0.6166$ and $\beta \approx 4.907$).

The final model equation obtained using Minitab 16 software was:

$$\text{TFC (log CFU)} = 4.67/(0.79 + 5.10 \exp(0.74 \log DF))$$

Eq. 5

Where $\alpha = 0.793518$, $\beta = 5.89016$ and $\gamma = 0.739028$. The experimental and predicted data scatterplot is presented in Fig. 4 and the RMSE of the fit (for the steeping treatment C+B) was 0.23 log CFU.
4. DISCUSSION

The inhibitory effect of *B. subtilis* on mould growth is well known and is often attributed to the presence of lipopeptides produced by *B. subtilis* species (Touré et al., 2004; Romero et al., 2007). Indeed, as shown in the results section, the *B. subtilis* strain used is capable of producing significant amounts of lipopeptides and inhibiting 7 moulds isolated from red sorghum grain. This inhibition (*in vitro*) is to differing degrees and dependent on the strain involved. However, during malting, the situation is far from reduced to simple mould inhibition by *B. subtilis*, but must still take into account the effect of the use of large amounts of microorganisms on grain metabolism. Indeed, it is known that plant-microbrial ecosystem interactions are complex and the molecular dialogue that occurs between microorganisms and plants has never been fully elucidated (Camphnhoui et al., 1998).
Our results (Fig. 5) show that the inhibition is not exclusively the effect of lipopeptide production. Indeed, when the culture is diluted to a dilution factor (DF) of 1/10000, for example, the lipopeptide concentration is extremely low and not sufficient to exert an effect on mould growth; but when both B. subtilis cells and cell-free supernatant or when B. subtilis washed cells are used in the steeping liquor, mould growth is still reduced due to the presence of B. subtilis cells. In other words, the increase in total fungal count is not proportional to the dilution of B. subtilis culture since the lipopeptide levels are above the minimal inhibitory dilution (minID) which corresponds – on Fig. 5 – to the region at the beginning of dilution (high B. subtilis population and high lipopeptide concentrations). Phase one is an area where the loss of efficacy of the culture is not proportional to the dilution. This area represents the range of dilution where the inhibitory effect is mainly due to the concentration of lipopeptides produced by B. subtilis. During the second phase, when B. subtilis cell-free supernatant is used alone as the steeping liquor, an increase in dilution is accompanied by a sudden jump in the total fungal count (Fig. 4: experimental data (B)). A third phase starts from the maximal inhibitory dilution (MaxID) – at which, the dilution of B. subtilis culture no longer affects the development of the total fungal count; this range of dilution is simply inefficacious. The advantage of such a model is to determine, based on the initial fungal load, the required dilution range to obtain certain levels of reduction in the development of the total fungal count. The inflection point (log DF = -2.51 and TFC = 2.94) of the function represented by the Eq. 4 can be obtained by solving the following equation:

\[ d^2y/dx^2 = \left[ 166.5152e^{-1.48x} - 10.3033xe^{0.72x} \right] \times \left[ 0.79 + 5.10xe^{0.74x} \right]^{-3} \]  
Eq. 6

Where y and x represent, respectively, the TFC and the log DF.

So, when we consider an infinitesimal variation of log DF (x) around the inflection point of the function represented by the Eq. 4 which corresponds to an infinitesimal variation in total fungal count (y), and we apply the principle of the right triangle, one obtains the slope of the line (y = -1.0935 x + 0.200) whose intersections with the asymptotes (y0 = α and y∞ = β) represent approximately the minID (log DF ≈ 0.540) and the MaxID (log DF ≈ -5.223), respectively. When B. subtilis is used as a starter, malts obtained have low levels of fungal contamination. The inhibitory effect of NaOH on mould growth has indeed been previously demonstrated (Lefyedi and Taylor, 2006). Thus, the advantage of using a biocontrol should not simply be reduced by decreasing the growth of mould. As shown in Table 1 – although soaking in alkaline solutions has given the best α- and β-amylase activities, total polyphenol compounds and condensed tannins reduction – the use of the biocontrol allows malts with high β-glucanase activities and low malting losses to be obtained. It is known that the hydrolysis of β-glucans and the reduction on malting loss are major concerns during the malting and brewing of sorghum (Etokakpan, 1992; Ogbonna, 2011). Kilned malt total fungal count, enzyme activities (α-amylase, β-amylase, β-(1,4)-β-(1,3), β-(1,3)-(1,4)-glucanases), total phenolic compounds, condensed tannins and losses are presented in Table 1.

Table 1 shows that the residual phenolics represented 84.3 % after NaOH and 86.6 % after Ca(OH)2, and residual condensed tannins represented 37.0 and 40.7 % respectively. Total phenolic compounds were unaffected and condensed tannins only slightly affected in the presence of the cell-free supernatant conditioned by B. subtilis S499, and the presence of bacterial cells (C+B, C) had no significant effect on the level of phenolics compared with steeping in distilled water (H2O). According to the Tukey’s HSD test, the level of condensed tannins was significantly lower in the presence of washed cells (C) than in the presence of a conditioned medium with or without cells (C+B, B).

The question remains, why does the use of B. subtilis not give good levels of α- and β-amylase activities? Several hypotheses can be made in relation to the effect of lipopeptides on malt enzyme activities and various interactions between the large bacterial population used in steeping liquor and grain germination. Biosurfactants produced by B. subtilis have antioxidant properties (Yalçın et al., 2010). In fact, it has been shown that amylase becomes less susceptible to enzymatic hydrolysis through the formation of complexes with surfactants and many surfactants can also interact with proteins and alter their secondary and tertiary structures (Rodríguez et al., 2006). The high levels of polyphenols and condensed tannins can also affect enzyme activities. The grain steep-out moisture in the biocontrol treatments is lower than those of grains obtained after soaking in dilute alkaline solutions, thus confirming the results of Dewar et al. (1997), according to which the grain moisture influences the malt diastatic power. Another mechanism involves the fact that when the steeping liquor is not aerated, the microbial ecosystem can compete with steeped grain for available oxygen (Lewis and Bamforth, 2006), which may have a significant effect on seed germination.

Malt obtained with the biocontrol presented enzyme activities higher than those obtained after steeping in
distilled water. Therefore, the results of Fig. 2 show that the dilution of the culture exerted an effect on the various enzymatic activities, thus confirming the effect of the microbial ecosystem on malt properties (Laitila, 2007; Camphnhou et al., 1998).

5. CONCLUSION

Bacillus subtilis can be used to control mould growth. The effect of B. subtilis dilution on total fungal growth can be modelled using a sigmoidal-like function. When B.-subtilis-based treatments are used during red sorghum malting – grain steeping conducted without aeration – mould growth is strongly inhibited compared with dilute alkaline steeping (NaOH and Ca(OH)₂), while malt α- and β- amylase activities are lower than those obtained after steeping in alkaline solutions. The use of the biocontrol also leads to the production of malts with high levels of phenolic compounds. Compared with steeping in dilute alkaline, the total malting losses obtained with the biocontrol treatment remained at lower levels. A B. subtilis starter used during red sorghum malting affected both the total fungal development and the grain physiology (the level of hydrolytic key enzymes). It is important to study the interactions between B. subtilis and seed germination to take advantage of the beneficial effects of the Bacillus-subtilis-based biocontrol.

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