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Bogotá, Colombia

Available in: http://www.redalyc.org/articulo.oa?id=49990204
STUDY OF THE STABILITY IN REAL TIME OF CRYOPRESERVED STRAIN BANKS

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ABSTRACT

The stability in real time of four strains cryopreserved in 10% v/v of glycerol was evaluated during a 6-month period. The strains studied were Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae and Aspergillus niger. The Master Cell Bank (MCB), cryopreserved at -70°C and -20°C, was activated using two thawing protocols, a fast one (F) and a slow one (S). A better cell recovery was achieved with the -70°C (F) protocol reaching a viability for Escherichia coli of 97.6% in the first 48 hours (p: 7.2x10^-4). The viability was retained in the 4th (p: 1.5x10^-5), 5th (p: 4.6x10^-3) and 6th months (p: 1.9x10^-3). Bacillus subtilis retained a viability of 92.5% after the 2nd (p: 4.7x10^-4), 4th (p: 1.76x10^-1), 5th (p: 3.4x10^-3) and 6th months (p: 3x10^-2). Saccharomyces cerevisiae demonstrated a viability of 95% in the second month (p: 9x10^-3) and for Aspergillus niger, the viability was 94.8%, in the first 48 hours (p: 7.2x10^-1) and after the 4th month (p: 2.79x10^-2). With the other protocols, -20°C F and S, some changes in viability were observed due probably to the formation of eutectic mixtures, nucleation and re-crystallization processes. The Master Bank microbial purity was maintained at 100% during the time of the study.

Key words: cryopreservation, eutectic, thawing, freezing.

RESUMEN

Se evaluó durante 6 meses la estabilidad a tiempo real de cuatro cepas criopreservadas en 10% v/v de glicerol; Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae y Aspergillus niger. Los Bancos de Células Primarios (BCP) fueron crioconservados a -70°C y -20°C; temperaturas que se combinaron con dos protocolos de descongelación rápido y lento (R y L). El protocolo -70°C R permitió mayor recuperación, manteniendo la viabilidad de Escherichia coli en 97.6%, a las 48 horas (p: 7.2x10^-3), a los 4 meses (p: 1.5x10^-5), 5 meses (p: 4.6x10^-3) y a los seis meses (p: 1.9x10^-3). Bacillus subtilis reportó viabilidad de 92.5%, a los dos meses (p: 4.7x10^-4), 4 meses (p: 1.76x10^-1), 5 meses (p: 3.4x10^-3) y a los 6 meses (p: 3x10^-2); Saccharomyces cerevisiae reportó 95% de viabilidad a los 2 meses de evaluación (p: 9x10^-3) y Aspergillus niger 94.8%, a las 48 horas (p: 7.2x10^-1) y a los 4 meses (p: 2.79x10^-2). Los otros protocolos -20°C R y L generaron cambios en la viabilidad probablemente a causa de la formación de mezclas eutécicas y el desarrollo de los procesos de nucleación y recristalización. La pureza microbiana de todos los bancos se mantuvo en 100% durante el tiempo de estudio.

Palabras clave: crioconservación, eutética, congelación, descongelación.
INTRODUCTION

A Cell Bank (CB) is a homogenous, microaerobic pure culture; stored under conditions that assure microbial viability and genetic stability (Amador, 1994). Microbial conservation systems in general must restrain microbial death, avoid adventitious agents and prevent changes in the biochemical and morphologic characteristics, nucleotide sequence and plasmid stability. In the case of metabolite producing strains, the biological activity and/or potency of the metabolite of interest (Kirsop, 1984), must be preserved. There are plenty of methods described for microbial conservation (Kirsop 1984; Simione 1991; Gherna 1994; Demain 1996; Hunter-Cevera 1996; NUNC 2000). The selection of the method depends on the economic resources of each laboratory. The following criteria must be consider for the selection; viability, purity, genetic stability, process costs, amount of culture (for producing microorganisms) and the frequency of use (Kirsop 1980).

Industries that use microorganisms for primary or secondary metabolite production, or biological controllers or modifiers of compounds, must have suitable microbial preservation methods. The purpose of the present study is to achieve a practical methodology, based on experience that can follow up and assess at real time, the stability of a CB cryopreserved in 10% (v/v) glycerol. It is important to highlight that the proposed methodology may be use in the stability evaluation of the Cell Banks conserved by any other means, just by making small modifications in each case.

MATERIALS AND METHODS

Microorganisms: The following strains; Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae and Aspergillus niger, were obtained from Microbiology Research Laboratory, Development Division, Bavaria S. A. Bogota, D.C. The microorganisms were culture in Plate Count agar (casein peptone 5g/l; yeast extract 2.5g/l; D-glucose 1g/l; agar-agar 14g/l; pH 7.0 ± 0. 2) or YGC agar (yeast extract 5g/l; D-glucose 5g/l; cloranfenicol 0.1g/l; agar-agar 14.9g/l; pH 6.6 ± 0. 2) depending upon the case. After growth, the bacterial strains were characterized by API 20E and API 50CHB ® Biomeriux S.A.; the yeast by API 20CAUX ® Biomeriux S.A., and the filamentous fungus according to Barnett and Hunter, 1982.

Performance of Cell Banks: The strains were cultivated in 500mL shake flask, with 100mL of nutritive broth (NB), at the following conditions; Escherichia coli and Bacillus subtilis, were cultivated at 37°C, 150 r.p.m, pH 7.0 ± 0.2 during 20 and 28 hours respectively. Saccharomyces cerevisiae was cultivated in NB at 30°C, 200 r.p.m, pH 6.6 ± 0.2, during 27h (Pedersen 2000; Rodriguez 2001). Batch culture was done until exponential phase (log) was achieve; then the axenic culture were mixed with equal volume of fresh media supplemented with 20% (v/v) glycerol. The mixture once homogenized was divided in fractions of 1ml in cryopreservation flasks, and stored immediately at -20 and -70°C (Kirsop 1984; Simione 1991; Gherna 1994; Demain 1996; Hunter-Cevera 1996; NUNC 2000). Aspergillus niger spore suspension, was recovered from the solid media in NaCl 0.85% w/v by loosening it with glass spheres; then mixed with fresh media, aliquoted and conserved in the same way describe for the other microorganisms (Simione, 1991).

Thawing Protocols: Two different thawing protocols were evaluated; a slow (S), and a fast (F) procedure. In the S protocol the temperature of the stock was change from -70°C to -20°C in a gradual time table,
waiting 20 minutes between temperature changes. At 0°C the time period was 45 minutes. In the fast method (F) the stocks temperature was change from -70°C to 37°C in 1 minute. After the thawing, serial dilutions from the stocks were made, seeded on plate-count agar and YGC modified agar (Park, 1997).

**Thermal Stability:** The combination of the two storage temperatures and the thawing protocols described previously (-70°C S, -70°C F, -20°C S and -20°C F) were determined monthly by cfu/mL, starting after 48 hours of storage and during the next 6 months. In addition for each microorganism the purity, the macroscopic and microscopic characteristics were also determined.

**Statistical Analysis:** The viability (cfu/mL), the recovery percent (%) and the microbial purity was measure in a unit of 10 stocks randomly selected from each CB and from each evaluation period. To determine the treatment differences, a variance analysis was made (ANOVA) using a completely randomized block design. All analysis were carry out with a confidence interval (CI) of 95%. The normality of the variables was verified by the Shapiro Wilk test. The Bonferroni test was carry out (α=0.05) for the detection of differences between the average viability of each thawing protocol. The analyses were made in Stata 6.0.

**RESULTS AND DISCUSSION**

**Cultures Preparation:** In order to increase the recovery of viable cells, the culture must be in optimal conditions, before exposing the microorganisms to low temperatures. Starting from batch cultures and spore recovery, suitable cellular concentrations are needed to initiate the conservation process (Table 1.); data that agrees with the reported by, Kirsop, *et al.*, 1984; Kirsop, 1980; Demain, *et al.*, 1996; Hunter-Cevera, *et al.*, 1996; NUNC 2000. Literature reports suggest that the cellular concentration must be between 10^6 and 10^7 cfu/mL and/or conidia/mL. Because the microscopic and macroscopic characteristics did not change, indicating that environmental conditions and media culture favored growth.

**Curves of Thermal Stability:** *Escherichia coli* results are shown in figure 1. The recovery percentage for the -70°C F protocol was 97.6% with a 95% of confidence interval (CI), thus indicating a recovery of 88-100%. The recovery for the -70°C S treatment was 95.2%, or else 82-100%. Statistical analysis shows that the *Escherichia coli* recovery with the -70°C F treatment was higher during the first 48 hours (p: 7.2x10^-4); fourth (p: 1.5x10^-4), fifth (p: 4.6x10^-4) and sixth month later (p: 1.9x10^-2). The storage at -70°C with fast thawing procedures provides better recovery conditions. The Master Cell Banks (MCB) stability of genetically modified *Escherichia coli* used in recombinant human alpha-interferon (α-INFhr) production, was evaluated by Amador *et al.*, (1994) maintained in glycerol

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Cell Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>15x10^7 cfu/mL</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>30x10^7 cfu/mL</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>95x10^4 cfu/mL</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>2.1x10^7 conidia/mL</td>
</tr>
</tbody>
</table>

100% of recovery. For the -20°C F treatment was 96.4%, a 85-100% of recovery and for the -20°C S treatment was 95.8%, a recovery of 82-100%. Statistical analysis shows that the *Escherichia coli* recovery with the -70°C F treatment was higher during the first 48 hours (p: 7.2x10^-4); fourth (p: 1.5x10^-4), fifth (p: 4.6x10^-4) and sixth month later (p: 1.9x10^-2). The storage at -70°C with fast thawing procedures provides better recovery conditions. The Master Cell Banks (MCB) stability of genetically modified *Escherichia coli* used in recombinant human alpha-interferon (α-INFhr) production, was evaluated by Amador *et al.*, (1994) maintained in glycerol
30% (v/v) and stored at -70°C during 5 years. As a result great stability was achieved, maintaining the population year after year over 10^8 cfu/mL (Amador, 1994; Poutou, 1994).

Figure 2 shows Bacillus subtilis results, indicating that -70°C F treatments gives 92.50%, corresponding to a 86-96% of recovery as compared with the -70°C S treatment, which has a 89.31%, or else a 82-95% of recovery. Conversely, 3x10^7 ufc/mL was the cell concentration that decreases logarithmically getting 85.47%, a recovery of 78-91%. Treatments of -20°C F and -20°C S gave 84.7% and 76-91% of recovery respectively; demonstrating that -70°C F treatments gives a greater recovery percentage. During the study period, Bacillus subtilis under the -70°C F treatment, at all times showed a higher recovery; after two (p: 4.7x10^-4), four (p: 1.76x10^-2), five (p: 3.4x10^-3) and six (p: 3x10^-2) months. Under the -20°C F treatment the best recovery appeared after two months of preservation (p: 2.9x10^-2). These results differ from the ones reported by Simione and Brown, 1991, which demonstrated that spore forming Gram positive bacteria are recovered in greater proportion than Gram negative. However, the outcome could be charge on the mechanical freezers storage at -20°C, because they can cause formation of eutectics mixtures, generating a viability loss. Nonetheless, this type of storage conditions can favor the cellular growth in conservation; as demonstrated by Rivkina et al., 2000 that metabolic activity of stored bacteria at temperatures between -5 and -20°C, may have a logarithmic phase from 200 to 350 days, followed by the lag phase where the substrate availability is limited.

The behavior of the yeast was stable during the storage time under the different treatments (Figure 3). Under -70°C F and -70°C S treatments the percentages achieve was 95.1%, and 94.9% respectively, a recovery of 91-99%. Treatment under -20°C F, achieve 94.7% and -20°C S, 94.50%, both between 89-98% of recovery. Similarly, the statistical analysis points out that the are no differences in the recovery of Saccharomyces cerevisiae between the treatments, even though under the -70°C F treatment, a higher viability was observed after two months of preservation (p: 9x10^-3). The same occurred after three months with the -20°C F (p: 4x10^-2) treatment.

Recovery percentages, assures that under the evaluated methods the viable cellular population is maintained near initial concentrations (Figure 3). These results agree with the reports of Ferreira et al., 1992, where several methods are compare as subcultures; the distilled sterile water conservation, liofilization and cryopreservation in liquid nitrogen. The best results were with cryopreservation having a range between 80-100% of viability; with cellular populations between 10^8 and 10^7cfu/mL. Lewis et al., 1994 compared different alcohols and carbohydrates like cryoprotectant agents for the freezing of diverse yeast types, establishing that glycerol was good enough as cryoprotectant allowing a high cellular recovery, as long as a fast thawing protocol is used.

William and Tamara, 1985 studied transformed Escherichia coli, Bacillus subtilis and Saccharomyces cerevisiae with replicatives plasmids, and followed their behavior in liofilization and cryopreservation procedures. In Escherichia coli was demonstrated that both methods maintained a stable viability and plasmid stability. Bacillus subtilis was preserved in a vegetative and sporulated form, demonstrating that both forms can be recovered in high proportion when cryopreservation was used; unlike liofilization, that favored the recovery of
sporulated form in greater proportion than the vegetative form. Similarly, *Saccharomyces cerevisiae* maintained better viability and plasmid stability at freezing temperatures after nine months of storage.

Figure 4 shows *Aspergillus niger* cellular recovery percentage. The storage temperatures and thawing protocols in this type of organisms did not exhibit significant differences in the recovery percentages. The values were 94.83%, a recovery of 89-98% when stored at -70°C F and -70°C S. Under -20°C F treatment the value was 94.85%, a recovery of 89-98% and under -20°C S, the value was 93.66%, a recovery of 91-94%. The statistical analysis demonstrates that recovery under -70°C F protocol was higher in the first 48 hours ($p: 7.2 \times 10^{-4}$) and in the next four months ($p: 2.7 \times 10^{-2}$).

The strains behavior under the four protocols; storage effect (-20°C and -70°C) and thawing (F and S) effect, are compared in Figure 5 after six months of conservation. In general, -70°C F showed higher cellular recovery, *Escherichia coli* 96.7%, *Bacillus subtilis* 92.5%, *Saccharomyces cerevisiae* 95% and *Aspergillus niger* 94.8%. *Aspergillus niger* and *Saccharomyces cerevisiae* display a similar response to stress generated by the freezing process, with an average viability percentage in the four treatments, near 95%; in contrast with the prokaryote strains, which presented different answers to the treatments. For *Escherichia coli* it was established that treatment at -70°C F allows a higher recovery, without despising the values obtained for the other treatments. The results are within the rank established by Hunter-Cevera and Belt, 1996; were it is describe that a conservation method must guarantee a storage recovery of 90%; on the contrary, *Bacillus subtilis* vegetative cells, neither the -20°C F nor -20°C S treatments was favorable; showing a significant decrease in viability, a recovery of only 84.7% as compare with the treatment of -70°C F.

**CONCLUSIONS**

The present study demonstrated that for the microbial genus assayed, the slow thawing procedures, decreases cellular viability (recovery %); probably by facilitating the nucleation processes and increasing the existing crystals as a result of the conservation mixture eutectics points, causing irreversible injuries on the cellular membrane (Wolstenholme and O’Connor, 1970).

**REFERENCES**


Recibido: 29-10-2003
Aceptado: 9-06-2004

![Figure 1. Escherichia coli recovery percentage vs. storage time.](image-url)
FIGURE 2. *Bacillus subtilis* recovery percentage vs., storage time.

FIGURE 3. *Saccharomyces cerevisiae* recovery percent vs., storage time.
**Figure 4.** *Aspergillus niger* recovery percent vs. storage time.

**Figure 5.** Microorganisms recovery percent under the 4 different treatment after 6 months of conservation.