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Improvement of the cryopreservation of the fungal starter *Geotrichum candidum* by artificial nucleation and temperature downshift control ☆

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Abstract

Food industry tends towards the use of controlled microorganisms in order to improve its technologies including frozen starter production. The fungus *Geotrichum candidum*, which is currently found in various environments, is widely used as ripening agent in some specific cheese making process. In order to optimize the cryopreservation of this microorganism, freezing experiments were carried out using a Peltier cooler–heater incubator, which permits to control the temperature downshift from +20 to −10 °C in time period ranges from 20 to 40 min depending on the experiments. Concomitantly, study of the effect of an industrial ice nucleator protein derived from *Pseudomonas syringae* (SNOMAX®) on the dynamic of freezing of *G. candidum* was carried out. Our results showed that the addition of this protein in the microbiological suspension has different complementary effects: (i) the synchronization of the different samples nucleation, leading to an homogeneous and earlier freezing, (ii) the increase of the freezing point temperature from −8.6 to −2.6 °C, (iii) a significant decrease of the lethality of *G. candidum* cells subjected to a freezing–thawing cycles challenge.

Keywords: *Geotrichum candidum*; Starter; Freezing; Nucleation; Stress; Cryopreservation

Any modification of environmental parameters that leads to a response by living organisms may be considered as a stress [1,33,35]. Currently, two types of stress are proposed: abiotic stresses including physical and chemical ones and biotic stresses. As a global phenomenon, it can be actually extended to anthropogenic pressure such as pollution or genetic engineering [24].

From prokaryotes [25,31] to humans [23], including plants [28], animals [32] and fungi [22] cold temperature is one of the major stresses that most of the living organisms have to confront [11]. At subzero temperatures, conditions considered to equate to 'moderate' or 'severe stress' according to Yousef and Courtney [41], the response of most microorganisms is passive, leading to a slow lethality of cells. Freezing stress is considered to be the combination of both osmotic and mechanical stresses, leading to cryoinjury of cellular structures and macromolecular damage.

Freezing is a transition from liquid to solid state by a mechanism called nucleation. Two types of nucleation can be observed: the first one, called homogeneous nucleation, occurs in pure liquid whereas the second one, called heterogeneous nucleation, occurs in a liquid which contains foreign substances around which the ice crystal develops. Those foreign substances act as nucleation sites at a relatively high sub-zero temperature [15,36,3,16] and are so called ice nucleators. Numerous ice nucleators have been described in literature such as silver iodide, river sand [37,4] and specific microbial proteins. Indeed, some microorganisms, particularly phytopathogenic ones such as *Pseudomonas syringae, Erwinia herbicola, Xanthomonas campestris* and *Fusarium moniliforme*, synthesize proteins which initiate nucleation and consequently induce frost

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damages to their hosts, in order to access to nutrients [21,14,17,26,12,40].

Geotrichum candidum is a filamentous yeast-like fungus. This ubiquitous microeucaryote, considered as the anamorph of *Galactomyces candidus* [5], is widely used as food starter to ripen soft and semi soft cheeses and in fermented milks [19,39,2]. More recently, *G. candidum* has been integrated to the malting process as a bioprotective agent [20]. Its optimal growth temperature is between 22 and 25 °C [9] and the average highest growth temperature around 35–36 °C even if some strains can tolerate higher temperatures and develop until 39 °C [5]. According to some authors, the lowest growth temperature is around 4 °C [8,27].

Most of the few studies related to the cryopreservation of *G. candidum* are focused on the physiological adaptation of this microorganism to freezing stress by mild stress pretreatment. This phenomenon, which is reversible and not heritable, leads to a transient increased resistance to the freezing challenge. Two types of physiological adaptation to negative temperatures have been observed with *G. candidum* depending on the type of pre-treatment: (i) Homologous adaptation when cells were pre-incubated at low positive temperatures [34]. (ii) Heterologous adaptation (cross protection) when cells were pre-exposed to specific chemicals [7]. Recently, cryopreservation experiments completed on an artificial microbial community (consortium) of dairy interest, including *G. candidum* and two prokaryotes, highlighted an interspecies cryoprotective phenomenon [6].

Our main goal is to improve the cryopreservation of *G. candidum*. The work presented in this manuscript has been carried out as a necessary step to increase our understanding of the fundamental mechanism related to the cold stress responses. We have developed a new freezing process, which brings together the control of the temperature downshift (Peltier cooler–heater) with the addition of an industrial nucleator (SNOMAX®) that is produced by *Pseudomonas syringae* and used in different cryobiological researches [30,18].

Materials and methods

Strain and culture conditions

The study was carried out with *Geotrichum candidum* ATCC204307, obtained from the laboratory collection (UCMA91). This strain initially isolated from a cheese (Pont l'Evêque, Protected Designation of Origin) made with raw milk, is cryopreserved (−80 °C) as a cell suspension in glycerol 15% (v/v).

Cells were spread on MEA (Malt Extract Agar) medium [29], incubated 48 h at 25 °C, and suspended in 3 mL of 0.9% (w/v) NaCl in order to prepare a preinoculum for further experiments.

One milliliter of the preinoculum was added to 100 mL of MEB (Malt Extract Broth) medium [29] in a 1 L Erlenmeyer flask, and incubated 48 h at 25 °C with orbital shaking at 150 rpm (AS850, LSL Biolafitte SA, St. Germain en Laye, France). Cells (stationary growth phase) were pelleted at 2400*g* for 10 min (Eppendorf centrifuge 5810 R, Hamburg, Germany), washed and centrifuged twice (2400*g*, 10 min) in 0.9% (w/v) NaCl. Cells were then resuspended in the NaCl solution, adjusted to an $OD_{620\ nm} = 1 \pm 0.05$ (3.5×10^6 TFU/mL) using a spectronic 301 spectrophotometer (Bioblock Scientific, Illkirch, France) and divided in 1 mL aliquots into 1.5 mL microtubes (Eppendorf).

Freezing procedures

Freezing was performed using a Peltier cooler–heater (PCH-2, Grant-bio, Cambridgeshire, England) which enables to control the temperature downshift from +20 to −10 °C in time period ranges from 20 to 40 min according to the experiments. This apparatus has a stand of 20 wells which contain 1.5 mL microtubes. The temperature of the samples was measured and recorded every 10 s using a thermic probe (Testo175-T3, Germany) inserted into one test microtube.

Freezing–thawing challenges on *G. candidum* were performed as follow: 1 mL aliquots were incubated 5 min at 20 °C then 40 min at −10 °C. This challenge was repeated five times per experiment. At different cycles, cells suspensions were sampled, diluted in NaCl 0.9% (w/v) and spreaded on MEA medium. The plates were incubated at 25 °C for 48 h before TFU counting.

Depending on the experiments, different concentrations (from 10 fg/mL to 1 mg/mL) of ice nucleant [SNOMAX® (York International, York, PA, USA)] were added to sterile distilled water or *G. candidum* suspensions. The effective freezing of the samples was evaluated by visual assessment. The results are the means of at least three experiments.

Results and discussion

Asynchronous freezing of G. candidum

In order to optimize the freezing–thawing process of *G. candidum*, cells suspensions were subjected to a controlled challenge using a Peltier cooler–heater as described above. Dynamic of freezing of *G. candidum* was followed concomitantly in 20 microtubes. Cooling and warming rate were continuously measured using a thermic probe inserted in a sample as control. The temperature reached 0 °C 10 min after the beginning of the controlled temperature downshift. Interestingly, as described in the Fig. 1(A), the 20 suspensions did not freeze simultaneously: some samples were frozen 20 min after the beginning of the experiment whereas others remained liquid 50 min after.

Fig. 2 shows, through the example of two samples, that the temperature profiles were clearly similar with a nucleating temperature of −8.6 °C. Nevertheless, time to reach the nucleation point was different between the two samples