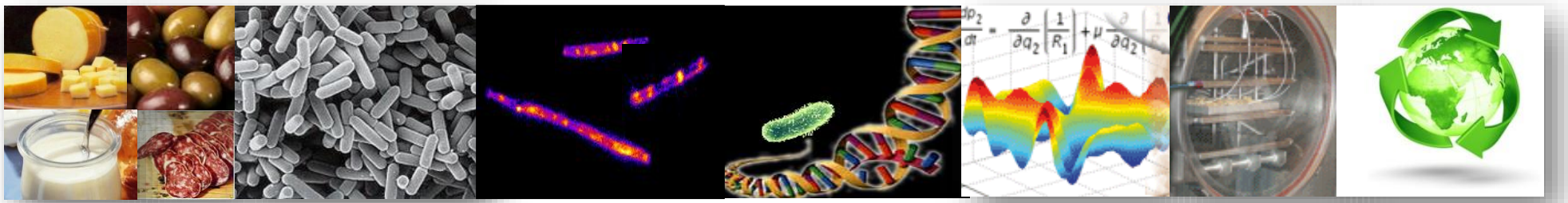
JCAT53– 53èmes Journées de Calorimétrie et d'Analyse Thermique

Palaiseau, France, 23-24 May 2023

Differential scanning calorimetry applied to the preservation of bacteria and mammalian cells

Fernanda Fonseca, Stéphanie Passot

UMR782 Paris-Saclay Food and Bioproduct Engineering, INRAE, AgroParisTech, Palaiseau, France



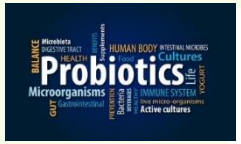
Preservation of bacteria and mammalian cells

Food applications



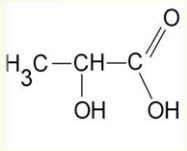




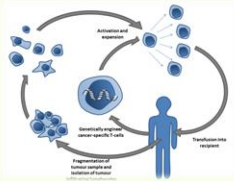



Medical applications

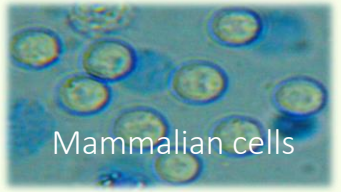
Chemical applications

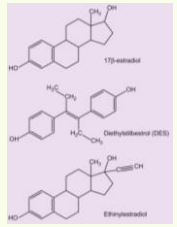


Cell therapies



Vaccines production





Antibodies, synthetic hormones and enzymes manufacturing

Starter market
~7% per year

Exploit the diversity of LAB's functionalities

High functionalities
Exploit new microorganisms

Innovative processes
High productivity

Low environmental impacts

Global Probiotic Market. Markets and Markets, 2020, 1-169.

Freezing & Freeze-drying

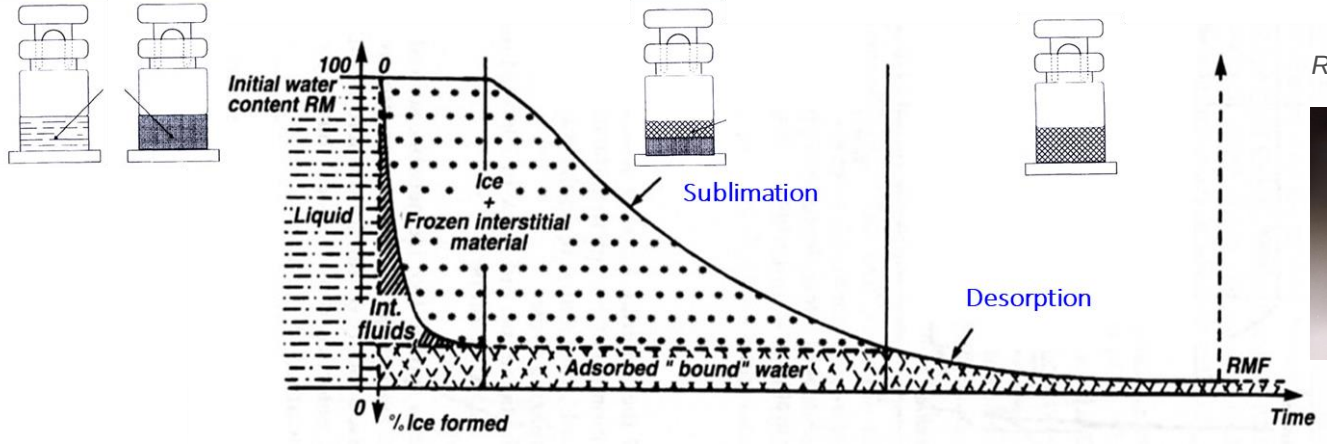
Preservation of **complex biological systems**

		Freezing	Freeze-drying
	Proteins	✓✓	✓✓
	Liposomes	✓	✓
	Viruses	✓✓	✓✓
	Prokaryotic cells	✓✓	✓
	Blood cells	✓	✗ ✓
	Eukaryotic cells	✓✓	✗ ✓
	Embryos	✓✓	✗
	Tissues	✗ ✓	✗
	Organs	✗	✗

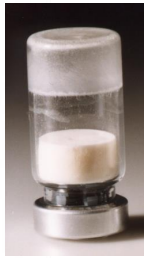
Freezing
Formation of ice crystals

Primary drying
Removal of ice crystals

Secondary drying
Removal of unfrozen water



Rey, L 1999

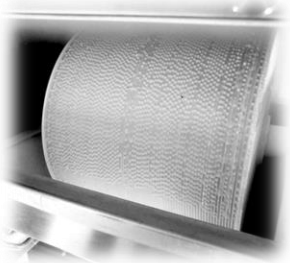


✓ Short, robust FD cycle
✓ Elegant, mechanically strong cake
✓ Rapid reconstitution
✓ Residual moisture content
✓ High yield of product activity
✓ Stability through the shelf life

Mammalian cells are preserved only by freezing
Freezing → The first and shortest step of the FD process

BUT important impact on the desired quality attributes of freeze-dried product

Stressful environment during freezing and freeze-drying



Microorganism

Fermentation

Cooling

Concentration

Protection

Stabilization

Storage

Final functionality

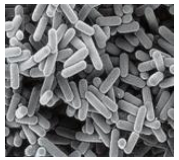
Freezing

Freeze-Drying

Stress

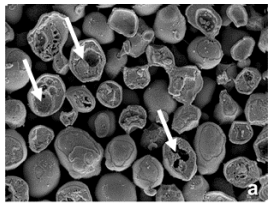
Functionality elaboration

Functionality degradation

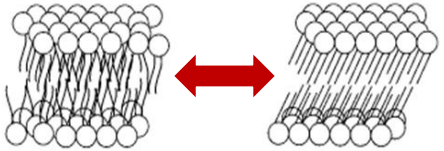


Acidification activity, production of aroma compounds, probiotics, exopolysaccharides, bacteriocins...

Intracellular ice formation



Membrane stiffness

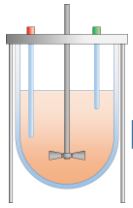


Extracellular ice formation and cell dehydration

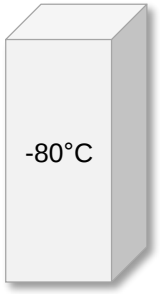
Cell production and functionalities' recovery

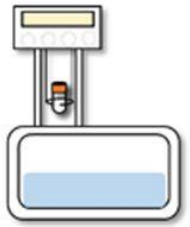
Physical properties

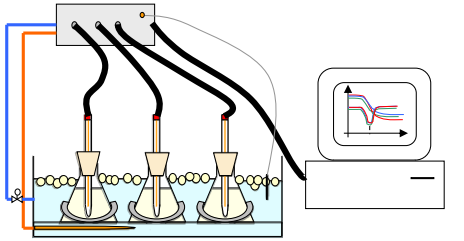
Lactic acid bacteria



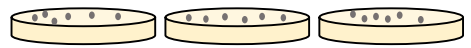
Culture: Lb bulgaricus
Different culture conditions





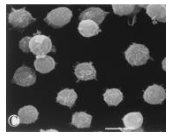


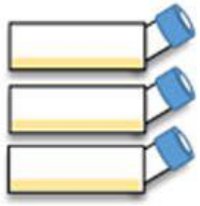
Acidifying activity



Viability

Mammalian cells



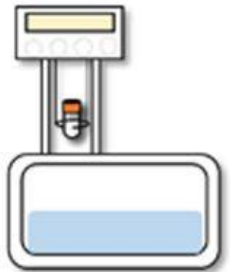


Cell culture
Jurkat cells, etc

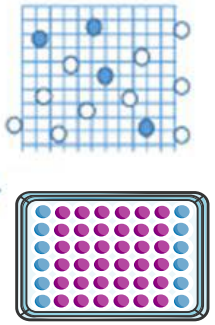
Formulation in cryopreservation media

Cooling cells

Storage



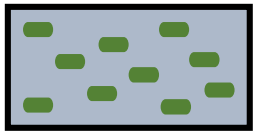
Thawing cells



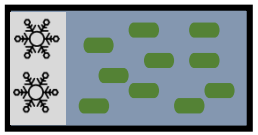
Cell analysis;
cell counts, proliferation and phenotype

Freezing

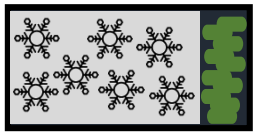
Ice crystals formation in the extracellular medium



Aqueous solution



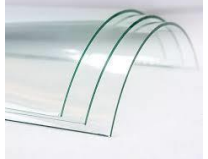
Ice crystallization

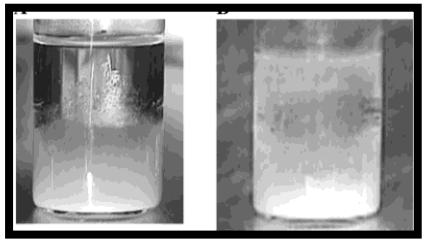


Ice crystal growth
Freeze-concentration

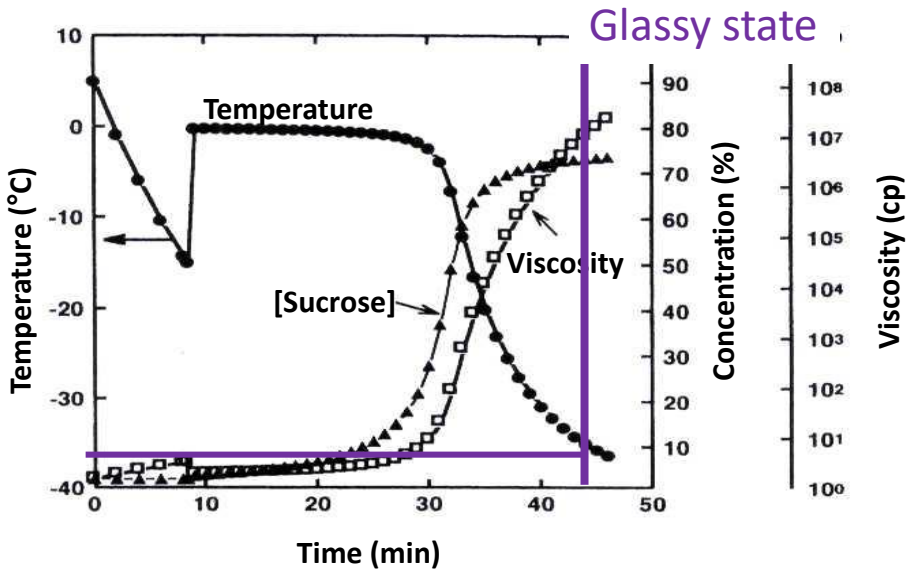
Solute crystallization

Vitrification
Glassy state

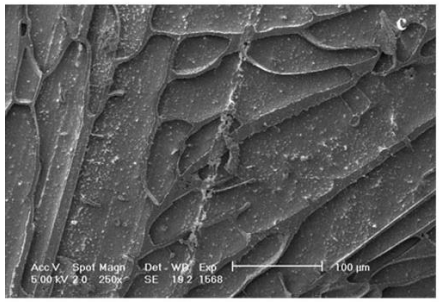


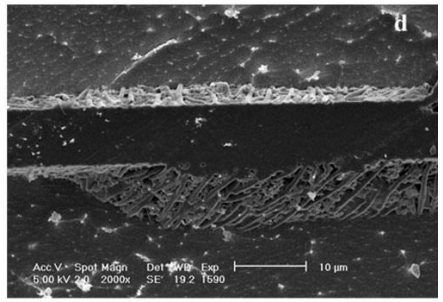


→ Drastic environmental changes



Pikal, M.J. 1999

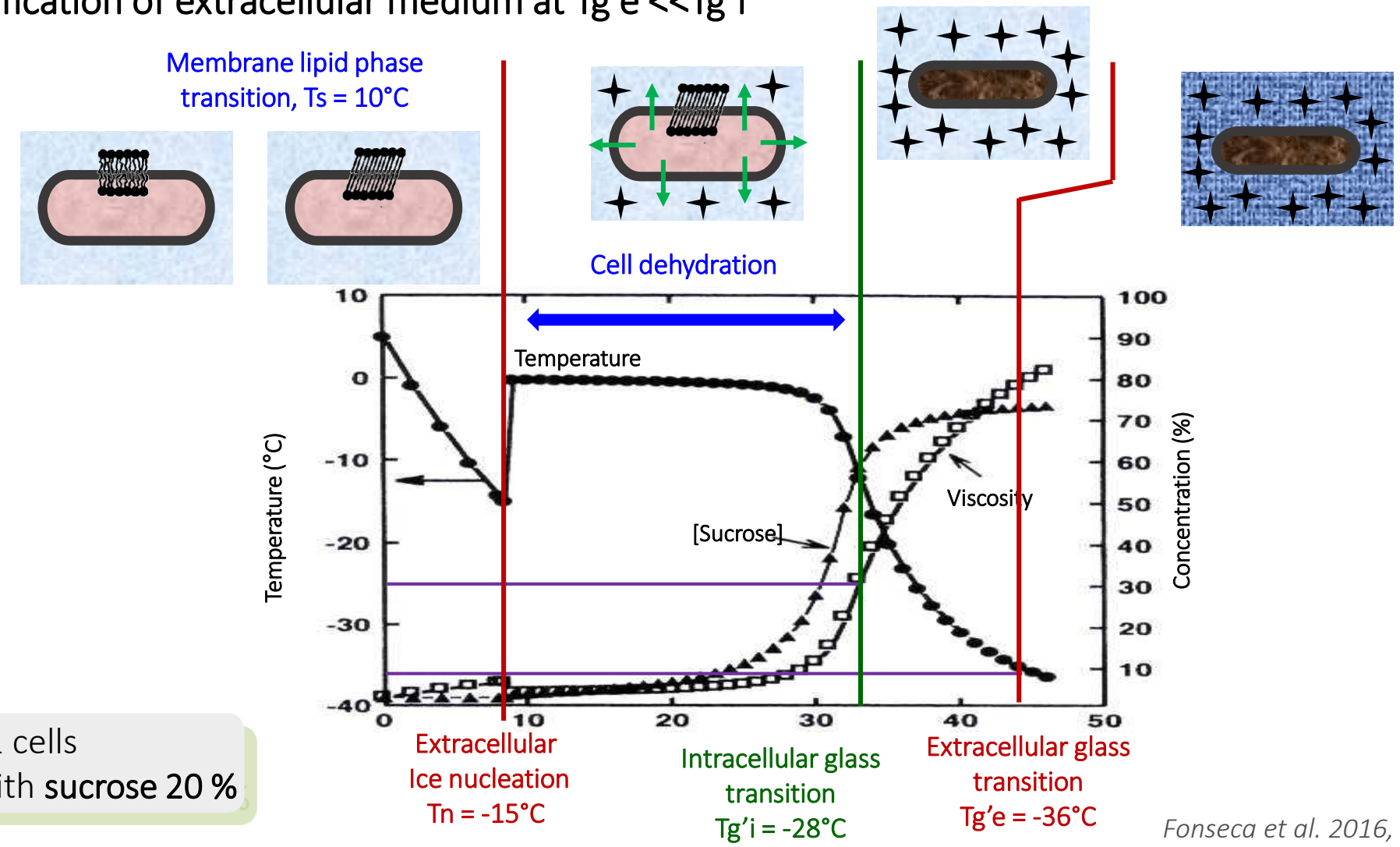




Fonseca. et al. 2006, AEM

The cell adventure during freezing

→ Vitrification of extracellular medium at Tg'e <<Tg'i

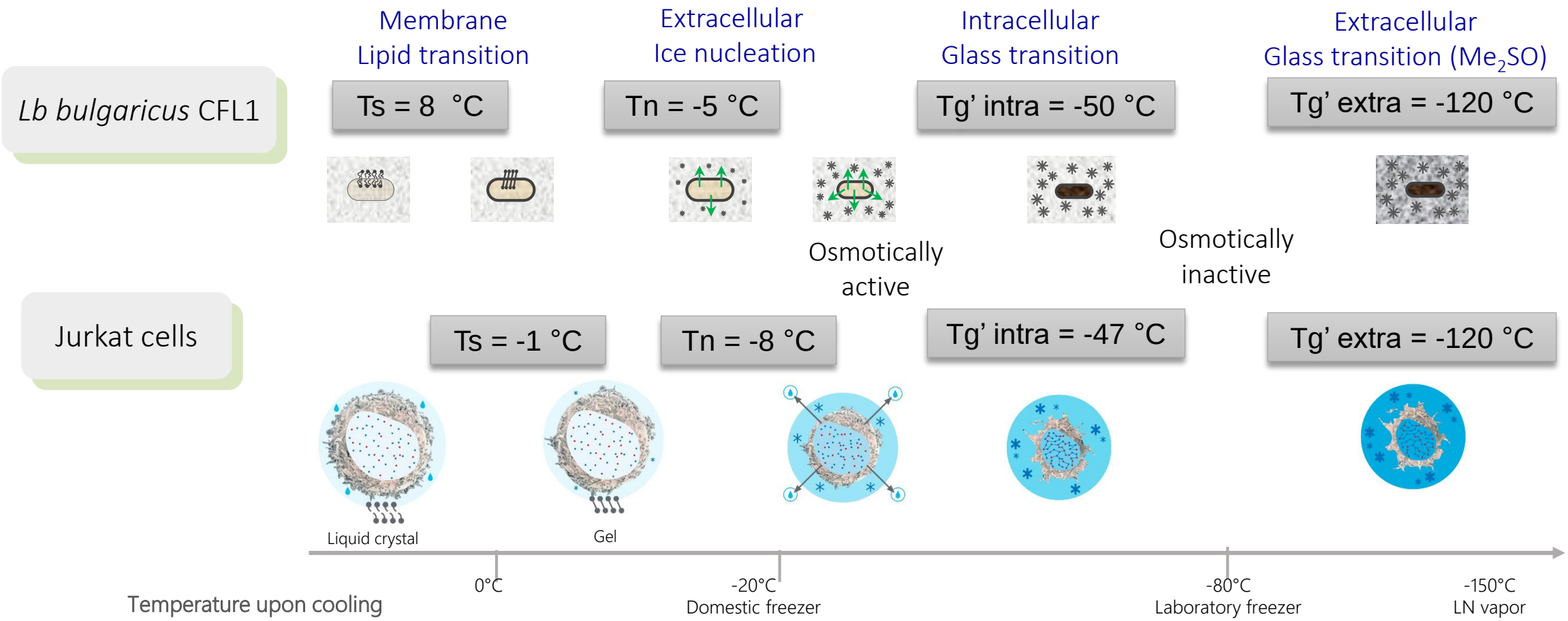


Lb bulgaricus CFL1 cells
Whey medium, protected with **sucrose 20 %**

Fonseca et al. 2016, PlosOne

The cell adventure during freezing

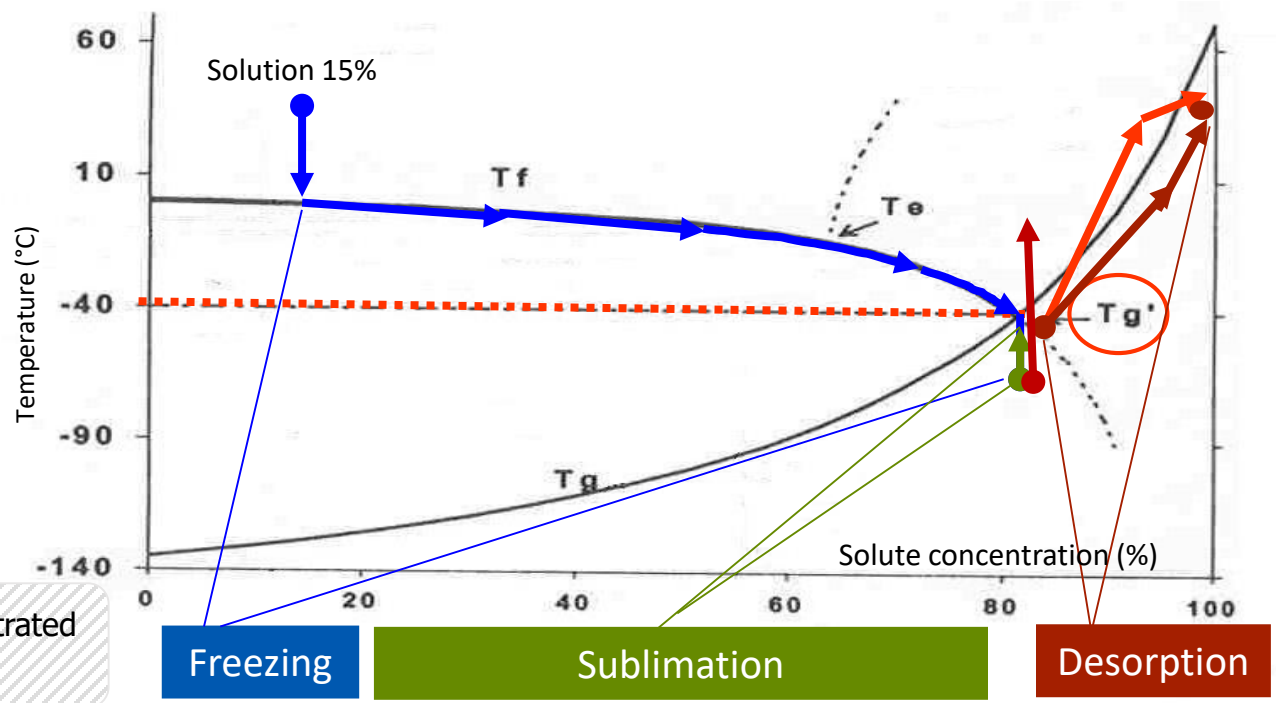
→ Similar approach applied to mammalian cells: cryoprotectant 0.58 M Me_2SO

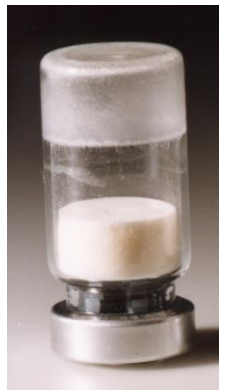


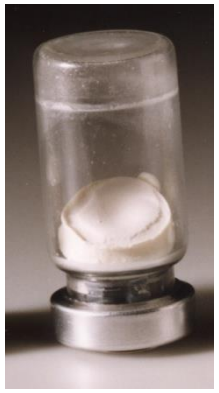
→ Relevance of Tg' intra and Tg' extracellular ?

Fonseca et al. 2016; Meneghel et al. 2019, PlosOne

The cell adventure during freeze-drying: the state diagram

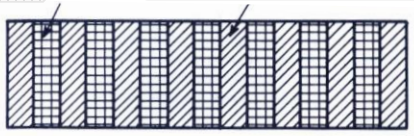


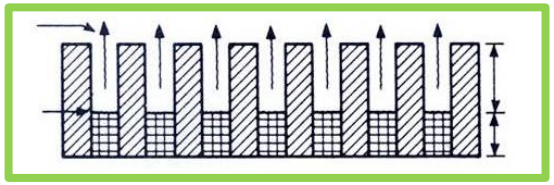




Ice

Freeze-concentrated matrix





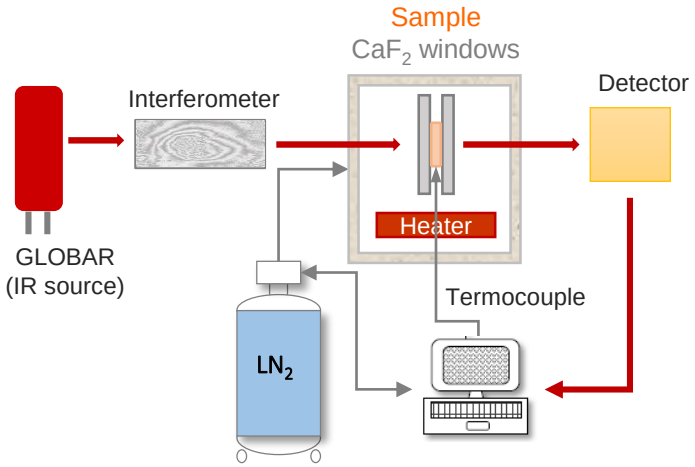
Characterisation of physical events

Lipid organisation, ice formation/structure, glass transition

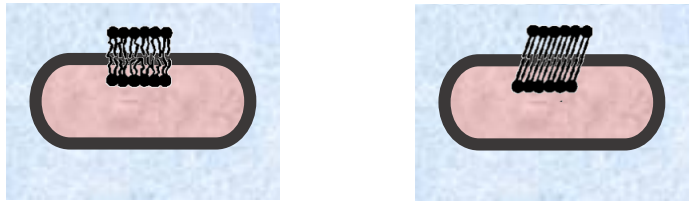
Cells in cryopreservation media

FTIR spectrosocopy

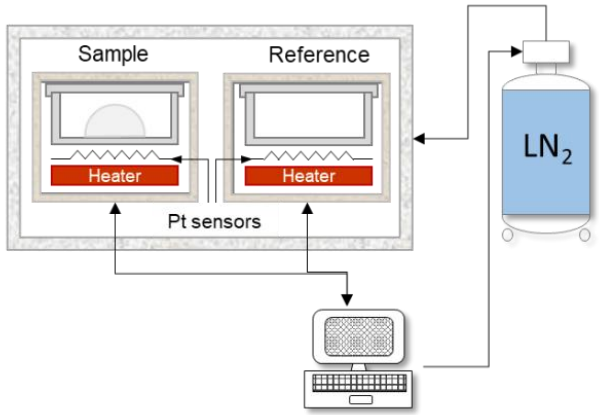
With controlled variable T° device



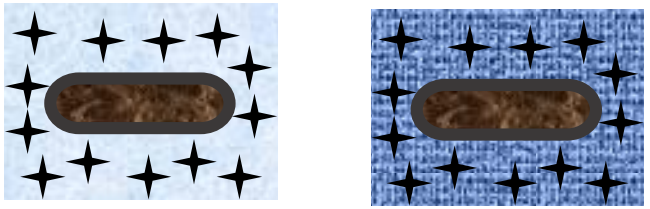
Lipid phase transition temperature
Lipid organization



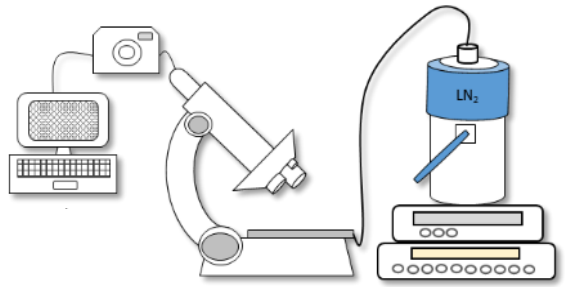
Differential Scanning Calorimetry



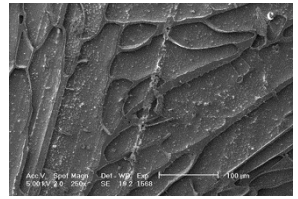
Intracellular ice formation, recrystallization
Glass transition temperature of
- Cellular content
- Cryprotective medium

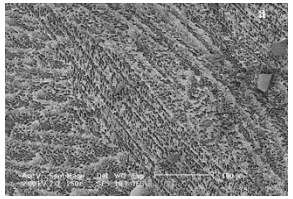


Cryomicroscopy SEM



Ice structure
Recrystallization

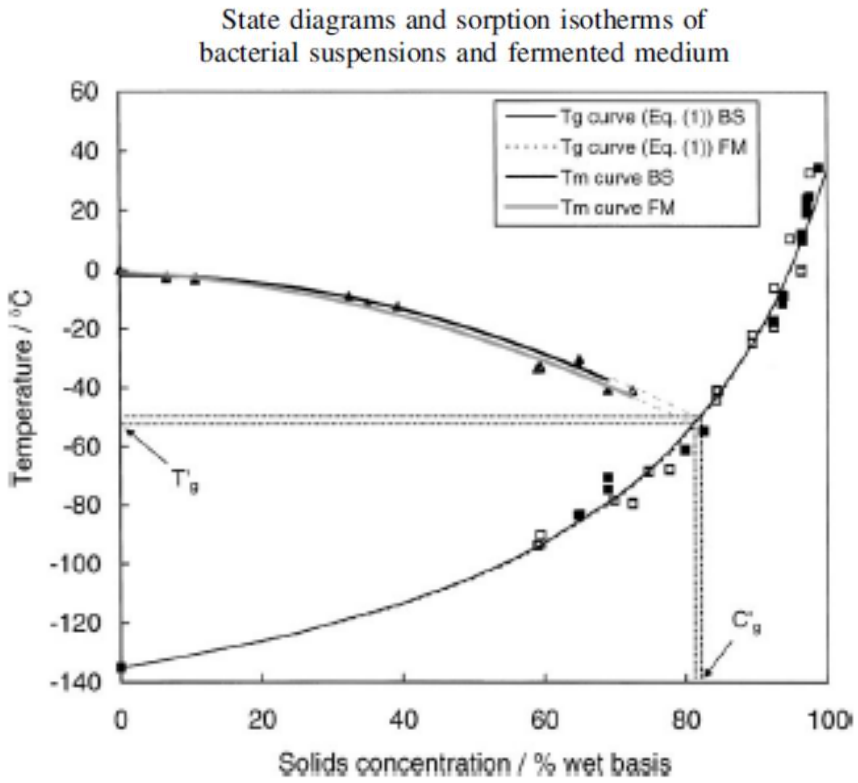




DSC = powerful tool

Physical properties: the state diagram of bacterial concentrates

Fermentation → Formulation → Freeze-drying → Product → Storage → Rehydration



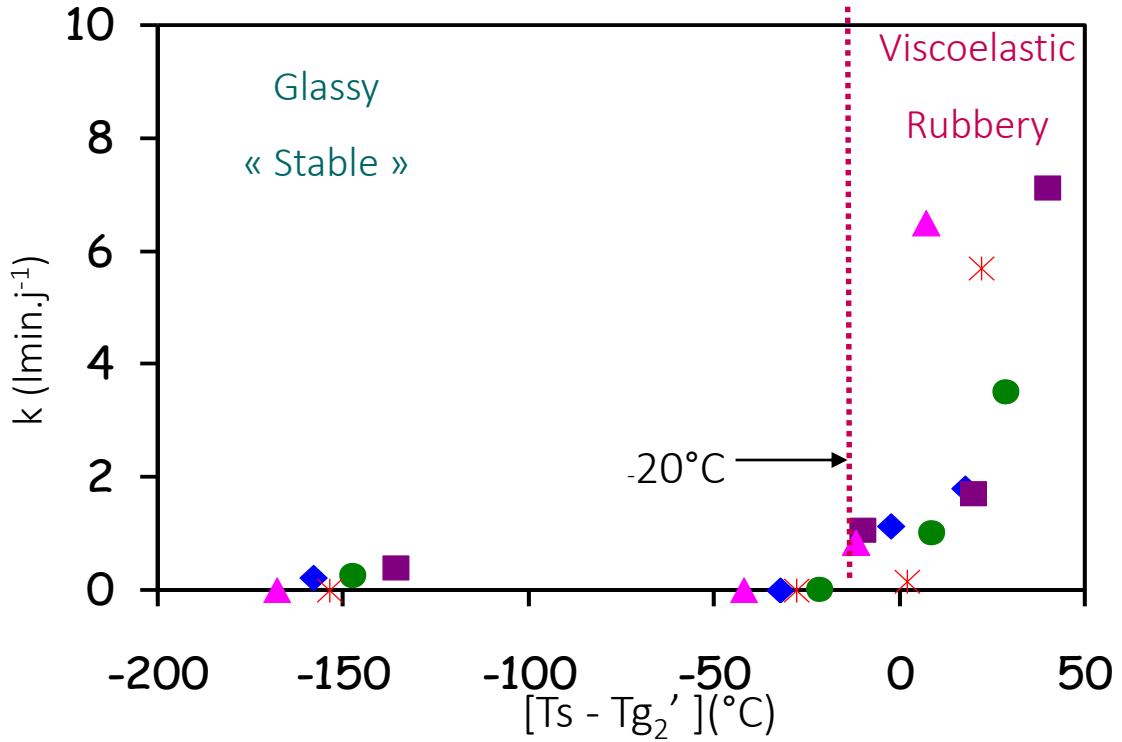
Fonseca et la. 2001, Thermochimica Acta

Lb bulgaricus CFL1
Ratio extracellular medium(g)/cell pellet (g) = 2/1

Bacterial concentrates exhibit a glass transition event, which is determined by the external medium and the water content (no visible effect of bacterial constituents)

Glass transition of the extracellular medium (Tg'e) and stability of frozen LAB

Lb bulgaricus CFL1



Cryoprotectants:
Sodium glutamate
Glycerol
Sodium ascorbate
Betaïne
Maltodextrin

The maintenance in a glassy state is a necessary condition for the stability of frozen LAB

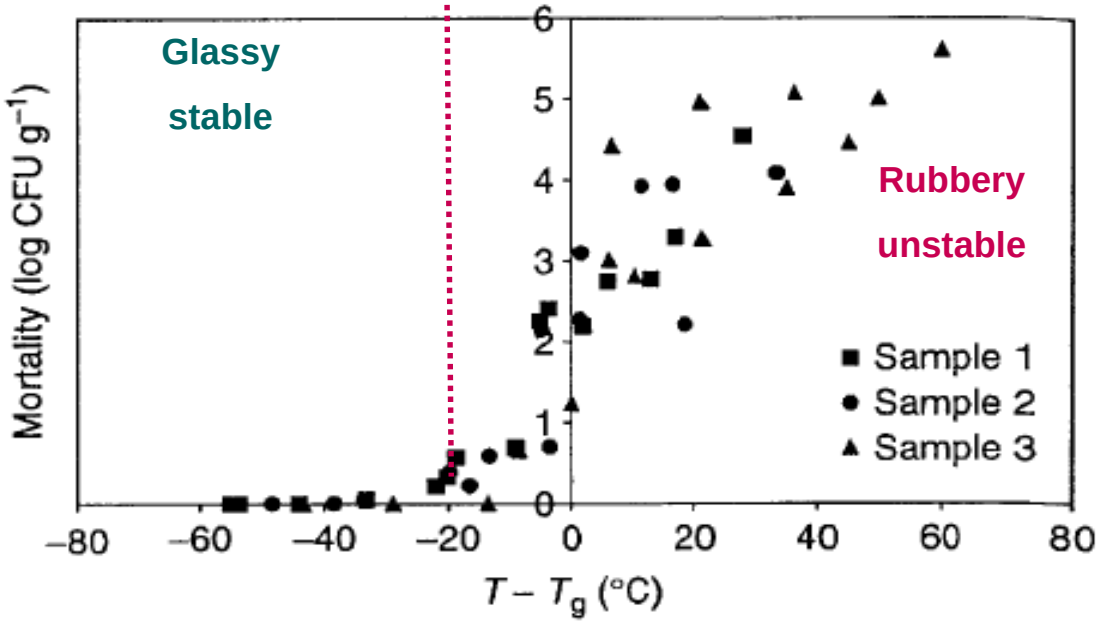
➔ If Ts < Tg_2' - 20°C, k low (< 1 $min.j^{-1}$) => ☺ glassy « stable » solid

➔ If Ts > Tg_2' - 20°C, k increases depending on the protective medium
☹ increase of molecular mobility, viscoleastic « unstable » material

Fonseca 2001, PhD thesis

Glass transition (Tg) and stability of dehydrated LAB

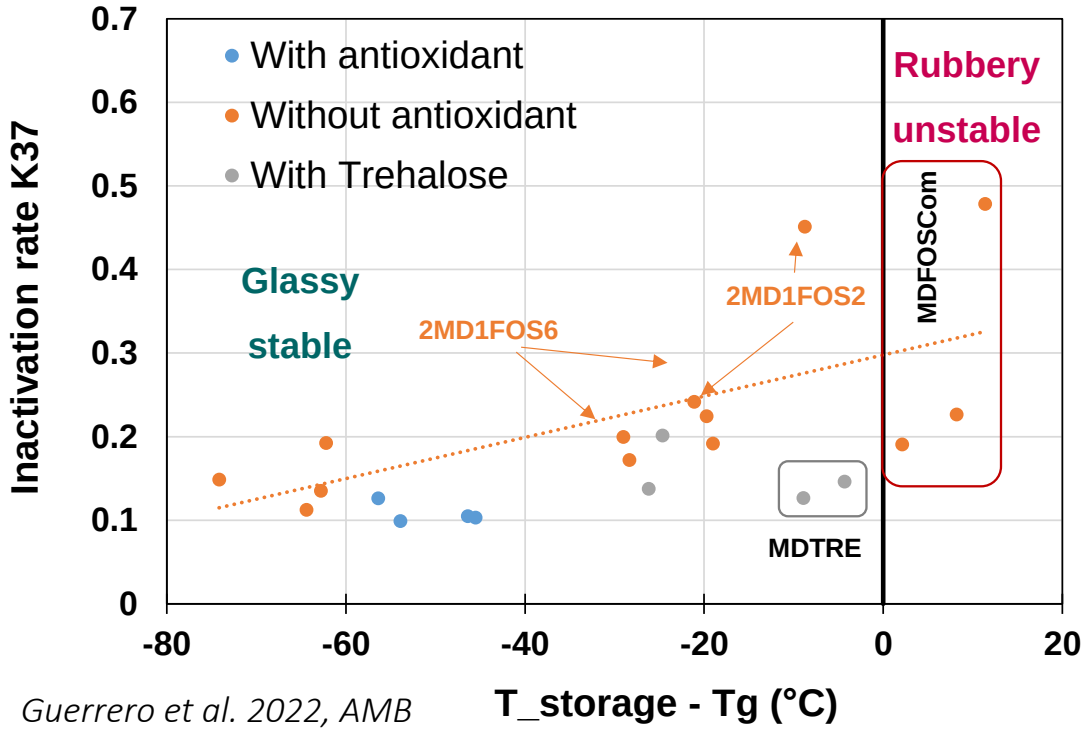
S. thermophilus



Selma et al., 2007, J Sci Food Agric

L. salivarius CECT57131

PREMIUM
Preserving bacteria with oligosaccharides and eco-friendly processes

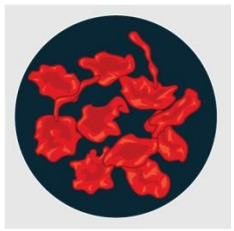


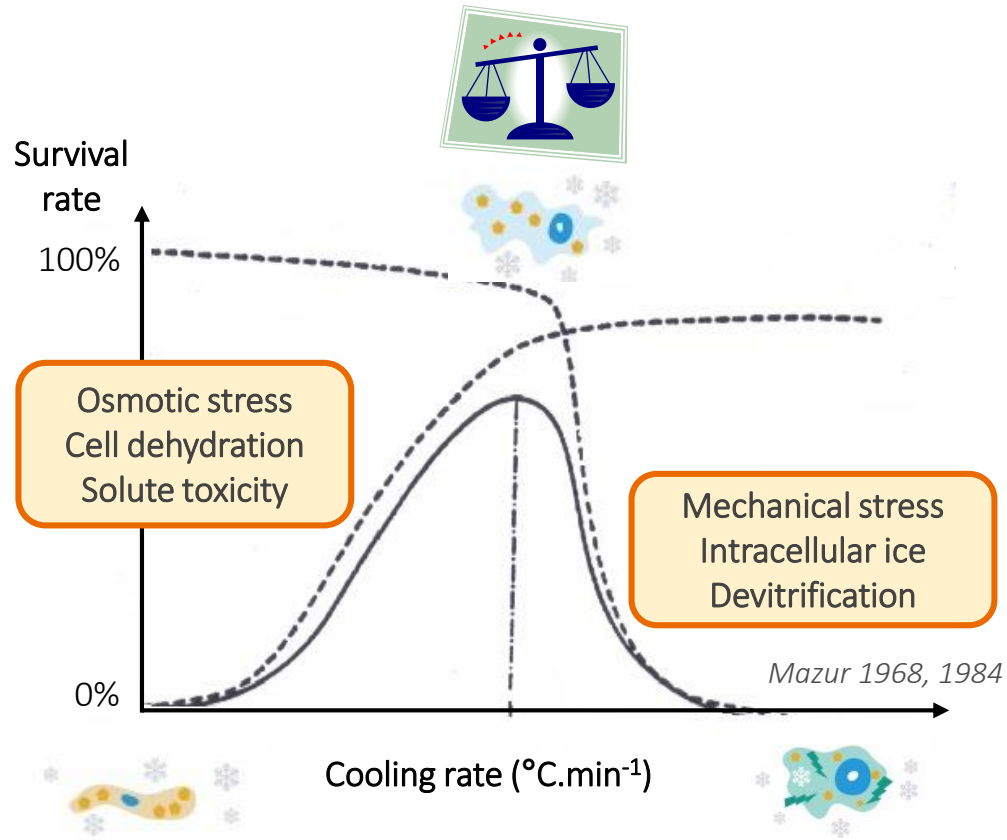
Guerrero et al. 2022, AMB

The maintenance in a glassy state is a necessary condition for the stability of dehydrated LAB

But …

Cooling rate and cell freezing resistance

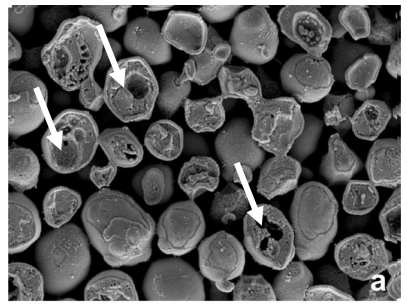




Mazur 1968, 1984

Intracellular ice formation
Mechanical stress

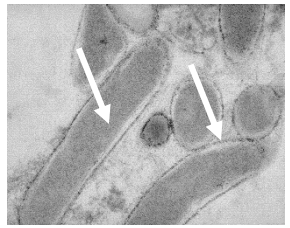
Yeast



Fonseca et al. 2016, PlosOne

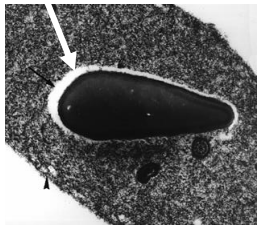
Or → Devitrification / plasmolysis

Lb bulgaricus



Fonseca et al. 2006, AEM

Sperm cell



Morris & Acton 1999, Human Rep

Avoid intracellular ice formation and control cell dehydration

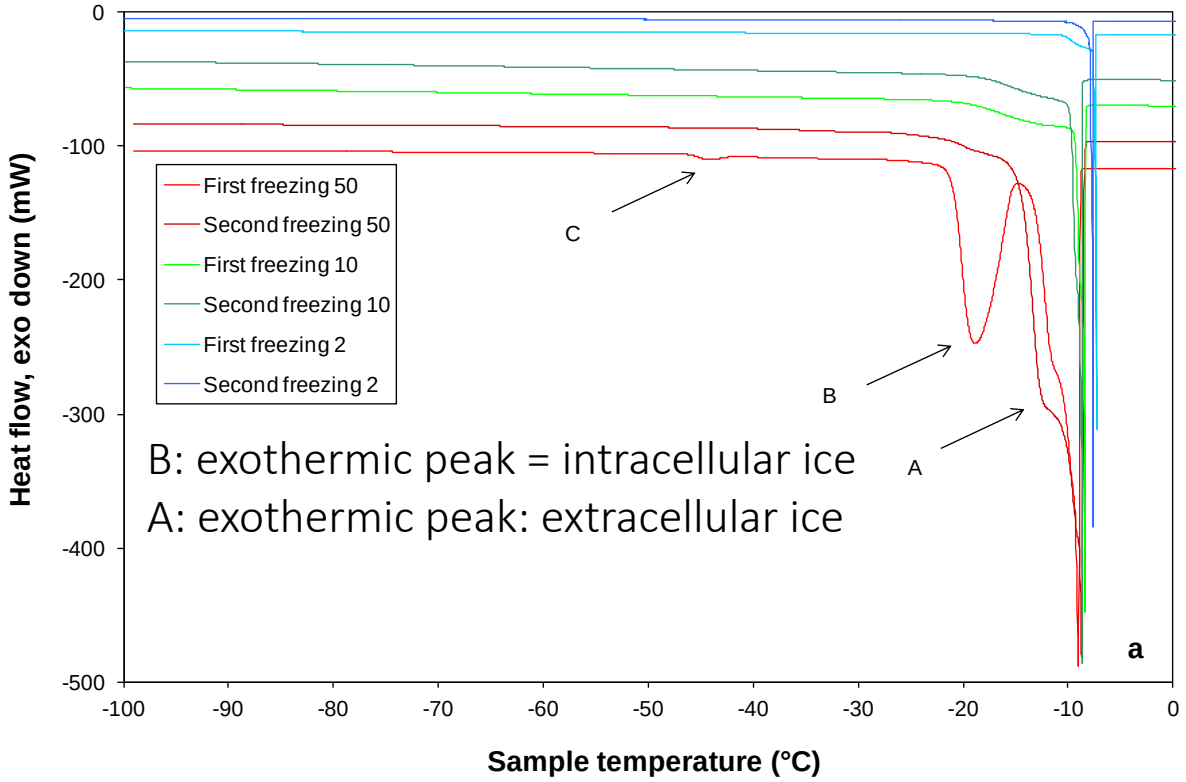
⇒ Control the cooling rate, apply optimal cooling conditions

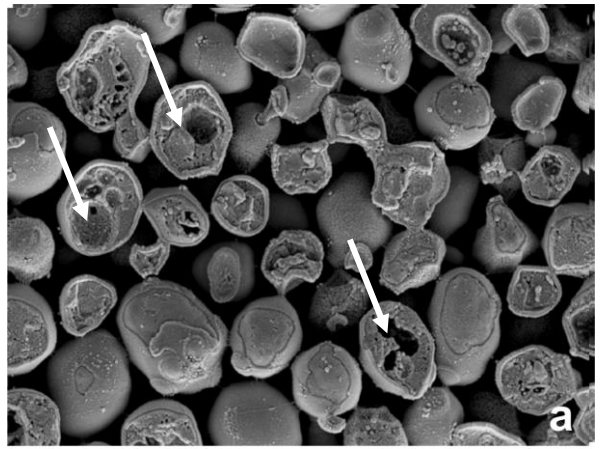
But, survival is measured after **storage** at a given T° and following **thawing**….

Intracellular ice formation: DSC and cryomicroscopy

S. cerevisiae

DSC scans of *S. cerevisiae* during cooling at 50 °C.min^{-1}, 10 °C.min^{-1} and 2 °C.min^{-1}



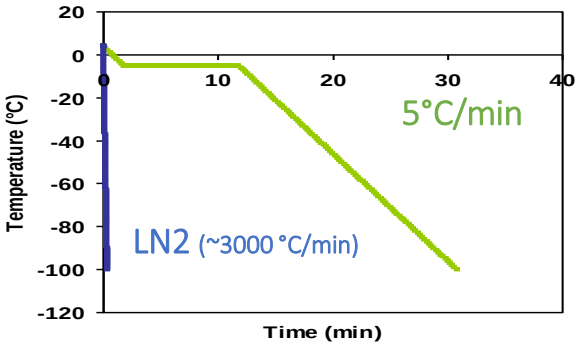


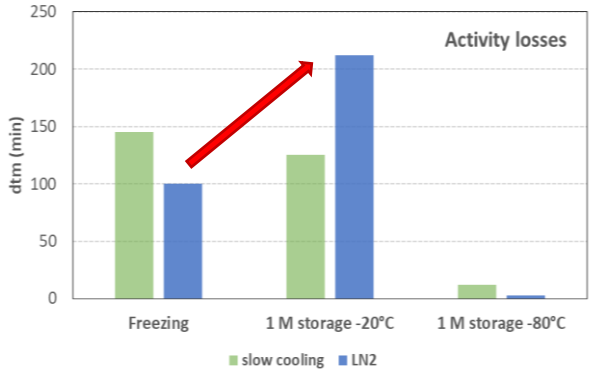
At 50 °C.min^{-1} intracellular ice is obvious within fractured cells as large voids

At 50 °C.min^{-1} intracellular ice is observed in the DSC trace (exothermic event during cooling)

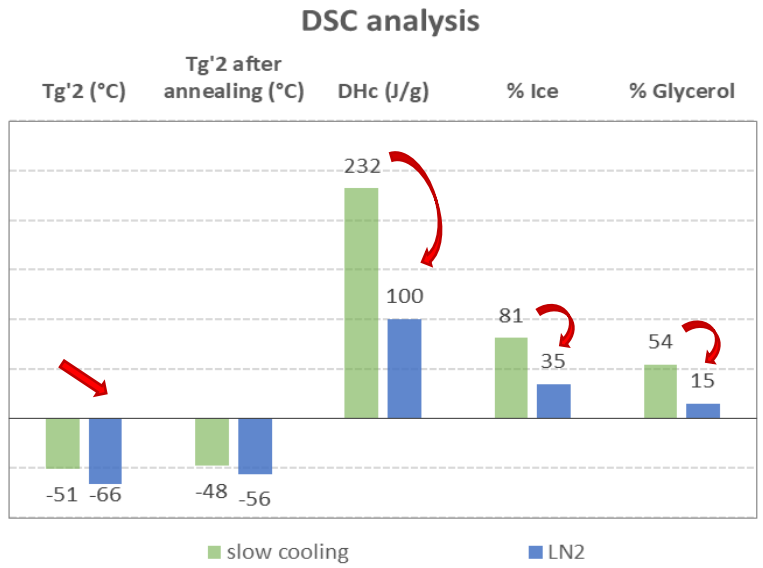
Fonseca et al. 2016, PlosOne

Ice recrystallization (devitrification): DSC + SEM & TEM

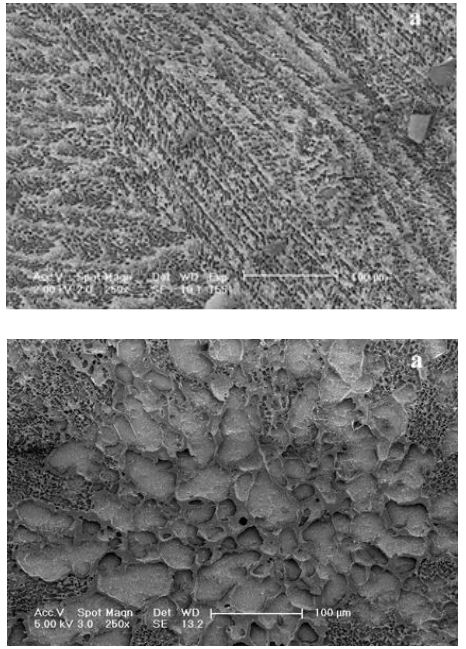




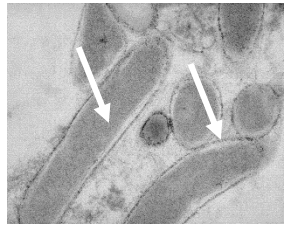
DSC:
LN2 cooling => decrease of: Tg', amount of ice and [glycerol];
Annealing -20°C => shift of Tg' to higher values => ice recrystallization



SEM: Ice recrystallization during storage at -20°c



TEM: Ice recrystallization => dehydration/plasmolysis



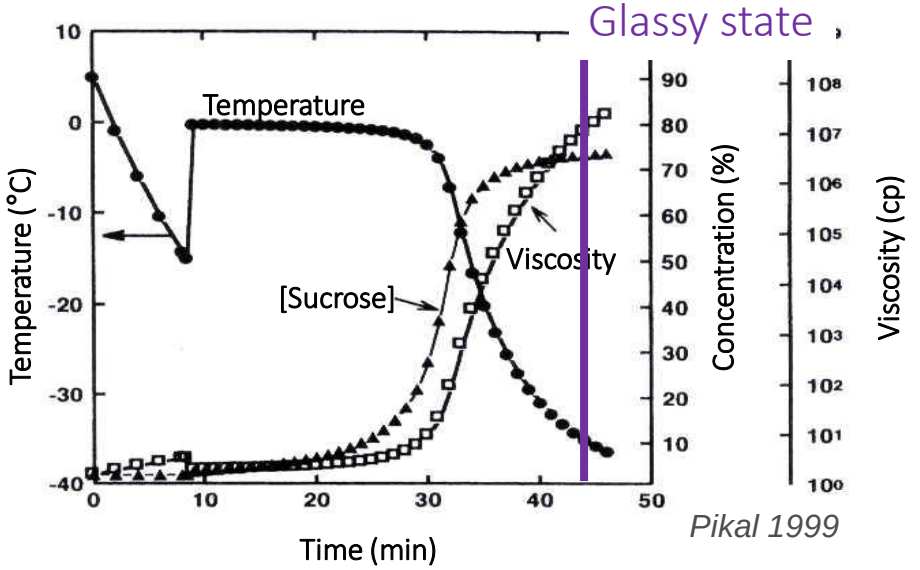
Fonseca et al. 2006,AEM; Morris et al 2007, Theriogenology; Baboo et al 2019, Sci. Rep.

Intracellular glass transition (Tg'i) determined by DSC

1. Cold stress (low T°): From ambient to -80°C or lower
2. Ice nucleation, crystal growth, solute concentration
3. Viscosity increase inside and outside the cell

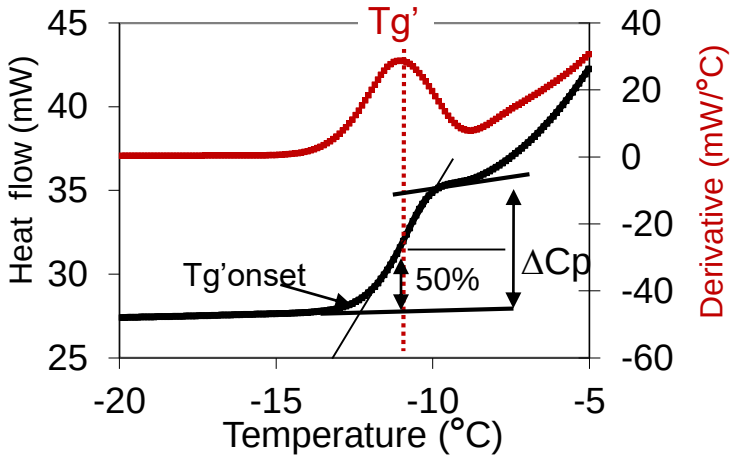
→ Vitrification of intracellular content at -10 to -26°C (Tg'i) without cryoprotectant

→ Vitrification of extracellular medium at Tg'e <<Tg'i

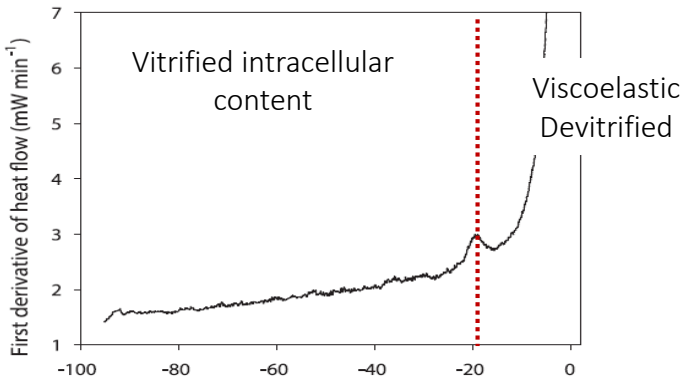


Pikal 1999

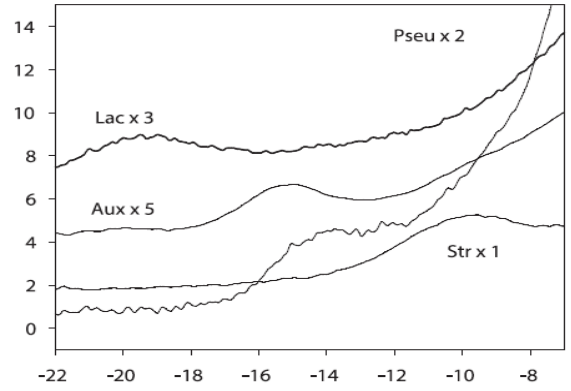
DSC cell pellets



Tg'i =-20°C



Lb bulgaricus CFL1

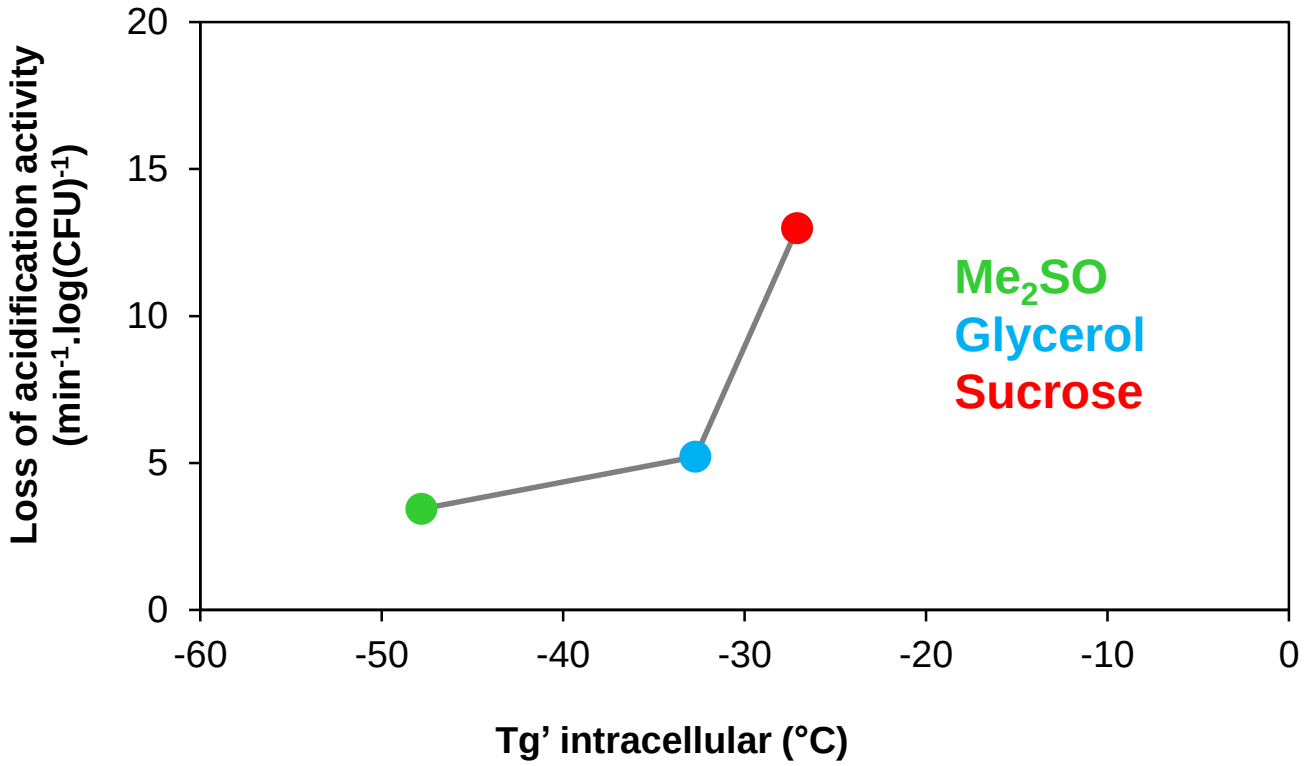


Bacteria, yeast, algae

Clarke et al. 2013, PLosOne

asymptote
Part of GE Healthcare Life Sciences

Intracellular glass transition and stability of frozen LAB



Lb bulgaricus CFL1
Cultured in whey medium

Low intracellular Tg' was associated with increased cryoresistance

BUT Incompatible with freeze-drying

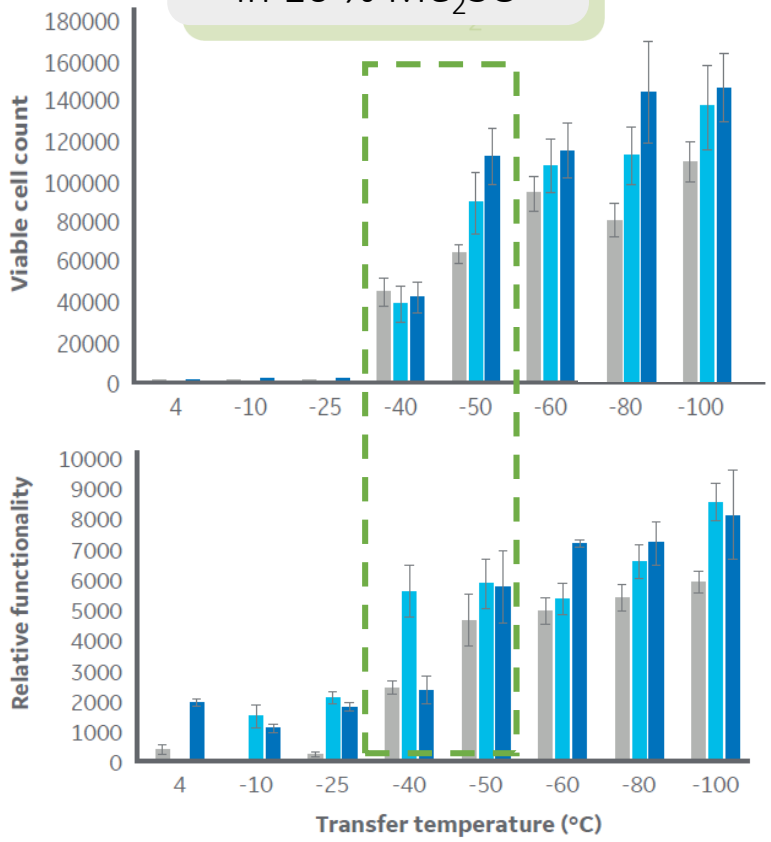
Fonseca et al. 2016, PlosOne

Intracellular glass transition and critical end point T°

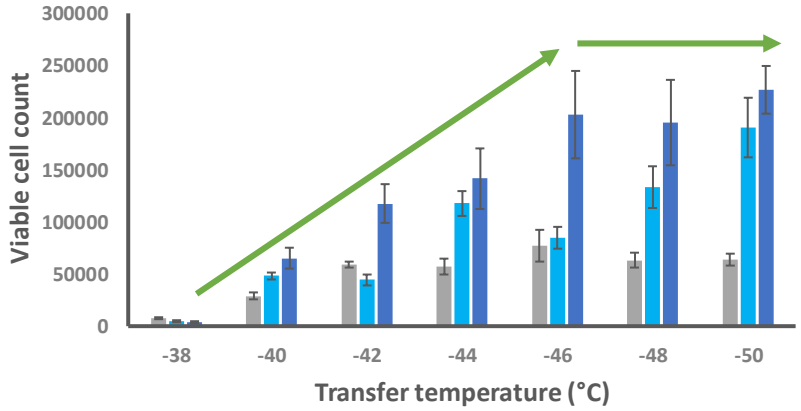
→ The critical controlled-cooling endpoint T° in Jurkat cells

day 1 day 2 day 3

Jurkat cells
in 10 % Me_2SO



Zooming in…



Cell type	Critical T (°C)	Tg'i (°C)
Jurkat	**-46**	**-47°C**

Jurkat cells:
Critical T° ~ Tg'i

Transferring cells to LN_2 at:

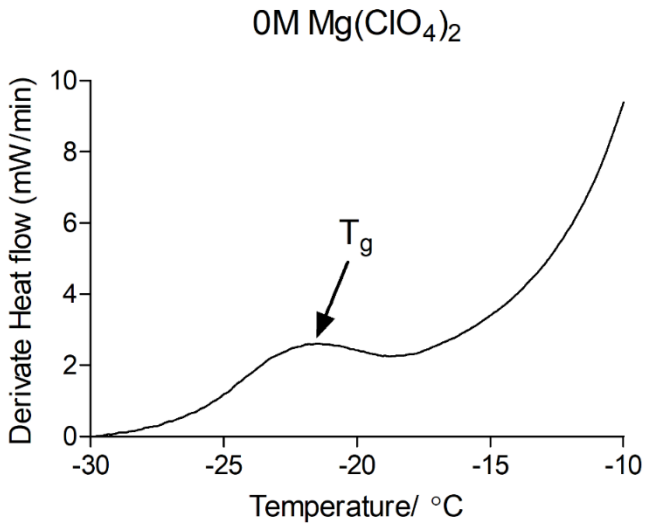
- T ≥ -40°C → loss of viability & functionality post-thaw
- T = -50°C → optimum viability and functionality post-thaw
- T < -50°C → no further improvement of cell viability and functionality post-thaw

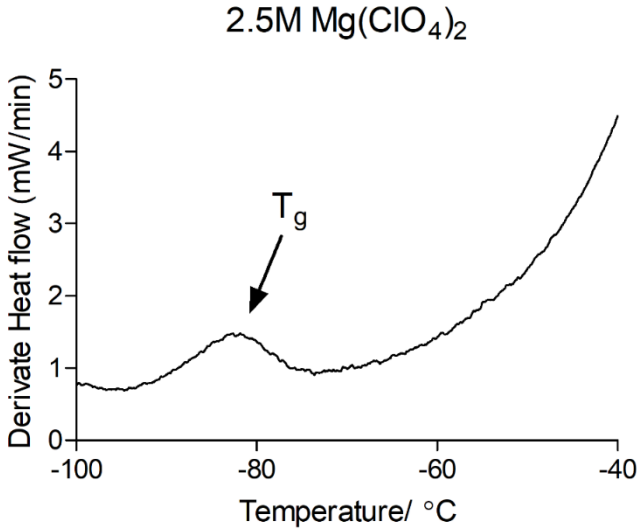
Same observations for HepG2, MG63 and CHO cells

PREMIUM
Preserving bacteria with oligosaccharides and eco-friendly processes

Meneghel et al. 2019, PlosOne

Implications of Intracellular glass transition for astrophysics

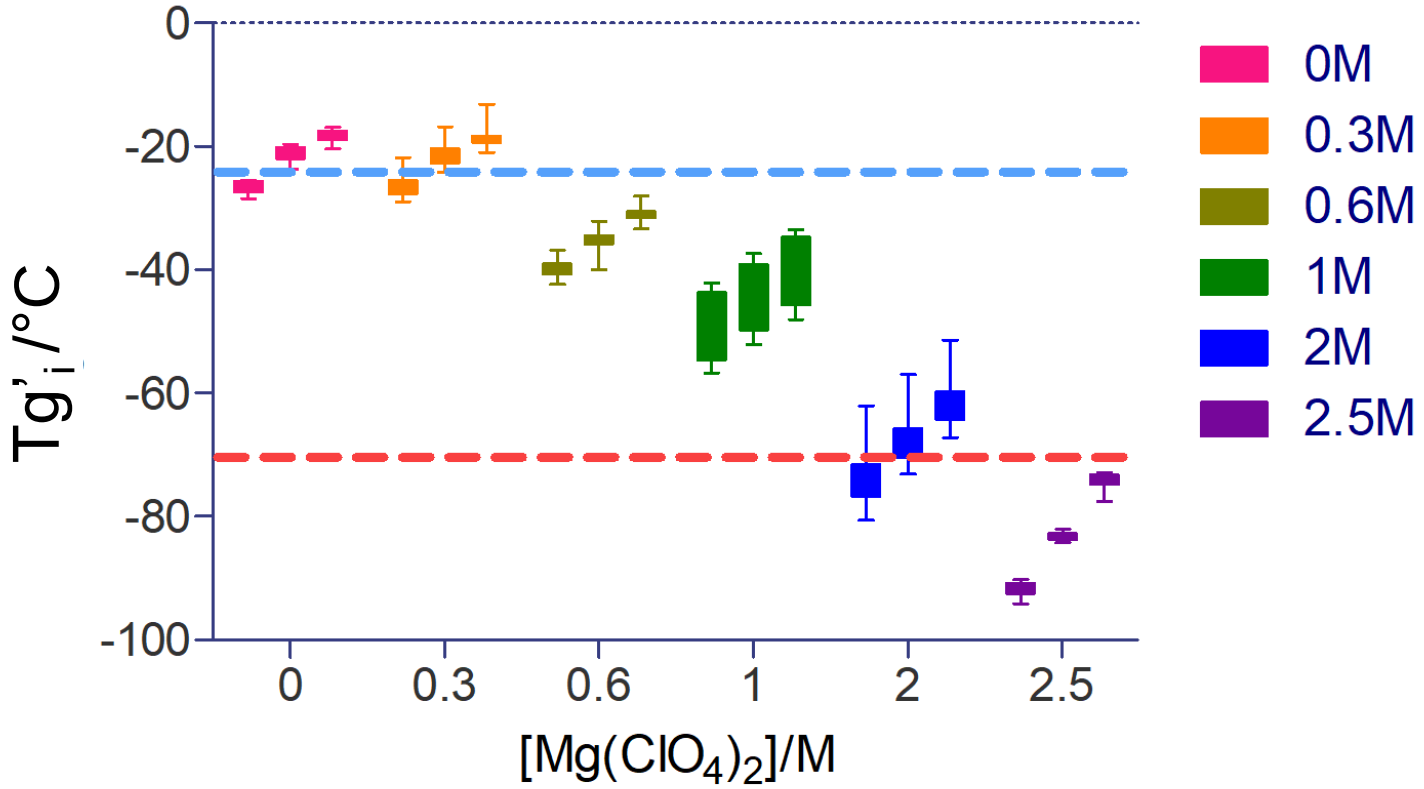




Bacillus subtilis

THE UNIVERSITY of EDINBURGH

Potential for active life on Mars?



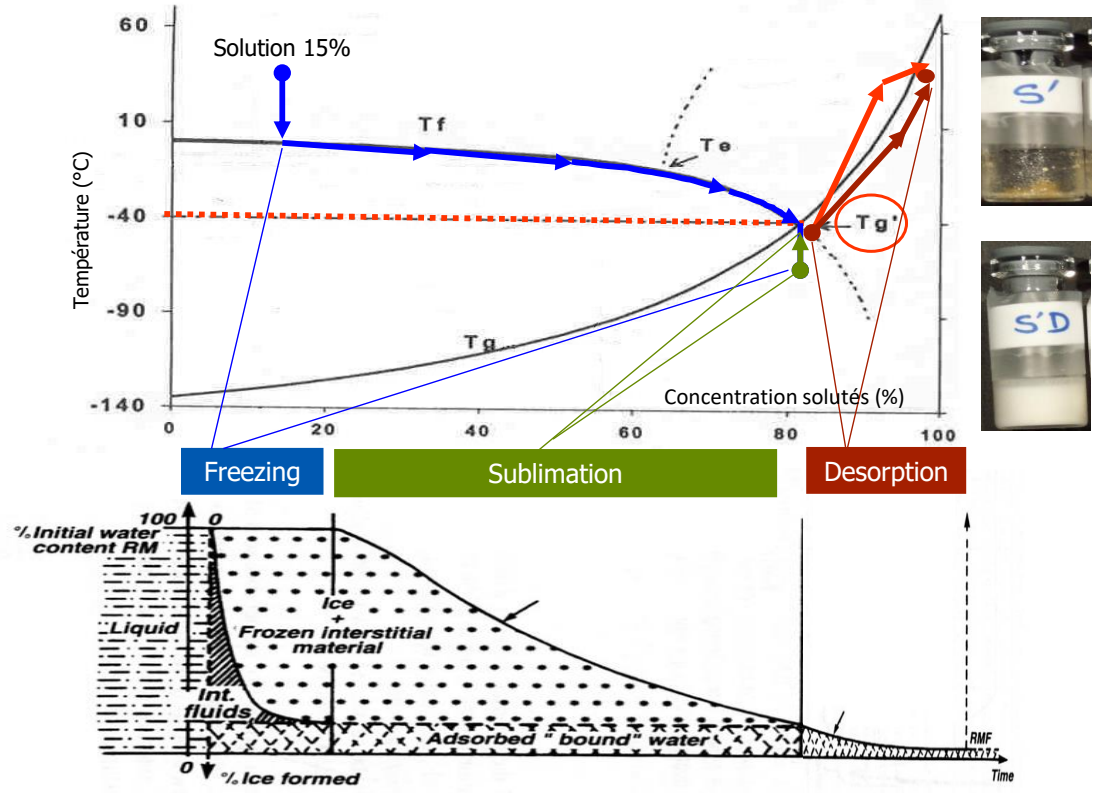
Perchlorate salts reduce the Tg'i of *Bacillus subtilis* cells
Life could be active in deep eutectic Martian environments

Gault et al 2022, Astrobiology Scie. Conf

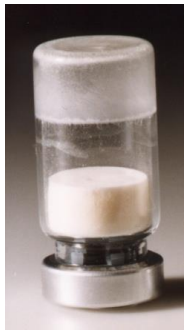
Physical properties & modelling – new tools

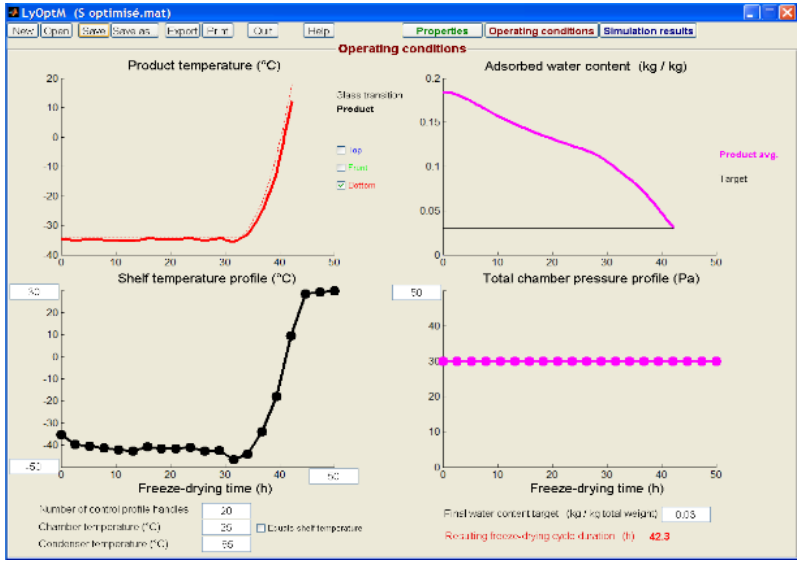
Fermentation → Formulation → Freeze-drying → Product → Storage → Rehydration

Importance of the product thermal history during freeze-drying process



⇒ Macroscopic structure of the FD cake
⇒ Biological activity of the bacteria





Tréléa et al. 2007, Drying Technology

Passot et al 2012, Food Chem.

2007- 2013

Tg and aw: key parameters for optimal preservation of freeze-dried LAB

Lb bulgaricus CFL1
sucrose 20%

△ Glass transition temperature

☐ Sorption isotherm

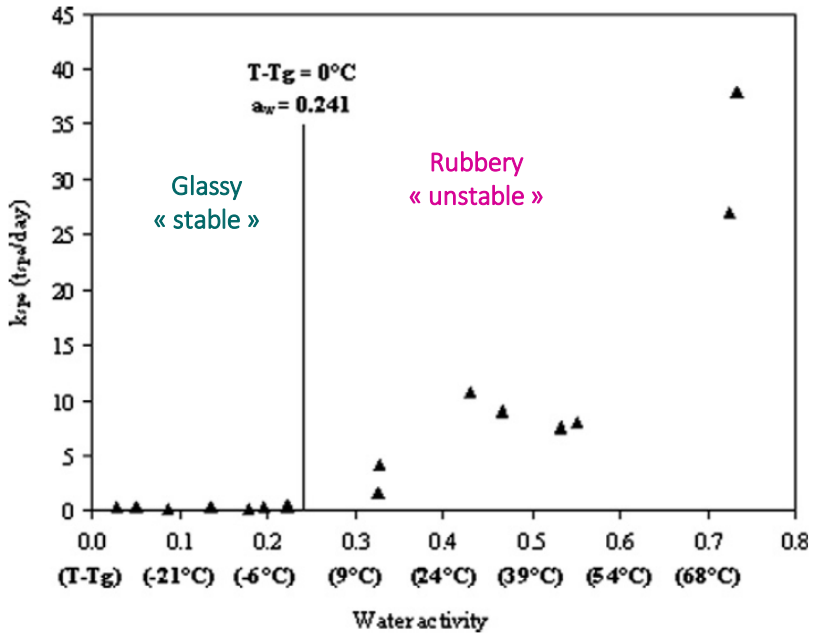


Fig. 4. Rate of loss of specific acidification activity during storage at 25 °C of lyophilised *Lactobacillus bulgaricus* CFL1 in a sucrose matrix (k_{spe}, in (min/(log(CFU/ml)))/day) as a function of water activity (a_w). A vertical line indicates the threshold value of a_w between the glassy and the rubbery states. In bold under the *x* axis, are reported the values of the temperature difference $T - T_g$ (with $T = 25$ °C) corresponding to the a_w values.

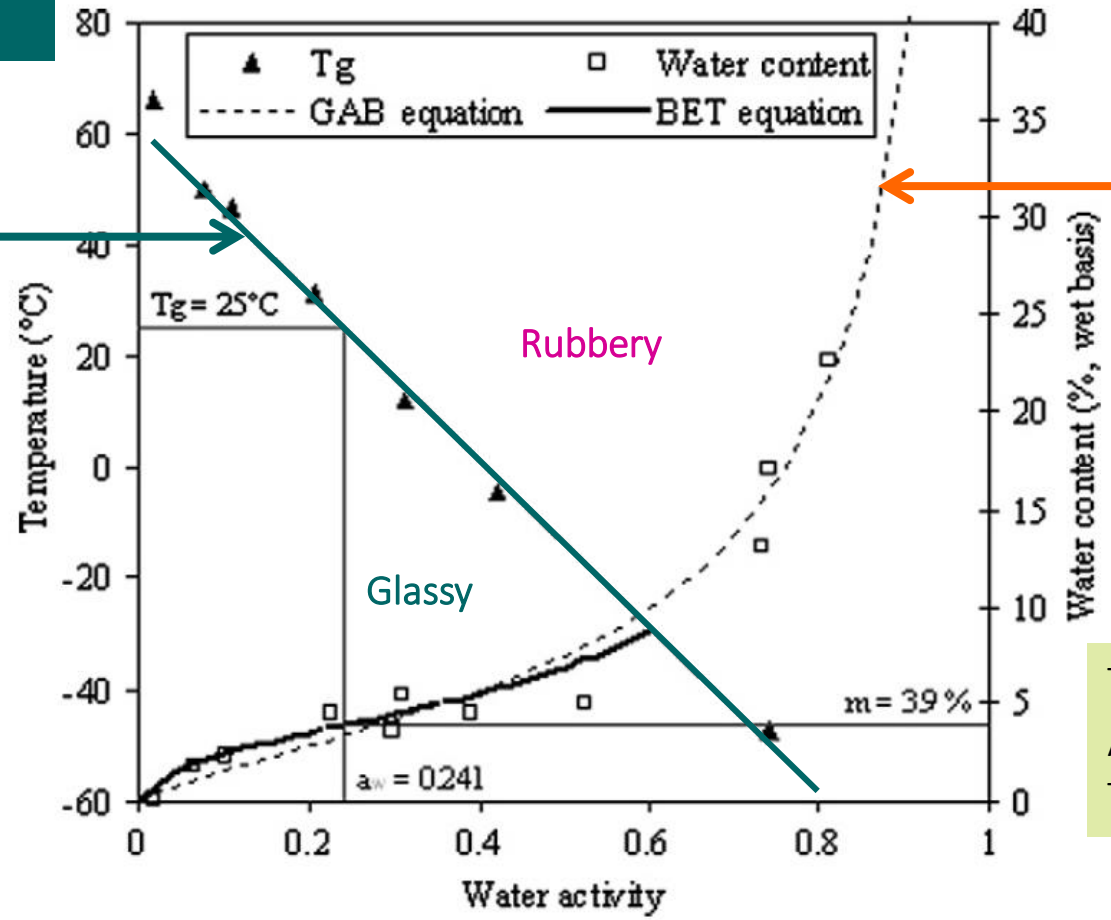


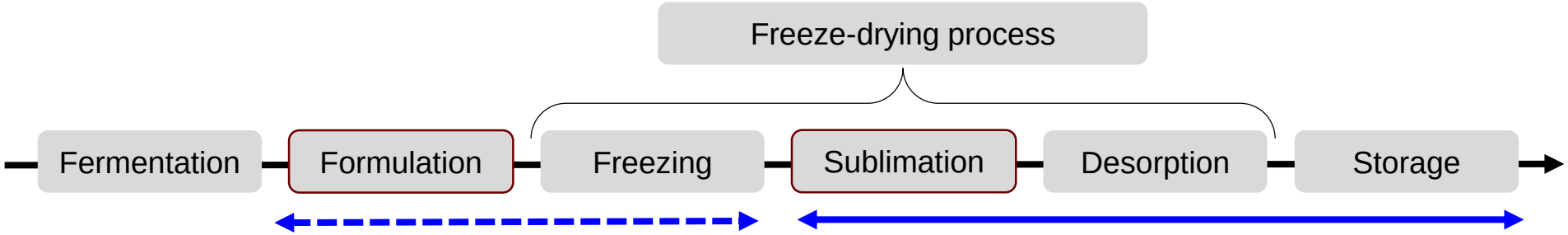
Fig. 1. Relationships between glass transition temperature (T_g), water activity (a_w) and water content (m) for bacterial suspension freeze-dried in a sucrose matrix. Lines indicate the location of critical T_g, a_w and m values at 25 °C.

Ts = 25 °C
Aw < 0,24
TE < 3.9 %

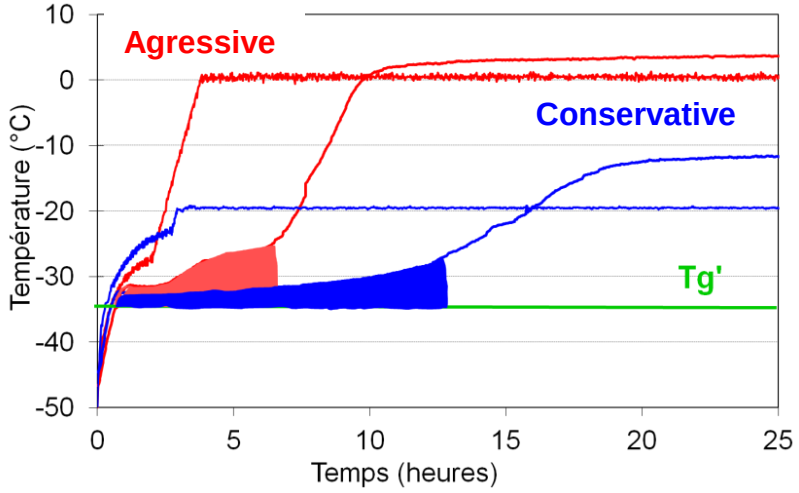
Passot et al 2012, Food Chem.

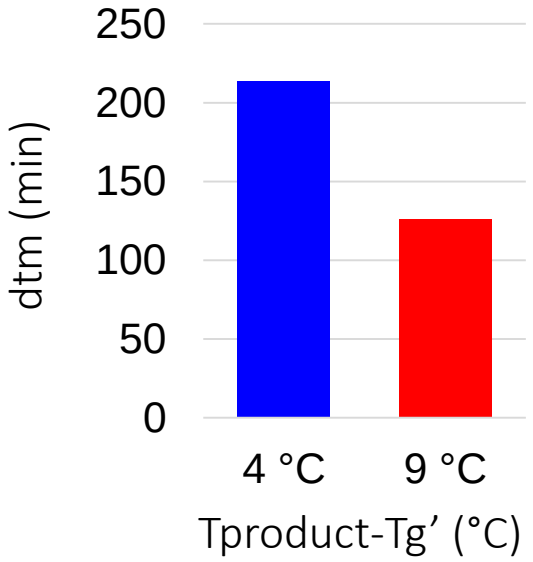
European project CAFE
Computer-aided Food processes for control Engineering

2007- 2013



Effect on the acidifying activity of operating conditions during sublimation

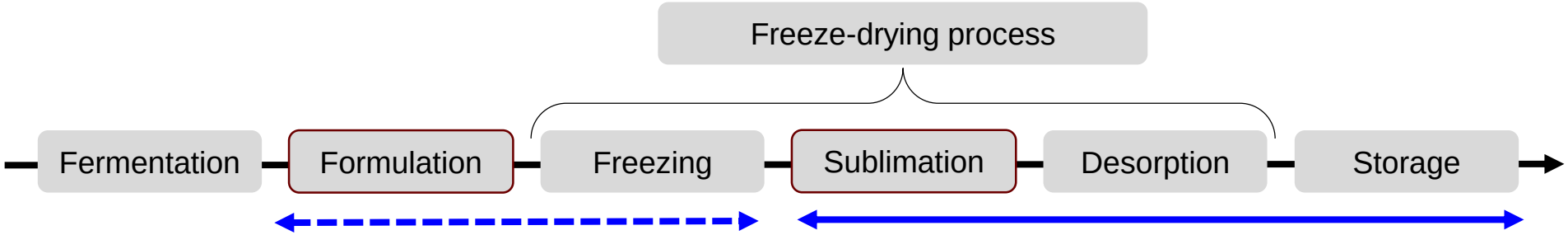




Passot et al. 2011, ICEF

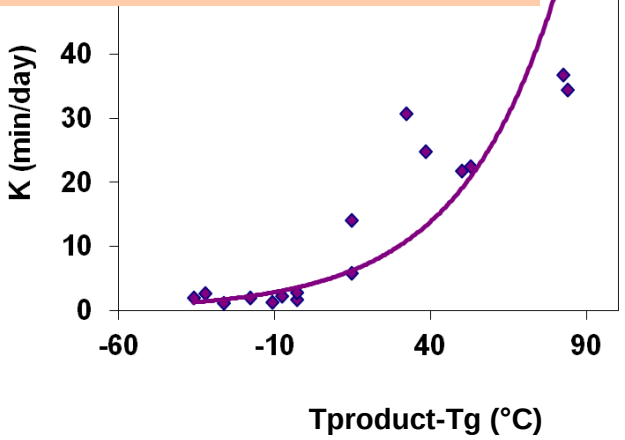
European project CAFE
Computer-aided Food processes for control Engineering

2007- 2013

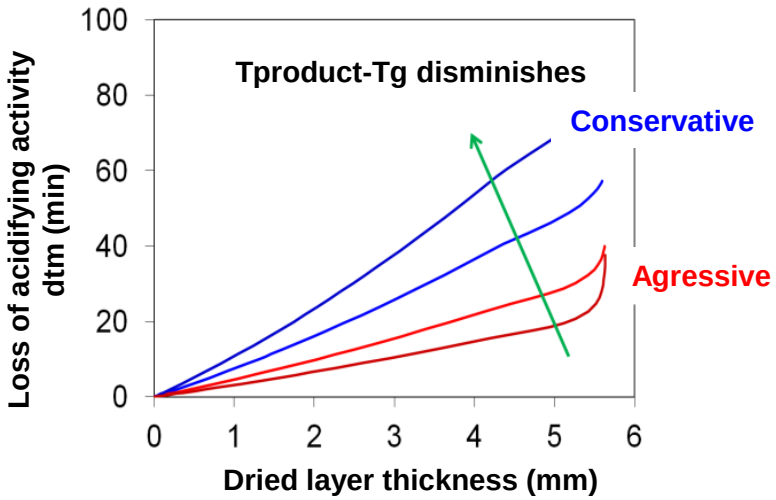


Predicting the loss of acidifying activity
=> It depends on the product thermal history (T°product – Tg)

$$K = k_0 e^{k_1 (Tproduct - Tg)}$$



LyOptM



Passot et al. 2011, ICEF

Thank you for your attention!

INRAE

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Talents d'une planète soutenable

asymptote
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Preserving bacteria with oligosaccharides and eco-friendly processes

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