Cryobiology 65 (2012) 308–318

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Evaluation of the relevance of the glassy state as stability criterion for freeze-dried bacteria by application of the Arrhenius and WLF model ☆

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ARTICLE INFO

Article history:
Received 14 March 2012
Accepted 24 August 2012
Available online 3 September 2012

Keywords:
Freeze-drying
Bacteria
Glassy state
Storage stability
Restricted diffusion
Arrhenius
WLF

ABSTRACT

The aim of this work was to describe the temperature dependence of microbial inactivation for several storage conditions and protective systems (lactose, trehalose and dextran) in relation to the physical state of the sample, i.e. the glassy or non-glassy state. The resulting inactivation rates k were described by applying two models, Arrhenius and Williams–Landel–Ferry (WLF), in order to evaluate the relevance of diffusional limitation as a protective mechanism. The application of the Arrhenius model revealed a significant decrease in activation energy E_a for storage conditions close to T_g. This finding is an indication that the protective effect of a surrounding glassy matrix can, at least, partly be ascribed to its inherent restricted diffusion and mobility. The application of the WLF model revealed that the temperature dependence of microbial inactivation above T_g is significantly weaker than predicted by the universal coefficients. Thus, it can be concluded that microbial inactivation is not directly linked with the mechanical relaxation behavior of the surrounding matrix as it was reported for viscosity and crystallization phenomena in case of disaccharide systems.

Introduction

Today, we face a situation with an ever-increasing demand for probiotics in stabilized form. It has been reported that a continuously growing global market will have developed from US$16 billion in 2008 to a predicted value of US$ 19.6 billion by 2013 [20]. The most common way to stabilize these cultures is freeze-drying [54]. These stabilized probiotics can be supplemented to different kinds of foods in order to achieve an additional value [49]. It has been extensively reported that the addition of protectants significantly reduces the vitality losses during drying and subsequent storage [7,12,38,42,56]. Typical protectants for the production of bacteria are di- and oligosaccharides. Usually the protectant is added to a highly concentrated cell suspension prior to drying. During the freeze-drying process the cell-protectant-suspension concentrates due to the removal of water. As a result the protectant fraction experiences a transition from the liquid to the glassy state. Thus, in the resulting product, microorganisms are embedded in a glassy matrix.

The inactivation of bacteria during drying and storage is a complex phenomenon. Several detrimental effects occur simultaneously and are sometimes even related with each other. As a result, the identification of a single mechanism and quantification of its contribution to a total inactivation is almost impossible. However, in the past a lot of effort has been expended to identify the most relevant mechanisms. It is generally acknowledged that every drying process poses a stress to bacteria and to some extend causes inactivation. The availability of sufficient water is a prerequisite not only for the stability of proteins, DNA and lipids, but also for the preservation of the cell structure [47]. Thus, removal of water generally goes hand-in-hand with a so-called dehydration inactivation. Here, it could be shown by implementing several techniques that the principal site of inactivation is the cell membrane [4,58,63]. The cell membrane which is mainly composed of phospholipids suffers a detrimental phase transition from the liquid crystalline to the gel phase [74]. Due to the fact that each biomembrane consists of lipids of different type and chain lengths, these phase transitions do not occur for all lipids simultaneously. Inhomogeneous phase transition causes packing defects from lateral phase separation between liquid-like and solid-like domains, a process known as leakage. Detrimental leakage may also occur during rehydration of the dried cells due to the phase transition from the gel to the liquid crystalline phase [14]. Besides a general dehydration damage, drying specific detrimental effects play a role. In the case of freeze-drying the formation of ice crystals during the

☆ This work was supported by Deutsche Forschungsgemeinschaft (DFG) Project Reference: KU 750/2-1.

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0011-2240/$ - see front matter © 2012 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.cryobiol.2012.08.005

freezing step causes so-called cryoinjuries. Whilst during spray-drying the high temperatures cause thermal injuries. Due to the fact that this work looks at storage stability of bacteria, the afore-mentioned drying specific effects are discussed not further in this paper.

Inactivation during storage, however, is not less complex. Here, oxidation is a big issue. Membrane lipid oxidation during storage leads to changes in membrane function and structure [3,28]. Due to lipid oxidation, the fraction of saturated lipids within the cell membrane increases and causes a decrease in membrane fluidity. This decrease finally leads to a more pronounced rehydration leakage. Another relevant effect is the formation of free radicals during oxidation of the biomaterial. It has been shown by several authors that free radicals are one of the major causes for cell death [15,71]. According to the current knowledge these free radicals attack fatty acid moieties and therefore cause a significant decrease in hydrophobicity. These hydrophobic interactions are, however, essential for the interaction between membrane and membrane proteins [10]. Additionally, free radicals are known to damage cell DNA directly [1,26,27].

In addition to oxygen, the storage parameters, namely temperature [2,19,32,64] and moisture [11,23] have a significant influence on bacterial stability. In this context the physical sample state is of the utmost importance. The high viscosity of a surrounding amorphous glass (>10^{12} Pa s) can slow down diffusion controlled deleterious reactions [66] and thus increase chemical and physical stability of embedded biomaterials. The most common parameter characterizing glassy material is the glass transition temperature (T_g). T_g is the temperature where the material undergoes a transition from the solid-like to the liquid state. A high viscosity and restricted mobility is only given at temperatures below T_g. For temperatures above T_g, viscosity significantly decreases and molecules gain translational mobility due to thermal plasticization [51]. Inactivation below T_g is low but not completely halted [23]. This may be due to some non-diffusion controlled inactivation mechanisms like the free radical reaction [40].

The prevalent storage conditions (temperature T and water activity a_w) for glassy material are of high importance. A dry sample of low a_w will absorb water from the surrounding environment until an equilibrium state is reached. Due to the fact that water acts as a plasticizer the uptake of water causes the glass transition temperature T_g of the protective matrix to decrease [66]. As a result, T_g can drop below the storage temperature. In this case the protective matrix turns from the glassy into the rubbery (non-glassy) state.

For amorphous materials the temperature dependence of viscosity can be described as a function of the temperature difference between the product temperature (T_p) and its T_g as $\eta = f(T_p - T_g)$. One equation which reflects this relationship is the Williams–Landel–Ferry (WLF) equation. It was first shown by Williams, Landel and Ferry [73] that the viscosity/relaxation of polymer blends above T_g can be predicted by means of the derived WLF equation with its universal constants (C_1 = 17.4 and C_2 = 51.6 K). Over the following years several publications confirmed that the WLF equation can also be applied to describe the temperature dependence of the system viscosity as well as chemical and physical reactions in the vicinity of T_g. Among these are: the viscosity behavior of mono-/disaccharide solutions [67], the formation of saccharide and ice crystals [22,39,53], the formation of Maillard products [5,13,30,51,52], the storage stability of seeds [69] and the heat resistance of bacterial spores [59].

A general protective effect of the glassy state on microorganisms and liposomes has been reported [6,8,9,36,68]. Furthermore, it was demonstrated that an absolute microbial stability can only be achieved at a temperature range 30–50 °C below T_g [23,43,61]. The identification of the underlying mechanisms, however, had not been the subject of further studies or a matter of debate. In most cases the relevance of the glassy state has been evaluated on basis of only few storage conditions, comparing for example only two samples where one is in and the other outside the glassy state. Results on the basis of this kind of investigations can only indicate a general protective effect but cannot explain the underlying protection mechanism.

Microbial inactivation is a very complex reaction [47,48,56]. Thus, it is more or less impossible to determine single protective mechanisms by classical analytical tools. Here, the relevance of limited diffusion in the glassy system can be evaluated by describing the temperature dependence of the microbial inactivation rate. Therefore, this study presents a comprehensive investigation with a set of storage conditions (T and a_w) in combination with three different types of protectants. Two disaccharides with similar T_g [50] but different chemical reactivity (reducing/non-reducing) and a polysaccharide with clearly elevated T_g. The resulting inactivation rates k were fitted with the models of Arrhenius and Williams–Landel–Ferry (WLF) in order to evaluate the relevance of diffusional limitation within the glassy state. If the microbial inactivation reaction is solely dependent on the system viscosity, the inactivation rates should be adequately described by applying the WLF equation with its universal constants. Moreover, in the case of diffusional limitation a significant change of the activation energy E_a (determined via Arrhenius) around T_g should be visible.

Material and methods

Fermentation and purification

For quantification of bacterial inactivation kinetics the probiotic starter culture *Lactobacillus paracasei ssp. paracasei* (F19) from Chr. Hansen A/S (Hørsholm, Denmark) served as a model microorganism. The culture was provided as a frozen concentrate with a titer of around 10^{11} colony forming units, cfu/g. The batch fermentation process using the de Man–Rogosa–Sharpe (MRS) broth (Scharlau, Barcelona, Spain) was conducted in 4 L reactors at 37 °C. Every fermentation process started at an optical density (OD_{600}) of about 0.3 and was carried out without neutralization. After 10 h the fermentation was stopped in the late exponential phase by cooling the cell suspension to 4 °C. Cell harvest was realized by three centrifugation (10 min; 4000 g; 4 °C) and washing steps. Washing was accomplished by means of phosphate buffer saline (0.1 M K_2HPO_4/KH_2PO_4 with 0.15 M NaCl, pH 7.0). A final cell density of 10^{11} cfu/mL could be achieved.

Addition of protectants

After washing, the protectants were added to the cell suspension with 50% (w/w) of the dry biomass. Therefore, the corresponding initial protectant concentration was 60 mg/mL. Preliminary experiments showed that above a protectant level of 50% (w/w) of the dry biomass further increase in protectant concentration did not lead to an additional protective effect. Three different saccharides were chosen as protectant: lactose monohydrate (Merck KGaA, Darmstadt, Germany), trehalose dihydrate (Cargill Inc., Wayzata, USA) and dextran T2000 (Pharmacosmos A/S, Holbaek, Denmark). The two disaccharides have identical molecular weights of 342.3 g/mol and similar T_g (trehalose 100 °C and lactose 101 °C) [50], but show different behavior in terms of chemical reactivity. Lactose is a reducing sugar whereas trehalose is not. Therefore, out of these protectants only lactose has the ability to take part in the Maillard reaction. This is especially important at higher storage temperatures [37]. Both disaccharides are known to be able to protect the cell membrane from dehydration damage during water removal thanks to their ability to replace water molecules between