THE EFFECT OF GROWTH PHASE, CRYOPROTECTANTS AND FREEZING RATES ON THE SURVIVAL OF SELECTED MICRO-ORGANISMS DURING FREEZING AND THAWING

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The effect of growth phase of cells, cryoprotectant agents and freezing rate on the survival of selected micro-organisms *(Bacillus cereus, Lactobacillus plantarum, Escherichia coli, Pseudomonas fluorescens, Saccharomyces cerevisiae, Kluyveromyces lactis, Candida utilis, Mucor racemosus, Aspergillus niger)* during freezing and thawing was studied. In cases where significant differences were observed stationary phase cells always survived better than exponential phase ones, while of the two cryoprotectant agents studied, horse serum + inositol was always superior to skimmed milk powder + inositol. The effect of freezing rate was different among the studied micro-organisms.

The inhibitory effects of reduced temperatures on physical, chemical and biochemical processes provide the bases of the long-term preservation of cells, tissues and organs (TAYLOR, 1987).

Parameters such as the age of the culture, the presence of cryoprotective agents, the rate of freezing are in many cases critical for the survival of micro-organisms. Although cryopreservation has been used extensively as a method for the maintenance of microbes for a long time, judgement of the effect of some parameters on survival during freezing is still subject to arguments.

According to HECKLY (1978), it is generally accepted that "cells from maximum stationary phase cultures are more resistant to damage by freezing and thawing than cells from the early or midlog phase of growth". Contrary to this, other authors found that some bacteria were more resistant against cold shock (MACKELVIE et al., 1968) and freezing (FRY, 1966), when cells were obtained from the exponential growth phase. From the usually applied cooling rates most authors consider slow cooling/freezing rates

more favourable in maintaining the viability of microbes (e.g. SMITH & ONIONS, 1994; DAVIS & OBAFEMI, 1985) than fast cooling rates, although SMITH and ONIONS (1994) demonstrated that the optimal cooling rate could be different for different fungi. However, there are other opinions as well. For instance DEÁK and BEUCHAT (1996) suggested fast freezing (coupled with fast thawing) for yeasts. Interestingly, despite of the abundance of results suggesting the opposite, INGRAM (1951) stated that the survival of micro-organisms directly after freezing is nearly independent of cooling rate and according to HECKLY (1978) freezing rate is not a critical factor for survival.

In the present paper we report the results of a study on the effect of the growth phase, cryoprotectants and cooling rates on the survival of some micro-organisms after freezing and thawing. All the selected strains belong to species with potential importance in the food industry either as starter cultures or as contaminants or spoilage organisms.

1. Materials and methods

1.1. Strains studied

The investigated strains with culture conditions are shown in Table 1.

Table 1

Microbial strains used

Name	Accession number	Medium	Incubation temperature
Bacillus cereus	NCAIM B 00076	TGE agar	30 °C
Lactobacillus plantarum	NCAIM B 01133	MRS agar	37 °C
Escherichia coli	NCAIM B 00200	TGE agar	37 °C
Pseudomonas fluorescens	NCAIM B 01154	TGE agar	26 °C
Saccharomyces cerevisiae	NCAIM Y 00200	GPY agar	26 °C
Kluyveromyces lactis	NCAIM Y 00231	GPY agar	26 °C
Candida utilis	NCAIM Y 00383	GPY agar	26 °C
Mucor racemosus	NCAIM F 00598	PDA agar	26 °C
Aspergillus niger	NCAIM F 00735	PDA agar	26 °C

NCAIM: National Collection of Agricultural and Industrial Micro-organisms, Budapest, Somlói út 14-16. H-1118, Hungary
TGE agar: Tryptone glucose yeast extract agar
GPY agar: Glucose peptone yeast extract agar
PDA agar: Potato dextrose agar

1.2. Experimental parameters

Except for the investigated mould strains (in which cases one week old cultures were used in order to have matured spores or conidia) cells from the late exponential growth phase (Ph1) and from the stationary growth phase (Ph2) were used. (In case of moulds the growth phase does not necessarily show direct correlation with the maturity stage of spores or conidia.) In order to determine the growth phase of the cultures grown on agar slants, a preliminary experiment was carried out. Sufficient number of slants of the given medium were inoculated with a loop of suspension made from young (overnight and 24 h in case of bacteria and yeasts, respectively) slant culture. Two slants of each strain were washed with 4–4 ml sterile water in regular intervals (in every hour in case of bacteria and in every second hour in case of yeasts). After homogenisation and dilution (if it was necessary) the absorbance of the suspensions was measured at 540 nm against the blind which was obtained by washing the same, but uninoculated medium. It was possible to fit a curve on the data in case of each strain with the aid of the modified Gompertz equation (ZWIETERING et al., 1990). The specific harvesting time for each strain was determined with the aid of these curves. The vast majority of stationary phase cells of the investigated *B. cereus* strain were spores. Comparing the survival of vegetative cells (Ph1) to spores (Ph2) had no theoretical but practical reason.

As cryoprotective agents two different suspending media were applied:

1) 8% skimmed milk powder + 4% inositol (Cr1)

2) inactivated horse serum + 5 g inositol/100 ml serum (Cr2)

Suspensions were made by pouring the suspending medium to the agar slant culture and stirring by vortex. They were homogenised before use. 0.2 cm^3 aliquots of the suspensions were distributed into sterile glass vials (about 48×6 mm with 1 mm thick wall). The vials were plugged with sterile cotton.

Three different freezing rates were applied. In the first treatment (T1) the suspensions were frozen on a centrifuge rack of a freeze-drying equipment (EDWARDS MODULYO) by the loss of latent heat (evaporative freezing) for 30 min.

In the second case (T2) the suspensions were kept in a commercial freezer at –25 °C for one hour, while in the third case (T3) the vials were submersed into liquid nitrogen (–196 °C) for one minute. The freezing rate for the T2 and T3 treatments were measured by copper-constantan thermocouple with the aid of a potentiometric recorder (RADELKIS OH-814/1) at 2 mV sensitivity. The freezing rates for T2 (freezing at –25 °C) and T3 (freezing in liquid nitrogen) were 2.1 °C min^{-1} and 233 °C min^{-1}, respectively.

Every experiment was done in duplicate. The experimental arrangement is shown in Fig. 1. Following every combination of the treatments described above, the samples were immediately thawed in a 37 °C water bath.
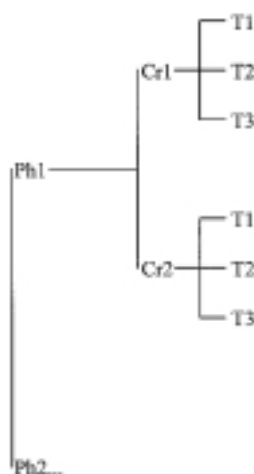
Fig. 1. Experimental arrangement. Ph1: Exponential growth phase, Ph2: Stationary growth phase, Cr1: 8% skimmed milk powder + 4% inositol, Cr2: Inactivated horse serum + inositol (5 g/100 ml), T1: Evaporative freezing, T2: Freezing at –25 °C, T3: Freezing in liquid nitrogen (–196 °C)

The viable count (colony forming unit, cfu) for every suspension was determined before freezing and after thawing. The viable count values obtained after freezing and thawing were normalised by dividing with the corresponding cfu values of the unfrozen suspensions. These normalised values are interpreted as survival ratio.

1.3 Mathematical-statistical evaluation

After logarithmic transformation of the normalised values multi-factor analysis of variance was applied to determine the significant effects of the treatments.

From the least significant difference (LSD) of the logarithmic values the critical survival ratios were calculated as follows:

$$LSD_R = 10^{LSD}$$

where:

LSD = the least significant difference between the logarithmic transformed survival ratios

LSD_R = the least significant ratio between the survival ratios

If the ratio of the survival belonging to the different treatments is higher than LSD_R, significant differences exist between the effects of the treatments. For the

simplest demonstration in tables the survival ratios together with LSD_R (critical ratio) values are summarised. For the mathematical statistical evaluation of the results STATGRAPHICS 5.1 program package (STATISTICAL GRAPHICS CORPORATION, USA) was used.

2. Results and discussion

2.1. The effect of the growth phase on the survival

The growth phase of the microbe had a significant effect on the survival in case of the *B. cereus, K. lactis* and *C. utilis* strains (Table 2). In all three cases the stationary growth phase (Ph2) resulted in a higher survival ratio than the late exponential one (Ph1). These results support the opinion of those authors who prefer stationary to exponential phase (e.g. HECKLY, 1978; OLASZ & TÖRÖK, 1988; STRANGE, 1976; KIRSOP, 1984) in order to ensure high survival ratio. In case of *B. cereus* the better survival of the culture from stationary phase may partly be explained by the spore formation of the species. (None of the investigated yeast strains formed spores under the culture conditions applied in this study.) The absence of significant differences in the survival in case of the other microbes can be – at least partly – considered to be the result of the high resistance to the freeze-thaw stress by some of the investigated micro-organisms. In case of the *S. cerevisiae* strain, for instance, the decrease in survival is within the accuracy of the applied pour plate method.

Table 2

Survival ratio (%) after freezing and thawing as a function of growth phases

Strains	Growth phase (%)		Critical ratio (LSD_R)
	Exponential (Ph1)	Stationary (Ph2)	
*B. cereus**	30.6	91.7	1.37
L. plantarum	78.9	89.7	1.19
E. coli	47.4	54.7	1.39
P. fluorescens	71.1	77.8	1.18
S. cerevisiae	99.8	96.4	1.14
*K. lactis**	57.8	71.3	1.19
*C. utilis**	21.2	34.6	1.33

* denotes significant difference at 95% level

LSD_R: the least significant quotient between the survival ratios

Table 3

Survival ratio (%) after freezing and thawing as a function of cryoprotectants

Strains	Cryoprotectants (%)		Critical ratio (LSD$_R$)
	Cr1	Cr2	
B. cereus	50.9	55.2	1.37
L. plantarum*	76.2	92.7	1.19
E. coli*	40.6	63.8	1.39
P. fluorescens	71.8	77.3	1.18
S. cerevisiae	97.1	99.5	1.14
K. lactis*	54.8	75.0	1.19
C. utilis*	22.2	37.6	1.33
M. racemosus	77.1	71.6	1.11
A. niger	75.3	81.2	1.31

* denotes significant difference at 95% level

Cr1: Skimmed milk + inositol

Cr2: Horse serum + inositol

LSD$_R$: the least significant quotient between the survival ratios

2.2. The effect of the cryoprotectants on the survival

The tested suspending media had significant effects on the survival in case of *L. plantarum*, *E. coli*, *K. lactis* and *C. utilis* (Table 3). In all cases the horse serum + inositol combination (Cr2) proved to be superior to skimmed milk powder + inositol (Cr1). These cryoprotectants were selected, because these or similar ones are widely used in the practice of microbial strain preservation.

2.3. The effect of freezing rate on the survival

In case of evaporative freezing from the time required for freezing (ca. 10 min.) the average freezing rate was estimated to be about 10 °C min^{-1}. This estimation was based on the following considerations. Taking into account the specific heat and latent heat of the water, the heat loss of the suspension during the cooling from room temperature (about 20 °C) to 0 °C is a quarter of the heat loss of the freezing at 0 °C. Counting with a constant heat transfer rate this ratio means that the time necessary for the cooling is about one fifth of the 10 min, required by the freezing of the suspension of 20 °C temperature. It would mean ca 10 °C min^{-1}. Although such calculation of the cooling rate from the time required for freezing is not suggested by MERYMAN (1966), we used it in the case of the other two freezing methods (T2 and T3) as well and in those cases we experienced good agreement with the data actually measured.

Table 4

Survival ratio (%) after freezing and thawing as a function of freezing rates

Strains	Survival ratio (%)			Critical ratio (LSD$_R$)
	T1	T2	T3	
B. cereus	52.8	46.7	60.5	1.47
L. plantarum * (T1-T2, T1-T3)	71.3	94.0	88.9	1.24
E. coli * (T1-T2, T2-T3)	33.3	92.0	43.2	1.50
P. fluorescens * (T1-T3, T2-T3)	62.1	69.3	95.7	1.22
S. cerevisiae	92.5	101.6	100.9	1.17
K. lactis * (T1-T3, T2-T3)	85.7	88.3	34.9	1.24
C. utilis * (T1-T3, T2-T3)	100.6	82.8	2.9	1.42
M. racemosus * (T1-T2, T2-T3)	78.2	63.4	82.8	1.14
A. niger * (T2-T3)	87.1	66.8	97.7	1.39

* denotes significant difference at 95% level

T1: evaporative freezing

T2: freezing at –25 °C

T3: Freezing in liquid nitrogen (–196 °C)

LSD$_R$: the least significant quotient between the survival ratios

The freezing rate had a significant effect on the survival in the case of *L. plantarum, E. coli, P. fluorescens, K. lactis, C. utilis, M. racemosus* and *A. niger* (Table 4). In the case of *Lactobacillus plantarum* the evaporative freezing (T1: 10 °C min^{-1}) resulted in a significantly lower survival than the other freezing rates (T2: 2.1 °C min^{-1}; T3: 233 °C min^{-1}). This result does not support the "two factor hypothesis" (MAZUR et al., 1972) and may indicate that it is not generally valid for every cell type and every freezing condition. In case of *E. coli* the slowest freezing (T2: 2.1 °C min^{-1}), while in case of *P. fluorescens* the fastest freezing (T3: 233 °C min^{-1}) resulted in the highest survival ratio. The judgement of the effect of the freezing rate on the survival is therefore contradictory. One of the reasons must be that different microbes react differently to different freezing rate. This is clearly demonstrated by the results for the two Gram-negative bacteria (*E. coli* and *P. fluorescens*).

From the studied yeasts the fastest freezing rate (T3: 233 °C min^{-1}) resulted in a significantly lower survival in the case of *K. lactis* and *C. utilis* than the other two freezing rates. The difference for *C. utilis* exceeded one order of magnitude. Our findings on the effect of freezing rate on the survival of yeasts are in good agreement with the data of SMITH (1993) and SMITH & ONIONS (1994), while they differ from the findings of DEÁK and BEUCHAT (1996) according to which, for yeast cells, fast freezing (coupled with fast thawing) is less lethal than slow freezing.

From the studied moulds *M. racemosus* had the lowest survival when frozen with the slowest cooling rate (T2: 2.1 °C min^{-1}). In case of *A. niger* the slowest freezing rate (T2: 2.1 °C min^{-1}) resulted in a significantly lower survival ratio than the fastest one (T3: 233 °C min^{-1}). These results are not in agreement with the findings of those authors who consider slow freezing rates more favourable in maintaining the viability of fungi than fast freezing rates.

Our data also reveal that Ingram's opinion (INGRAM, 1951) which was accepted also by JAY (1992) can be negotiated, that is, it is not supported by these investigations that the survival ratio of micro-organisms after freezing is always nearly independent of the cooling rate. During our study out of the 9 investigated microbes the different freezing rates resulted in significantly different survival ratios in 7 cases. From the remaining two, in case of *S. cerevisiae* the small decrease in viability (less than 10%) may have masked the effect of different freezing rates (Fig. 2). We have to add that in the present study not only the freezing rates were different, but the final temperatures as well and this may also have influence on the survival ratio.
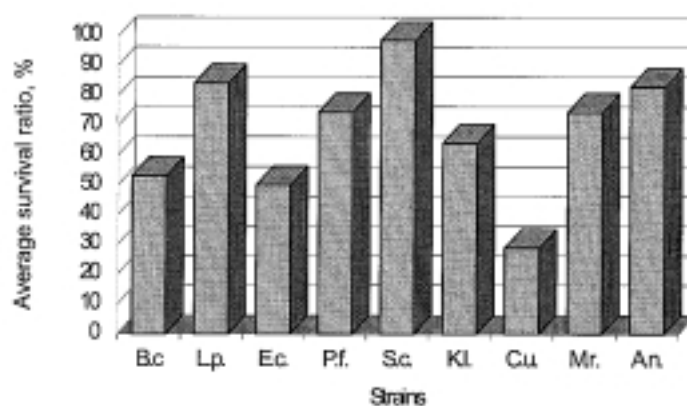

Fig. 2. The average survival ratio (calculated as the average of different treatments) of micro-organisms after freezing and thawing. B.c.: *Bacillus cereus*, L.p.: *Lactobacillus plantarum*, E.c.: *Escherichia coli*, P.f.: *Pseudomonas fluorescens*, S.c.: *Saccharomyces cerevisiae*, K.l.: *Kluyveromyces lactis*, C.u.: *Candida utilis*, M.r.: *Mucor racemosus*, A.n.: *Aspergillus niger*

Conclusions

Under the present experimental conditions the microbial cells from stationary growth phase showed usually higher survival ratios than cells from late exponential phase. This difference was significant in three cases out of the studied seven.

For four of the nine investigated strains horse serum and inositol proved to be better cryoprotectant than the skimmed milk + inositol combination.

The effect of freezing rate was different for the different microbes. As a consequence, if the aim is to achieve the possible highest survival rate, the above mentioned parameters should be optimised for each species.

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