Ten year Storage of the freeze-dried *Mycobacterium* sp MB-3683 strain

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ABSTRACT

The mutant of *Mycobacterium* sp. MB-3683 employed in the biotransformation of sterols for the pharmaceutical industry was freeze-dried vacuum-sealed and stored for 10 years at 10 ± 2 °C and 30 ± 2 °C. The formulations for freeze-drying were A (2.4 g KH_2PO_4, 0.24 g $MgSO_4$. $7H_2O$, 0.6 g magnesium citrate, 12 ml glycerol, 3.6 g L-asparagine, 20 ml malachite green solution (2%), distilled water until 1L), B (10% skim milk) and C (5% sucrose + 5% sodium glutamate). The freezing temperatures were –20 °C and –196 °C, the primary drying was carried out for 18 h with a product temperature of –20 °C. During the secondary drying, the product temperatures were maintained at 20 °C for 3 h and 20 °C for 8 h (2^2 factorial design). The results indicate that freezing at –20 °C and secondary drying at 20 °C for 8 h were the best conditions for freeze-drying the strain regardless the protective medium employed. The ability to produce androstenedione (AD) during sterol biotransformation was not affected.

Key Words: freeze-drying, mycobacteria, sterol biotransformation

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RESUMEN

Micobacterial strain freeze-dried for ten years. El mutante de *Mycobacterium* sp. MB-3683 que se emplea en la biotransformación de fitosteroles para obtener esteroles de interés farmacéuticos se liofilizó y se almacenó durante 10 años a 10 ± 2 °C y 30 ± 2 °C. Tres formulaciones de protección fueron estudiadas: A (KH_2PO_4 2.4g, $MgSO_4$. $7H_2O$ 0.24g, citrato de magnesio 0.6g, glicerol 12ml, L-asparagina 3.6g, solución de verde malaquita (2%) 20ml, agua destilada 1L), B (leche descremada 10%) y C (sacarosa 5% + glutamato de sodio 5%). Las temperaturas de congelación fueron –20 °C y –196 °C, el secado primario duró 18 horas con una temperatura del producto de –20 °C. Durante el secado secundario, la temperatura del producto se mantuvo a 20 °C durante 3h y 8h (diseño experimental 2^2). Los resultados indican que la congelación a –20 °C y el secado secundario a 20 °C por 8h fue la mejor condición para liofilizar la cepa independientemente del medio de protección utilizado. La producción de androstendiona (AD) a partir de los fitosteroles no se afectó después de almacenada la cepa por 10 años.

Palabras claves: liofilización, micobacteria, biotransformación de esteroles

*I*ntroduction

Freeze-drying exhibits more practical applications than any other microorganism preservation method. However, there are some species of *Mycobacterium* that may prove difficult to freeze-dry resulting in relatively poor survival rates [1]. Also, if the storage period of the strain is very long, viability could be significantly affected [2].

Certain factors can affect the viability of freeze-dried microorganisms. Among them: the nature of the strain, growing conditions, growth phase, cell concentration, formulation composition, freezing and freeze-drying parameters, rehydratation method, etc [3].

An adequate formulation, one of the most important aspects, is very difficult to establish given the lack of information on the protective mechanisms of additives. In addition, each biological material has its own behavior. Besides, the composition of the lyophilization medium determines the thermophysical characteristics of the system and imposes the operational parameters during the freeze-drying process [4].

There are certain substances which are commonly used to freeze-dry microorganisms such as skim milk [3-6] and sodium glutamate [3, 4, 7]. Other compounds as peptones, inositol, lactose and sucrose have also given successful results.

The mutant *Mycobacterium* sp. MB-3683 degrades the side-chain of sterols, hence, it is used to biotransform these substrates to obtain 1,4-androsten-3,17-dione (AD) as the main product [8-10].

The aim of this study was to select an adequate freeze-drying medium and the conditions of the sublimation phase that can maintain a high survival level in this strain after lyophilization for a long time.

*M*aterials and Methods

Microorganism

Mycobacterium sp. MB-3683 was maintained on enriched nutrient Agar with glycerol (2%, w/v).

Freeze-drying conditions

The bacteria were propagated in a nutrient broth (OXOID) at 30 ºC on an orbital shaker (200 r/min) until reaching 2/3 of the exponential phase and 10^{10} cfu.ml^{-1}. Cells were harvested by centrifugation at 7000 x g for 10 min. and washed twice with distilled water. The pellet was suspended in 10 ml of the following variants of the protective media: A (composed of 2.4 g KH_2PO_4; 0.24 g $MgSO_4$. $7H_2O$; 0.6 g magnesium citrate; 12 ml glycerol; 3.6 g L-asparagine; 20 ml malachite green solution (2%); distilled

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water until 1l), B (skim milk, 10%) and C (sucrose 5% + sodium glutamate 5%). Aliquots of 0.2 ml of cell suspensions were dispensed in sterile cotton-plugged ampoules. All the samples were sublimated in a SERAIL RP 1.5 freeze-drier (France) using a plating temperature of –20 ºC and a pressure chamber of 100 microns for 18 h. The effect of the freezing temperature and secondary drying conditions on survival were analyzed through a 2^2 factorial design (Table1).

A group of ampoules was stored for 10 years at 10 ± 2 ºC and 30 ± 2 ºC.

Table 1. Freeze-drying conditions for each type of protective medium.

Experiments	Freezing temperature (X_1)	Secondary drying (X_2)
1	-1 (-196 ºC)	-1 (3 h at 20 ºC)
2	1 (-20 ºC)	-1
3	-1	1 (8 h at 20 ºC)
4	1	1

Estimating viability

The number of colony-forming units (cfu.ml^{-1}) was determined using the Agar plate method initially, after freeze-drying and after 10 years of storage at 10 ± 2 ºC and 30 ± 2 ºC. Three replicate plates were spread for each dilution in the Agar nutrient medium. The freeze-drying ampoules (tripled) were hydrated using 0.1 ml of distilled water for 15 min.

Biotransformation

For each variant, three colonies obtained after estimating of viability were suspended in a Conner medium [11]. The cultures were incubated at 30 ºC and 200 rpm for 48 h. Then, they were centrifuged at 7000 x g for 10 min. and the pellets were washed with sterile Tween 80 solution (0.7%, w/v) and centrifuged again under similar conditions.

Each biomass was suspended in the sterile Tween 80 solution and homogenized by shaking. A synthetic medium with glycerol at 1% [12] and a cholesterol suspension [10] was inoculated to obtain a cellular concentration corresponding to 2.5 x 10^{10} cfu.ml^{-1}. The cultures were incubated at 30 ºC, 200 rpm for 5 days.

The method employed for steroid determination was that described previously by Borrego et al. [13].

Evaluating the experiments

Survival after 10 years of storage was analyzed by the ANOVA-1 and Duncan tests [14].

Results and Discussion

Table 2 shows the effect of different formulations and freeze-drying conditions. Before freeze-drying the bacterial concentration was 2.5 x 10^{10} cfu.ml^{-1} and after lyophilization the survival in all cases was reduced. Nevertheless, the results depended on the formulation composition. For this bacterium the best results were found in medium A, where the lowest losses of survival were obtained. Although, this medium has glycerol as the carbon source, this substance appears to be a good additive for this strain. Tsukamura [2] reported that glycerol is a good additive to preserve the mycobacteria. Actually, this compound is widely used in the formulation of many protective media for freeze-drying different microorganism because it is a good preservant [15].

Table 2. Effect of formulations and freeze-drying conditions on the survival of strain *Mycobacterium* sp. MB-3683.

	Medium A		Medium B		Medium C	
Experiment	cfu. mL 1 after freeze drying*	ΔS ± SD*	cfu. mL 1 after freeze drying*	ΔS ± SD*	cfu. mL 1 after freeze drying*	ΔS ± SD*
1	2.8 x 10^9	0.95 ± 0.01	6.2 x 10^8	1.60 ± 0.09	3.0 x 10^8	1.92 ± 0.10
2	5.0 x 10^9	0.69 ± 0.01	4.2 x 10^7	2.77 ± 0.12	3.1 x 10^7	2.64 ± 0.15
3	5.4 x 10^9	0.66 ± 0.02	3.2 x 10^8	1.89 ± 0.12	3.1 x 10^8	1.90 ± 0.09
4	7.3 x 10^8	1.53 ± 0.13	3.5 x 10^7	2.85 ± 0.08	2.5 x 10^7	3.00 ± 0.16

* = Each result represents the mean of three experiments

ΔS = log Cb – log Ca

SD = standard deviation

Cb = cfu.ml^{-1} before freeze-drying

Ca = cfu.ml^{-1} after freeze-drying

Note: Before freeze-drying the bacterial concentration was 2.5 x 10^{10} cfu.ml^{-1}

A polynomial was obtained for each freeze-drying medium indicating the relative influence of the freeze-drying parameters (X_1 and X_2) on survival (Y):

(1) $Y_A = 0.95 + 0.15X_1 + 0.13X_2 + 0.28X_1X_2$

(2) $Y_B = 2.27 + 0.53X_1 + 0.09X_2 + 0.05X_1X_2$

(3) $Y_C = 2.36 + 0.45X_1 + 0.08X_2 + 0.09X_1X_2$

The polynomials obtained indicate that freezing (X_1) at -20 ºC and a secondary drying (X_2) of 8h at 20 ºC were the best conditions for freeze-drying this strain regardless the protective medium employed.

The ability of the strain to biotransform the cholesterol after freeze-drying was evaluated (Table 3). The yields of AD and ADD were similar to the control regardless the survival obtained in the different formulations. Hence, the freeze-drying did not affect the mutation of the strain.

After storing the mutant for 10 years at 10 ± 2 ºC the survival was very good regardless the protective medium used (Tables 4 and 5) The loss of viability in all cases was less than one potency. These results contrast with those of Tsukamura [2], who reported that viability is maintained for more than two years when storing lyophilized cultures at –20 ºC. But if storage is at room temperature, viability could be affected after one month. In general, significant differences were observed with the highest protection obtained in medium B (skim milk), with the exception of experiment 3. Nevertheless, medium **A** has continuously been the best protector with the highest viability in all experiments

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Table 3. Production of steroidal precursors after freeze-drying the mutant *Mycobacterium* sp. MB-3683 under different conditions and protective media.

	Medium A		Medium B		Medium C	
Experiment	Y (AD) (%)	Y(ADD) (%)	Y (AD) (%)	Y(ADD) (%)	Y (AD) (%)	Y(ADD) (%)
1	37.3 ± 1.0	1.5 ± 0.2	38.1 ± 1.2	1.2 ± 0.3	38.2 ± 1.5	1.1 ± 0.1
2	37.8 ± 1.2	1.3 ± 0.2	37.4 ± 1.3	1.4 ± 0.2	37.5 ± 1.2	1.4 ± 0.1
3	38.1 ± 1.5	1.3 ± 0.1	37.5 ± 1.0	1.1 ± 0.1	38.3 ± 1.5	1.2 ± 0.3
4	38.4 ± 0.9	1.4 ± 0.3	38.5 ± 1.4	1.3 ± 0.3	38.5 ± 1.1	1.1 ± 0.1
Control *	38.5 ± 1.3	1.4 ± 0.3				

* - Fresh culture

Y = (mg precursor obtained/mg cholesterol added) x 100

AD – Androstendione

ADD – Androstadienedione

Note: Each value of Y(AD) or Y(ADD) is the means of three replicates.

Table 4. Survival of the mutant *Mycobacterium* sp. MB-3683 after 10 years of storage at 10 ± 2°C.

	Medium A			Medium B			Medium C		
Exp.	cfu. mL 1 after freeze drying (0 h)	cfu. mL 1 10 years storage	ΔS ± SD*	cfu. mL 1 after freeze drying (0 h)	cfu. mL 1 10 years storage	ΔS ± SD*	cfu. mL 1 after freeze drying (0 h)	cfu. mL 1 10 years storage	ΔS± SD*
1	2.8×10^{9}	1.5×10^{9}	0.28 ± 0.02 (c)	6.2×10^{8}	4.6×10^{8}	0.13 ± 0.01(hi)	3.0×10^{8}	2.0×10^{8}	0.18 ± 0.02 (g)
2	5.0×10^{9}	3.1×10^{9}	0.20 ± 0.01 (f)	4.2×10^{7}	3.2×10^{7}	0.12 ± 0.02 (ij)	3.1×10^{7}	3.7×10^{7}	0.19 ± 0.01 (fg)
3	5.4×10^{9}	3.2×10^{9}	0.23 ± 0.03 (e)	3.2×10^{8}	1.6×10^{8}	0.30 ± 0.04 (b)	3.1×10^{8}	1.1×10^{8}	0.45 ± 0.03 (a)
4	7.3×10^{8}	4.7×10^{8}	0.19 ± 0.01(fg)	3.5×10^{7}	2.7×10^{7}	0.11 ± 0.01 (j)	2.5×10^{7}	1.4×10^{7}	0.25 ± 0.01 (d)

* - Each value is the mean of three results

ΔS = log Cb – log Ca

SD = standard deviation

Cb = cfu.ml^{-1} before freeze-drying

Ca = cfu.ml^{-1} after freeze-drying

Note: Before freeze-drying the bacterial concentration was 2.5×10^{10} cfu.ml^{-1}.

The values (b), (c), (d), (e), (f), (g), (fg), (hi), (ij) and (j) are significantly different from (a) (Test of Duncan, $p \leq 0.05$).

Table 5. Survival of the mutant *Mycobacterium* sp. MB-3683 after 10 years of storage at 30 ± 2°C.

	Medium A			Medium B			Medium C		
Exp.	cfu. mL 1 after freeze drying (0h)	cfu. mL 1 10 years storage	ΔS ± SD*	cfu. mL 1 after freeze drying (0h)	cfu. mL 1 10 years storage	ΔS ± SD*	cfu. mL 1 after freeze drying (0h)	cfu. mL 1 10 years storage	ΔS ± SD*
1	2.8×10^{9}	1.3×10^{8}	1.33 ± 0.12 (cd)	6.2×10^{8}	7.7×10^{7}	1.25 ± 0.09 (d)	3.0×10^{8}	1.5×10^{7}	0.80 ± 0.05 (ef)
2	5.0×10^{9}	2.5×10^{8}	1.30 ± 0.10 (cd)	4.2×10^{7}	5.3×10^{6}	1.30 ± 0.11(cd)	3.1×10^{7}	2.1×10^{6}	0.77 ± 0.02 (ef)
3	5.4×10^{9}	2.4×10^{8}	1.35 ± 0.09 (c)	3.2×10^{8}	6.3×10^{7}	1.20 ± 0.08 (d)	3.1×10^{8}	2.0×10^{7}	0.86 ± 0.05 (e)
4	7.3×10^{8}	3.4×10^{7}	2.33 ± 0.16 (a)	3.5×10^{7}	7.5×10^{6}	1.43 ± 0.09 (b)	2.5×10^{7}	1.3×10^{6}	0.74 ± 0.01 (f)

* - Each value is the mean of three results

ΔS = log Cb – log Ca

SD = standard deviation

Cb = cfu.ml^{-1} before freeze-drying

Ca = cfu.ml^{-1} after freeze-drying

Note: Before freeze-drying the bacterial concentration was 2.5×10^{10} cfu.ml^{-1}.

The values (b), (c), (cd), (d), (e), (ef) and (f) are significantly different from (a) (Test of Duncan, $p \leq 0.05$).

(compared with survival before freeze-drying). These results indicate that the glycerol present in the medium could be responsible for preserving the freeze-dried mycobacteria mutant for a long time.

The ability of the freeze-dried strain to biotransform the cholesterol was the same after 10 years of storage at 10 ± 2 ºC and 30 ± 2 ºC (Table 6). It means that the protective media employed have the proper additives to preserve the *Mycobacterium* mutant for a long time, which is mutationally very stable in time. Similar results were obtained [6] with other mycobacteria strains.

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Table 6. Production of AD after 10 years of storage at 10 ± 2°C and 30 ± 2°C of the mutant *Mycobacterium* sp. MB-3683.

	Medium A			Medium B			Medium C		
Exp. *	Y(AD) after freeze drying (%) ± SD	Y(AD) 10 years storage (%) ± SD 10°C	30°C	Y(AD) after freeze drying (%) ± SD	Y(AD) 10 years storage (%) ± SD 10°C	30°C	Y(AD) after freeze drying (%) ± SD	Y(AD) 10 years storage (%) ± SD 10°C	30°C
1	37.3 ± 2.1	37.8 ± 2.7	37.5± 2.3	38.1 ± 3.0	38.3 ± 2.7	38.0 ± 3.1	38.2 ± 3.0	38.5 ± 3.2	37.8± 2.8
2	37.8 ± 1.9	38.0 ± 1.8	38.3 ± 2.0	37.4 ± 2.8	38.0 ± 1.5	37.8 ± 1.9	37.5 ± 1.5	38.5 ± 2.7	38.3 ± 1.8
3	38.1 ± 2.0	37.5 ± 3.0	37.9 ± 2.7	37.5 ± 2.3	37.8 ± 3.1	38.1 ± 2.5	38.3 ± 2.6	37.5 ± 1.9	37.9 ± 2.0
4	38.4 ± 1.5	38.1 ± 2.5	38.0 ± 2.1	38.5 ± 2.0	37.9 ± 2.5	38.0 ± 2.0	37.5 ± 2.0	37.8 ± 2.2	37.4 ± 1.9

* - Each experiment was carried out three times.

SD = Standard deviation

$Y_{(AD)}$ = (mg AD obtained/mg cholesterol added) x 100

AD = Androstenedione

ADD = Androstadienedione

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