International Journal of Food Microbiology 155 (2012) 217–221

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Effect of physical properties on the stability of *Lactobacillus bulgaricus* in a freeze-dried galacto-oligosaccharides matrix

E. Elizabeth Tymczyszyn [a,1], Natalia Sosa [b], Esteban Gerbino [a], Ayelen Hugo [a,1], Andrea Gómez-Zavaglia [a,*,1], Carolina Schebor [b,1]

[a] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Conicet La Plata, UNLP, (1900) La Plata, Argentina
[b] Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, UBA, Ciudad Universitaria (1428) CABA, Argentina

ARTICLE INFO

Article history:
Received 11 October 2011
Received in revised form 16 January 2012
Accepted 16 February 2012
Available online 21 February 2012

Keywords:
Galacto-oligosaccharides
Freeze drying
Water activity
Lactobacillus
Membrane damage
Molecular mobility

ABSTRACT

The ability of galacto-oligosaccharides (GOS) to protect *Lactobacillus delbrueckii* subsp. *bulgaricus* upon freeze drying was analyzed on the basis of their capacity to form glassy structures. Glass transition temperatures (T_g) of a GOS matrix at various relative humidities (RH) were determined by DSC. Survival of *L. bulgaricus* in a glassy GOS matrix was investigated after freezing, freeze drying, equilibration at different RHs and storage at different temperatures. At 32 °C, a drastic viability loss was observed. At 20 °C, the survival was affected by the water content, having the samples stored at lower RHs, the highest survival percentages. At 4 °C, no decay in the cells count was observed after 45 days of storage. The correlation between molecular mobility [as measured by Proton nuclear magnetic resonance (^{1}H NMR)] and loss of viability explained the efficiency of GOS as cryoprotectants. The preservation of microorganisms was improved at low molecular mobility and this condition was obtained at low water contents and low storage temperatures. These results are important in the developing of new functional foods containing pre and probiotics.

1. Introduction

Development of new functional foods containing probiotics and prebiotics, is of great interest because of their significant health benefits (Gibson et al., 2004; Wang, 2009). For this purpose, the preservation of lactic acid bacteria is essential to obtain concentrated viable starters, being freeze drying one of the most used methods (Meng et al., 2008; Morgan et al., 2006). During the preservation processes, the decrease of water activity produces damages on the cellular structures, decreasing the bacterial viability. To prevent these damages, sugars are usually incorporated as cryoprotectants (Carvalho et al., 2004). The protective effect of sugars has been ascribed to their ability to replace water during the dehydration, maintaining the biological structures in hydrated conditions (Crowe et al., 1992; Leslie et al., 1995; Santivarangkna et al., 2008). Another mechanism of protection is the ability of sugars to form glassy matrices, in which the high viscosity and low mobility restrict molecular interactions (Lodato et al., 1999). In this regard, the long term storage of dried products must be optimized taking into account the glass transition temperatures (T_g) at different water contents (Higl et al., 2007; Miao et al., 2008). The T_g value separates the supercooled from the glassy state (Roos, 1995) and is indicative of the degree of molecular mobility of the amorphous matrix. However, mobility of small molecules within the matrix is possible (*i.e.:* water, gases and small organic molecules) (Tromp et al., 1997; Schoonman et al., 2002). NMR relaxation has been proposed as a valuable method to understand the relationship between the molecular mobility of water and sugars, the moisture content and temperature (Hills et al., 2001).

GOS are carbohydrate-based well-known food ingredients with prebiotic properties, chemically synthesized by transgalactosylation of lactose (Playne and Crittenden, 2009; Neri et al., 2009; Vera et al., 2011). They are composed of a variable number of galactose units linked to a glucose unit, with a range from two to eight monomeric units (Neri et al., 2009). In addition to the prebiotic properties, the ability of GOS to act as cryoprotectants has been recently reported (Tymczyszyn et al., 2011). Tymczyszyn et al. (2011) demonstrated that commercial GOS preparations are very efficient in the cryopreservation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Starters of *L. bulgaricus*, usually preserved by freezing, freeze drying and spray drying, are widely used in the elaboration of dairy products (Texeira et al., 1997). *L. bulgaricus* is known by its high sensitivity toward any kind of stress (Texeira et al., 1997) and according to our previous studies, strain CIDCA 333 is particularly sensitive (Tymczyszyn et al., 2007; 2011).

From a physical perspective, GOS are polyhydroxilated compounds and their efficiency as cryoprotectants could be explained on the basis of the vitrification and water replacement hypotheses.

* Corresponding author at: Calle 47 y 116 La Plata, Buenos Aires, (1900) Argentina. Tel.: +54 221 4890741; fax: +54 221 4249287.
E-mail address: angoza@qui.uc.pt (A. Gómez-Zavaglia).
[1] Members of CONICET, Argentina.

0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2012.02.008

Nomenclature

ANOVA	Analysis of variance
CFU	Colony forming units
DP	Degree of Polymerization
DSC	Differential scanning calorimeter
FC	Flow cytometer
FID	Free induction decay analysis
GOS	Galacto-oligosaccharides
^{1}H NMR	Proton nuclear magnetic resonance
k	Inactivation constant
MRS	de Man, Rogosa, Sharpe
N	CFU of the sample under study
N_0	CFU of sample at time = 0
PI	Propidium iodide
RH	Relative humidity
t	time of storage
T	temperature of storage
T_2	Relaxation times
T_g	Glass transition temperature

However, the thermophysical properties of GOS have been little explored (Torres et al., 2011). Considering this, the aim of this paper was to get an insight on the thermophysical properties of GOS matrices containing *L. bulgaricus* CIDCA 333 as a support for future developments of new functional foods. For this reason, we determined the ability of commercial GOS to protect *L. bulgaricus* upon freeze drying and storage at different RHs, taking into account their effect on bacterial membranes and the formation of glassy structures. In order to perform a complete evaluation of the protectant properties of GOS, the recovery of cells after different times of storage at various RHs was correlated with the T_g and molecular mobility.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a fermented product (Gómez-Zavaglia et al., 1999). The strain was maintained frozen at −80 °C in 120 g L^{-1} non-fat milk solids. Microorganisms were grown in MRS broth at 37 °C (De Man et al., 1960).

2.2. GOS

A commercial syrup, Cup Oligo H-70® (Kowa Company, Tokyo, Japan) kindly donated by Kochi S.A. (Santiago, Chile) was used for the experiments. The syrup contained 75% of GOS of different DP: 4% of high-molecular-weight oligosaccharides (DP≥5); 21% of tetrasaccharides DP 4; 47% of trisaccharides DP 3; 23% of disaccharides (DP2) and lactose, and 5% of monosacharides, including glucose and galactose (Tymczyszyn et al., 2011).

2.3. Preparation of samples for freeze drying

One-milliliter cultures in the stationary phase (grown in MRS broth at 37 °C overnight to obtain approximately 10^9 CFU/mL) were harvested by centrifugation at 4000 *g* for 10 min. The pellets were washed twice with sodium chloride 0.85% w/v, and resuspended in 1 mL of 20% (w/w) aqueous solutions of GOS, previously sterilized using 0.2 μm sterile filters. As reported before (Tymczyszyn et al., 2011), this GOS concentration allows the highest bacterial recovery.

2.4. Freeze drying procedure

Aliquots of 1 mL containing cell suspensions in the presence or absence of GOS and pure GOS solutions were transferred into 5 mL glass vials under aseptic conditions and frozen for 48 h at −20 °C. A freeze-drier Alpha 1–4 LD/2–4 LD-2 (Martin Christ, Gefriertrocknungsaniagen GmbH, Osterode, Germany) operated with the condenser at −84 °C at a chamber pressure of 0.04 mbar was used. The freeze drying process lasted for 48 h.

2.5. Humidification procedure

Freeze-dried matrices were equilibrated for 15 days in atmospheres of the following saturated salts: LiCl, KCH_3COO, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaCl and $(NH_4)_2SO_4$, giving relative humidities (RH) 11, 22, 33, 44, 52, 75 or 80%, respectively.

2.6. Glass transition temperatures

Glass transitions were determined by DSC (onset values, heating rate: 10 °C min^{-1}) using a DSC 822^e Mettler Toledo calorimeter (Schwerzenbach, Switzerland), calibrated with indium, lead and zinc. Hermetically sealed 40 μL medium pressure pans were used (an empty pan served as reference). Thermograms were evaluated using Mettler Star^e program. An average value of at least two replicates was reported. The standard deviation for the glass transition temperature measurement was ±1 °C.

2.7. Bacterial plate counts

Viable bacterial plate counts were determined before and after freezing, after freeze drying and after equilibration at different RHs. Dried microorganisms were rehydrated in 1 mL of 0.85% (w/v) sodium chloride for 15 min. Bacterial suspensions were serially diluted and plated on MRS agar plates. Bacterial counts were determined after 48 h of incubation at 37 °C.

2.8. Storage

Equilibrated samples at 11, 22 and 33% RH were sealed and stored at 4, 20 or 32 °C for 45 days. The recovery of cells after different times of storage was analyzed by plate counts.

2.9. Water content determination

Karl Fischer (KF) titration was carried out at 25 ± 1 °C with a Karl Fischer titrator DL 31 from Mettler Toledo (Zurich, Switzerland), applying the one-component technique with Hydranal Titrant Composite 5 from Riedel-de Haën (Seelze, Germany). Methanol/formamide mixture 95 (1:1) was used as solvent, and they were purchased from Merck (Darmstadt, Germany). Sample sizes were approximately 100 mg. The water content for samples equilibrated at 11, 22 and 33% was 4.84 ± 0.03, 6.20 ± 0.01 and 7.53 ± 0.01% (dry basis), respectively.

2.10. Membrane damage

Cells were incubated with the DNA-binding probe propidium iodide (PI), which only penetrates bacterial cells when membranes are damaged. Stock solutions of PI (Molecular Probes, Leiden, The Netherlands) were prepared in distilled water to a final concentration of 10 mg/mL and stored in the dark at 4 °C. PI was added to a final concentration of 0.5 mg/mL. For flow cytometric analysis, the concentration of microorganisms in the samples was adjusted to approximately 10^6 CFU/mL. The cells were incubated with the probe for 5 min at room temperature and the PI uptake was performed by flow cytometry (FACSCalibur, CellQuest software; Becton Dickinson,