DOI:10.1021/jf901044u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Browning of Freeze-Dried Probiotic Bacteria Cultures in Relation to Loss of Viability during Storage

Lone Kurtmann, Leif H. Skibsted, and Charlotte U. Carlsen*

Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Freeze-dried cultures of *Lactobacillus acidophilus* (La-5) showed visible brown discoloration even after a short storage at relatively mild conditions (a_w = 0.22 and 30 °C), and the browning processes were found to coincide with bacteria inactivation. It was demonstrated, by using high-pressure treatment for obtaining bacteria samples with different ratios of live/dead bacteria, that death of bacteria is not a prerequisite for the browning processes. Furthermore, it was shown that hydroxymethylfurfural (HMF) (or condensation products of HMF) introduces accelerated viability loss when HMF is added to the freeze-drying medium. Discoloration of bacteria cultures containing only sucrose/maltodextrin or lactose/maltodextrin in the freeze-drying matrices is suggested to be related to various types of nonenzymatic browning reactions, including carbonyl−protein (or carbonyl−DNA) interactions and carbohydrate condensation/polymerization (without involvement of proteins), the latter proceeding at low a_w following hydrolysis of the peptidoglycan layer in the bacteria cell wall. More than one single type of browning reaction is accordingly concluded to be related to bacteria death, and the loss of viability in freeze-dried bacteria seems to be influenced by oxidation reactions, browning reactions, and the physical instability of the bacteria membrane/cell wall.

KEYWORDS: Dried bacteria cultures; discoloration; browning reactions; protein−carbohydrate interactions; oxidation; DNA damage; viability

INTRODUCTION

Intake of probiotic bacteria is associated with various health benefits (*1*, *2*), and new probiotic food products are entering the market. To preserve probiotic bacteria cultures during transportation and storage, they are usually dried in an aqueous matrix of solutes, which serve to embed the bacteria and ensure a long-term delivery of active bacteria upon rehydration. However, during storage in the dry state, loss of bacteria viability is still observed, and selection of optimal matrix components has been the scope of many investigations (*3*−*5*). Furthermore, a more complete understanding of the chemical and physical processes leading to decrease in survival is receiving increased attention (*6*).

Coincident with the loss of bacterial viability, a few studies have reported that discoloration of dried bacteria cultures develops during storage in the dried state (*7*−*9*). Both the viability loss and browning of the otherwise white to light yellow dried bacteria cultures are clearly unwanted. Similar browning during storage is observed for dried foods such as milk powder and infant formulas and has been found to result mainly from sugar−protein or sugar−peptide interactions in the so-called Maillard reactions (*10*−*12*). With the aim of achieving more detailed knowledge of the processes leading to bacteria instability in freeze-dried matrices, a correlation between discoloration and loss of viability was recently suggested by Kurtmann et al. (*8*, *9*) and Carvalho et al. (*7*). In plant seed science, an interaction between the formation of Maillard reaction products during storage and a decreased enzymatic activity has been demonstrated (*13*, *14*) and explained by protein modification resulting from glycation with reducing sugars or reaction with aldehydes from lipid oxidation processes. Such a relationship is in agreement with the indications previously obtained for freeze-dried *Lactobacillus acidophilus* cultures (La-5), in which interactions between discoloration, radical formation, and viability loss during storage were demonstrated (*9*).

The present study explores accordingly more specifically the correlation between viability loss and discoloration in freeze-dried La-5. Different matrices (including reducing/nonreducing carbohydrates) are compared, and viability and browning are both followed during storage.

MATERIALS AND METHODS

Preparation of Probiotic Bacteria Cultures. The freeze-dried system was a noncommercial laboratory-scale production of a matrix-encapsulated concentrate of *L. acidophilus* (La-5) from Chr. Hansen A/S, Hørsholm, Denmark. All bacterial concentrate used was from the same fermentation process, and pH and temperature were controlled during the fermentation. After harvest of cells in the stationary growth phase and concentration by centrifugation, the bacterial concentrate was divided into four portions, and each was mixed with an equal volume of a protective solution. The protective medium consisted of sucrose (Danisco Sugar, Copenhagen, Denmark)/lactose (Rovita GmbH, Engelsberg/Wiesmühl,

*Corresponding author (telephone +45 35 33 32 90; fax +45 35 33 33 44; e-mail cac@life.ku.dk).

 Published on Web 07/10/2009

Germany), maltodextrin (Glucidex IT12, DE 11-14, Roquette, France), and water with or without the addition of hydroxymethylfurfural (HMF) (Sigma-Aldrich, Steinheim, Germany). The dry ingredients were dissolved in water at room temperature before addition to the bacterial concentrate. The protective medium and bacterial concentrate was mixed for 30 min before freezing. The bacterial suspensions were frozen in pellets of approximately 2 mm in liquid nitrogen and placed at −50 °C until freeze-drying. For freeze-drying (Hetosicc freeze-dryer, CD-10-1, Heto Lab Equipment, Heto-Holten A/S, Allerod, Denmark), the pellets (approximately 6.5 kg) were placed in a chamber that had been precooled to −40 °C at atmospheric pressure. Afterward, the pressure was reduced to 0.3 mbar, and subsequently the temperature was raised at 0.5 °C/min until an end temperature of 32 °C was reached. After freeze-drying, the water activity of the dried samples was below 0.1. The samples were stored at −40 °C in sealed metalized bags until further use. On a dry basis the composition of samples was 40% bacteria cells/10% maltodextrin/50% sucrose/lactose or 40% bacteria cells/10% HMF/10% maltodextrin/40% sucrose/lactose (w/w).

High-Pressure Treatment of Freeze-Dried Bacteria. Freeze-dried bacteria was vacuum-packed in plastic bags to remove any headspace before high-pressure treatment. The samples were submerged in the pressurizing chamber of a QUINTUS Food Processing Cold Isostatic Press QFP-6 (Avure Technologies AB, Västerås, Sweden) with water thermostated at 5 °C as the pressure transmission fluid. The bacteria samples were exposed to hydrostatic pressures at either 200 or 800 MPa for 15 min.

Storage Experiments. The bacteria cultures were placed in glass desiccators with saturated salt solutions in the bottom of the desiccators with $a_w = 0.22$ (CH_3COOK) and $a_w = 0.32$ ($MgCl_2 \cdot 6\ H_2O$) and stored at 25/30 °C for a period of up to 13 weeks. Survival of the bacteria (CFU/g) was followed during the storage period, and for each storage condition colony-forming units (CFU) per gram was measured twice for each sampling time. Following sampling for survival analysis, a_w of the sample material was measured, and a_w of the saturated salt solution used for equilibration was controlled. Additionally, the surface color of the dried bacteria samples was measured.

UV−Vis Spectroscopy. A 0.25 mg freeze-dried bacteria sample was resuspended in 2.50 mL of Milli-Q water. The suspension was centrifuged for 5 min at 20000*g*, and the supernatant was used for absorbance measurements using an HP8453 UV−vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). Absorbance spectra were recorded from 200 to 700 nm for appropriate dilutions of the samples to obtain the absorbance characteristics in the UV as well as the visible area.

Water Activity Measurements. a_w values of freeze-dried bacteria material and saturated salt solutions were measured at room temperature using an Aqua Lab CX-2 instrument (Aqua Lab, Pullman, WA).

Color Measurements. Surface color of the dried cultures was measured using a Hunter *L**, *a**, *b** color system with a Byk-Gardner color guide (Byk-Gardner GmbH, Geretsried, Germany). In this study the color changes were expressed by the *b** value, which measures yellowness (+) or blueness (−); an increase in the *b** value reflects a change in color toward yellow and brown. The Hunter values were determined as the mean of three measurements each at two different places on the surface of the dried bacteria sample.

Colony-Forming Units. The CFU were determined by rehydrating the bacteria samples (10^{-2} dilution) for 20 min at room temperature in a sterile diluent 0.90% NaCl (Merck, Darmstadt, Germany) using a magnetic stirrer, and appropriate dilutions were prepared afterward. One hundred microliters from the selected dilutions was transferred into duplicate sets of Petri dishes and mixed thoroughly with 12−15 mL of melted MRS agar (45 °C) (Merck). The inverted dishes were incubated under an anaerobic atmosphere established by using Anaerocult A (Merck) at 37 °C for 3 days, and the colonies were counted on plates on which the dilution gave between 30 and 300 colonies. The results were expressed as CFU per gram of dry material (CFU/g). The survival was determined twice for each storage condition. The coefficient of variations (CV %) was always below 15%.

Furosine Analysis. Furosine was determined in solid-phase extracted hydrolysates of the freeze-dried bacteria samples using liquid chromatography separation. An ion-pair reverse phase column (Synergy, 4 μm Fusion- RP 80 Å, 250 mm × 4.6 mm, Phenomenex ApS, Allerod, Denmark) connected to an HPLC system with UV detection at 280 nm (Agilent 1100, Agilent Technologies, Santa Clara, CA) was used with a mobile phase of 0.11% (w/v) acetic acid with 0.10% (w/v) ammonium hydrogencarbonate, pH 5.1. Isocratic elution at room temperature was used with a flow rate of 0.7 mL/min and an injection volume of 20 μL. For quantification of furosine in the samples a calibration curve was established using dilutions (in 3.0 M HCl) of a furosine standard (ε-*N*-(2-furoyl)-L-lysine from NeoMPS S.A., Strasbourg, France). The freeze-dried bacteria samples were prepared for the HPLC analysis according to the method of Resmini et al. (*15*) by hydrolyzing 100 mg in 8.0 mL of 8.0 M HCl for 23 h at 110 °C and with a subsequent filtration on a Sep-Pak C18 cartridge (Mallinckrodt Baker B.V., Deventer, The Netherlands). The Sep-Pak cartridge was prewetted with 5.0 mL of methanol and 2 × 5 mL of Milli-Q water. A 500 μL sample (supernatant of the hydrolysate) was finally added and eluted with 4.5 mL of 3.0 M HCl.

RESULTS

When freeze-dried *L. acidophilus* (La-5) samples were stored at room temperature and water activities controlled in the interval of 0.1−0.4 for up to 3 months, increased browning of the bacteria culture was observed during the storage periods (*9*). A change in color is clearly visible even after short storage at relatively mild conditions ($a_w = 0.22$ and 30 °C), as may be seen from **Figure 1**.

The rate of discoloration increased with storage time and with increasing a_w and storage temperature, and discoloration seems to coincide with a significant loss of viability for La-5 freeze-dried in sucrose/maltodextrin or lactose/maltodextrin and stored at $a_w = 0.22$ and 30 °C as shown in **Figure 2**.

A similar coincidence of browning and bacterial inactivation was observed at higher water activity (see **Figure 3**), and it seems that the composition of the freeze-drying matrix (sucrose/lactose) also has an impact on the rate of inactivation of the bacteria at this higher a_w. The samples containing the reducing sugar lactose as a main part of the freeze-drying matrix exhibit a higher loss of viability during the storage period than the samples with a freeze-drying matrix with the nonreducing sugar sucrose (**Figure 3A**). This difference was, however, not reflected in the surface color measurements as rather similar values for the *b** parameter were observed for the two sample types (**Figure 3B**).

Carvalho et al. (*7*) suggested that death of freeze-dried bacteria is a prerequisite for their participation in browning reactions, most probably by making protein side chains more available for reaction with reducing matrix sugars in Maillard reactions. To test this hypothesis, a storage experiment with La-5 freeze-dried in a lactose/maltodextrin matrix was performed, in which the initial amount of viable cells was varied by subjecting the freeze-dried bacteria samples to high-pressure treatments just before onset of the storage period. Hence, time profiles from the storage of these samples would indicate if a higher number of dead cells leads to more pronounced discoloration. From **Figure 4** it may be seen that high-pressure treatments at 200 and 800 MPa both resulted in inactivation of approximately 90% of the bacteria in the dried samples and that the viability loss and discoloration during storage at a_w 0.23 had the same time profile for the

Figure 1. Browning processes in dried bacteria cultures during early storage: *Lactobacillus acidophilus* freeze-dried in lactose/maltodextrin matrix before (left) and after 6 weeks of storage at 30 °C and a_w 0.22 (right).

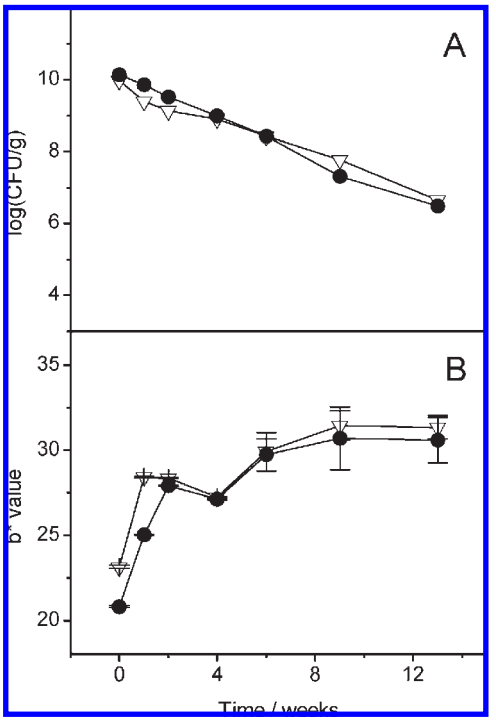

Figure 2. (**A**) Survival (log(CFU/g)) and (**B**) development in b^* value during storage at 30 °C and a_w 0.22 of *L. acidophilus* freeze-dried in a sucrose/maltodextrin (▽) or lactose/maltodextrin matrix (●).

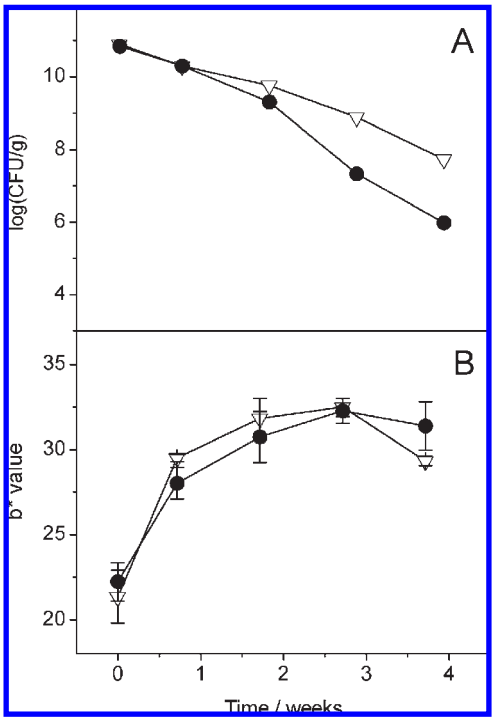

Figure 3. (**A**) Survival (log(CFU/g)) and (**B**) development in b^* value during storage at 30 °C and a_w 0.32 of *L. acidophilus* freeze-dried in a sucrose/maltodextrin (▽) or lactose/maltodextrin matrix (●).

pressurized samples as for the nonpressurized samples. Hence, the initial number of viable and nonviable cells is concluded to have no influence on bacteria inactivation (**Figure 4A**) or discoloration (**Figure 4B**). A similar behavior was observed for storage of high-pressurized bacteria samples at $a_w = 0.33$ (data not shown).

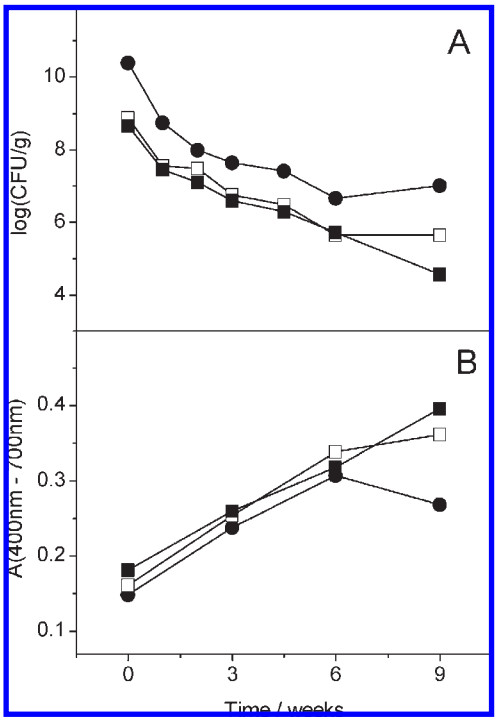

Figure 4. Storage at 25 °C and a_w = 0.23 of high-pressure-treated *L. acidophilus* freeze-dried in a lactose/maltodextrin matrix: (□) 200 MPa, 15 min; (■) 800 MPa, 15 min; (●) no treatment; (**A**) survival (log(CFU/g)) during storage and (**B**) absorbance at 400 nm for supernatants of resuspended samples as corrected for sample turbidity ($A_{400-700nm}$).

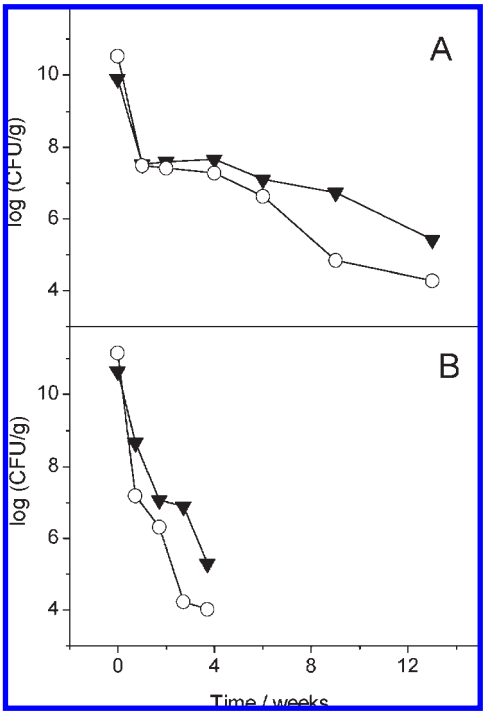

Figure 5. Survival (log(CFU/g)) of *L. acidophilus* freeze-dried in a sucrose/maltodextrin matrix with HMF (▼) or lactose/maltodextrin matrix with HMF (○): (**A**) storage at 30 °C and a_w = 0.22; (**B**) storage at 30 °C and a_w = 0.32.

The correlation between discoloration and viability loss was studied further by adding hydroxymethylfurfural (HMF), as a colorless nonenzymatic browning reaction product, to the

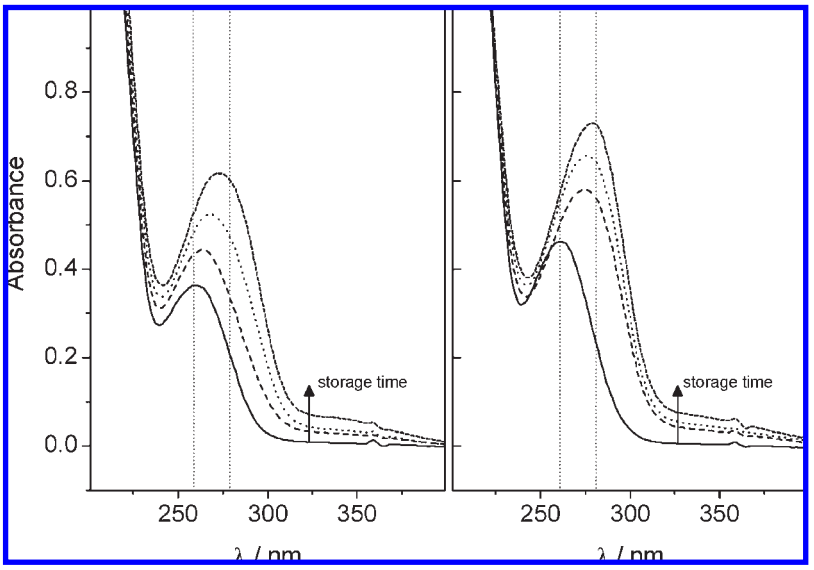

Figure 6. UV spectra of supernatants from resuspensions of freeze-dried *L. acidophilus* in sucrose/maltodextrin matrix (left) or lactose/maltodextrin matrix (right). Samples were stored at 30 °C and $a_w = 0.22$ and taken out for measurements at weeks 0, 2, 4, and 9. Vertical lines indicate λ_{260} and λ_{280} in each panel.

freeze-drying matrix. Thereby, samples of freeze-dried La-5 with high amounts of HMF in the matrices were produced. The impact of HMF on bacteria stability during storage was very clear, as shown in **Figure 5** for bacteria with a sucrose/maltodextrin/HMF or a lactose/maltodextrin/HMF matrix. HMF (and/or its degradation products) was very detrimental to the bacteria, and fast bacteria inactivation was observed at all conditions (cf. **Figures 2** and **3**). Inactivation was, as expected, faster at $a_w = 0.32$ than at $a_w = 0.22$, and it is noteworthy that the viability loss is highest for bacteria samples containing lactose rather that sucrose. All HMF-containing bacteria samples had a very dark color already after freeze-drying and turned almost black during storage (data not shown). Additionally, it was observed that the addition of HMF to bacteria suspensions during the fermentation step did *not* affect the viability (data not shown), and the addition of a very high amount of HMF to the freeze-drying medium for production of freeze-dried bacteria samples with HMF was based on this observation.

The formation of HMF in biological systems is often followed by UV spectroscopy as HMF absorbs at 280 nm, and by resuspending the dried bacteria samples containing added HMF in their matrices and using the supernatant (after a centrifugation step) for UV spectroscopy, a characteristic HMF spectrum with intense absorption maximum around 280 nm was obtained (not shown). The same analysis was applied for some of the freeze-dried bacteria samples from the storage experiment shown in **Figure 2**, that is, bacteria samples without added HMF, and the resulting UV spectra are shown in **Figure 6**. For both sample types the initial UV spectrum (storage time zero) has a clear absorbance maximum at ~260 nm, a maximum that is characteristic for nucleic acids from, for example, RNA and DNA (*15*). As the storage proceeds it was, however, observed that the UV absorption increased and that the spectrum is red-shifted. These changes were most significant for bacteria samples freeze-dried in lactose/maltodextrin (right panel), but also notable for bacteria samples freeze-dried in sucrose/maltodextrin (left panel).

As HMF is formed both in Maillard reactions involving reducing sugars and amino groups from protein/peptides and in sugar isomerization/condensation without involvement of amino groups, the bacteria samples were also subjected to furosine analysis. Furosine is formed by acid hydrolysis of biological samples if Amadori products are present. Amadori products accumulate in the early stage of the Maillard reaction through reactions between reducing carbohydrates and lysine side chains in proteins/peptides (*17*). The applied HPLC analysis showed only very little furosine formation from the bacteria samples stored at $a_w = 0.22$, and no changes in concentration with storage time were measured (data not shown). Skimmed milk powder was analyzed for comparison and validation of the method, and in these samples an amount of furosine comparable to what is reported in the literature was found.

DISCUSSION

Browning of freeze-dried *L. acidophilus* (La-5) cultures has a significant influence on the appearance of the products after prolonged storage even at relatively mild conditions (low water activity and room temperature). A correlation between the browning processes and loss of bacteria viability during storage is, however, more unclear, and the exact nature of the browning reactions involved in such bacteria inactivation remains to be identified.

The results from the current and a previous study (*8*) show that the presence of higher amounts of reducing sugar in the freeze-drying matrix results in a slightly higher rate of bacteria inactivation during storage, suggesting involvement of peptide/protein−carbohydrate interactions (Maillard reactions) in the process of bacteria inactivation. The investigation of the effect of the ratio between dead and live bacteria cells at the beginning of the storage period (using high pressure as a nonthermal inactivation) clearly showed that death of the cells is not a prerequisite for the discoloration process (**Figure 4**).

As a logical continuation of the experiment with nonthermal bacterial inactivation, the effect of adding HMF as an intermediate nonenzymatic browning product to the freeze-drying medium was investigated (**Figure 5**). These experiments showed that browning reactions *can* have an effect on bacteria viability under some conditions. The high amount of HMF significantly promoted bacteria inactivation when compared to the rate of inactivation in the storage experiments with bacteria freeze-dried in sugar matrices without added HMF (**Figures 2** and **3**). However, the observation that the bacteria samples dried with HMF had a much darker color already after the freeze-drying process compared to samples dried without the addition of HMF illustrates that the added amount of HMF was very high. It

should, however, be noted that HMF was nontoxic to bacteria suspensions when added immediately after the fermentation step. Accordingly, the experiments provide the mechanistic information that not HMF as such or alone but, rather, HMF degradation/condensation products are detrimental to the bacteria in the freeze-dried state.

Surprisingly, analysis of furosine formation in acid-hydrolyzed samples of bacteria freeze-dried in carbohydrate matrices of sucrose/maltodextrin or lactose/maltodextrin showed very small furosine levels, which, moreover, were constant with time for storage at $a_w = 0.22$. Hence, the observed browning in the freeze-dried bacteria samples stored at $a_w = 0.22$ does not seem to result from Maillard reactions, because Amadori products formed between reducing carbohydrates and lysine side chains in proteins/peptides will be converted to furosine upon acid hydrolysis of the samples (*17*). The low a_w and storage temperature (0.22 and 30 °C, respectively) do most probably not favor browning through carbohydrate condensation and polymerization in the bacteria matrices (without involvement of proteins), and *if* this was the cause of the observed browning, one would have expected a larger difference between samples with high and samples with low amounts of reducing sugar. Accordingly, we suggest the following two hypotheses for the observed browning during storage of freeze-dried bacteria at low a_w, which both are further supported by the UV spectroscopic analysis:

(i) The red shift of the UV spectra of the supernatants from centrifuged bacteria suspensions toward $\lambda_{max} = 280$ nm (**Figure 6**) indicates formation of HMF in the dried bacteria samples as the storage time proceeds. As this HMF formation is apparently not related to Maillard chemistry and most probably not to condensation reaction in the carbohydrate matrices, it may be assigned as reaction products of transformations occurring in the bacteria cells, which, in contrast to the matrices, is the same for the two investigated types of freeze-dried bacteria cultures. The peptidoglycan layer, which constitutes the cell wall of the Gram-positive *L. acidophilus*, is suggested to partly degrade and hydrolyze during freeze-drying and storage, forming reducing carbonyl groups, which may slowly undergo condensation reactions resulting in browning of the freeze-dried samples. Hydrolysis of the peptidoglycan layer may be enzymatically assisted by lysozyme from the bacteria cytoplasm (*18*). A somewhat similar hypothesis was proposed for the browning processes occurring in dried soybean seeds, which initially do not contain reducing carbohydrates (*19*).

(ii) The UV spectra of the supernatants from bacteria samples prior to storage ($t = 0$) indicate the presence of nucleic acids (**Figure 6**). Such presence requires a leak of DNA/RNA (or degradation products hereof) from the bacteria cells and could be due to damages to the cell wall/cell membrane occurring during drying and storage or during the resuspension and centrifugation. Consequently, the red shift and the increase of the UV spectra with the storage time for both sample types may not solely be due to HMF formation as suggested above, but may, alternatively, be due to DNA/RNA oxidation. Oxidation of DNA has been shown to result in red-shifted absorption (at least for some oxidation products) (*20*, *21*), and under oxidizing conditions condensation reactions between nucleophilic groups in DNA/oxidized DNA and free carbonyl groups are very likely to occur (*22*), eventually leading to browning. Such condensation reactions may also involve reducing sugars from the freeze-drying matrix, which may explain the slightly higher absorbance in supernatants from bacteria in a maltodextrin/lactose matrix (compared to a maltodextrin/sucrose matrix). Further studies, including samples stored in anoxic atmosphere and more specific detection of DNA/oxidized DNA, should be encouraged.

Oxidation-mediated DNA damage has been suggested as a mechanism leading to bacteria cell death (*23*−*25*), and oxidation of other cell components such as the membranal lipids has also been in focus (*26*). Furthermore, lipid carbonyls as formed as secondary oxidation products from lipid oxidation have been suggested to participate in browning reactions through reactions with amino groups in Maillard-like reactions (*14*). Hence, oxidation and browning processes in freeze-dried bacteria may indeed be inter-related processes, and it is very possible that hypothesis (i) above does not rule out hypothesis (ii). They may both contribute to the observed browning during storage at $a_w = 0.22$ in the present study.

In our previous study the impact of a_w on viability loss during the storage of freeze-dried *L. acidophilus* was studied in some detail (*9*). It was observed that an increase of storage a_w from 0.11 to 0.22 had relatively less negative impact on the viability of the bacteria than did an increase in a_w from 0.22 to 0.32. A further increase from 0.32 to 0.43 also had a relatively less negative effect that the increase from 0.22 to 0.32. The increase in a_w from 0.22 to 0.32 may result in acceleration of detrimental processes, but also new reactions may be introduced. It is possible that the conditions for Maillard reactions are favored when a_w is increased from 0.22 to 0.32 and that Maillard reaction products formed at this a_w are related to the increased negative effect on the bacteria viability. Notably, both the rate and extent of browning in the freeze-dried bacteria are higher at $a_w = 0.32$ than at $a_w = 0.22$ (cf. **Figures 2** and **3**), indicating that another type of browning reaction, such as Maillard reactions, could have been introduced.

In conclusion, the browning of freeze-dried bacteria seems to be related to various types of browning reactions, the relative importance of which depends on a_w and to a lesser degree on the composition of the freeze-drying matrix. The correlation between browning reactions and viability loss during storage is complicated, and one type of browning reaction seems not to be solely responsible of bacteria death. Losses of viability seem to involve both oxidation and browning reactions and the physical stability of the bacteria membrane/cell wall, and the interplay should be subject to further investigations.

ACKNOWLEDGMENT

The technical assistance of Kirsten Sjøstrøm is gratefully acknowledged, and we thank Chr. Hansen A/S, Hørsholm, Denmark for providing the bacteria samples.

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Received March 30, 2009. Revised manuscript received June 18, 2009. Accepted June 25, 2009. The research was supported by the Danish Dairy Research Foundation, Aarhus, Denmark, the Danish Research Council (The FELFO program committee), Chr. Hansen A/S, Hørsholm, Denmark, and the LMC graduate school FOOD, Copenhagen, Denmark.