PRELIMINARY STUDIES REGARDING THE BEHAVIOUR OF SELECTED *STREPTOMYCES* STRAINS AS XYLANASES PRODUCERS PRESERVED BY LYOPHILIZATION

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Abstract: Lyophilization is an effective method for conservation of most bacteria and fungi started cultures. Through the combination of low pressures and low temperatures, the water has been removed until residual moisture of 1 - 3%. The aim of this study was to evaluate the behavior and stability of lyophilizated selected *Streptomices sp.* strains, selected by for xylanases producing, included in the collection of micro-organisms Bioaliment Research Platform (acronym MIUG). Behavior of freeze-dried cultures after reactivation varies depending on the nature and concentration of protective agents during lyophilization.

Keywords: *Streptomyces sp., xylanases, lyophilization, conservation stock cultures, collection of micro-organisms.*

INTRODUCTION

In biotechnological practice is necessary and useful to establish simple methods of microorganisms' conservation to ensure the maintaining of the initial metabolic activity. Thus, for each group of microorganisms is the most indicated to establish the proper conservation methods that improve the survival of the cultures over long periods of storage [1].

There have been developed several methods of conservation. Classical methods supposed microorganisms growing on specific media, followed by keeping them at low temperature (0 ... 40 °C) and assignment on fresh medium at regular intervals of time (replication method). Disadvantages of this method consist of increasing the risk of the contamination and loosing of the stability of the genetic characteristics. Cryopreservation assumes keeping microorganisms on temperatures in the freezing dominium in liquid nitrogen (– 196 °C). This is a very effective method of preservation, especially for bacteria, but is costly and additionally requires liquid nitrogen [3 – 4].

Lyophilization is the method of preservation most often used for storage and preservation of stock cultures, which is based on water removal by sublimation in a cycle of freezing and drying of the frozen cellular material. Lyophilization process involves three stages:

- *prefreezing*, which ensure obtaining of the "start" material frozen and solidified;
- *primary drying* - which removes most of the water;
- *secondary drying* – the bound water is removed about [1].

Primary drying involves water removing by sublimation from the frozen samples, a process facilitated by the presence of vacuum. Secondary drying ensures sample drying, residual moisture had to reach to 2 ... 3% to ensure a good preservation of the freeze-dried samples [2]. To get a good freeze drying, especially when it is pursue to keep intact the viability of bacteria need to be taken into account several factors such as protective substance used, the rate of freezing, the mode of drying, residual humidity, the method of conservation of the product after lyophilisation [4 – 5].

Taking into account that streptomycetes represents an important class of microorganisms with biotechnological potential for obtaining economic value products (antibiotics, enzyme preparations, etc.), finding effective methods of streptomycetes conservation represents a permanent interest for manufacturers. This study presents the researches regarding the assurance of preservation and metabolic stability of selected active streptomyces cultures producing xylanases, through qualitative and quantitative variation of the protective medium.

MATERIALS AND METHODS

Microorganisms

Streptomyces sp. P7-72, *Streptomyces sp*. P11-118, *Streptomyces sp.* P12-137 strains isolated from soil in the period November 2007 – March 2008, selected as active producers of xylanases.

Chemicals

Cryoprotective agents: 10% glycerol, 20% skim milk, 10% polyethyleneglycol (PEG), 10% gelatin. Solutions were prepared in 0.9% NaCl solution and then were sterilized at 121°C, for 20 min.

Growth conditions of streptomycetes

Growing medium used for multiplication and preservation of pure cultures had the following composition (%): potato starch 1.0; yeast extract 0.25; meat extract 0.5; peptone 1.0; $MgSO_4 \cdot 7H_2O$ 0.05; $CaCl_2 \cdot 6H_2O$ 0.005; NaCl 0.5; agar 1.5. In mature cultures, the air mycelium was mixed with 0.9% NaCl solution and then cells (vegetative and spores) were separated by sedimentation for 2 hours at –4 °C.

Lyophilisation conditions: Lyophilisation has been achieved on a LD Plus Alpha 1-4 Lyophilisator (Martin Christ, Germany), with a capacity of condensing maximum 4 kg/24 h, room temperature of with condensation around –25 °C and a sublimation temperature in the range of –55 … 85 °C. Separated cells were mixed in equal proportions with cryoprotective agent (v/v). After homogenization from suspension were extracted 0.1 – 0.3 mL to test viability. Cells suspension and cryoprotective agent were distributed in sterile glass vials in doses of 2 – 5 cm^3 frozen during 30 minutes in ultrafreezer (–70 °C), after that lyophilisated, in the following conditions: primary drying at –42 °C, for 100 hours; secondary drying at –20 °C, for 20 min. Lyophilisation was considered complete when the suspension of cells turned into a white or slightly yellow powder depending on the pigments produced by the mycelium. Lyophilisates were stored at temperatures between 0 ... 40 °C until further use.

Lyophilisates cultivation in submerged conditions

Cultivation of lyophilized cultures was done on the medium described above, without agar, on the orbital shaker at 28 °C, 150 rpm for 120 hours. Biomass separation was achieved by centrifugation at 10000g, at the temperature of –4 °C, for 15 min.

Assessing streptomycete cultures biological stability after lyophilisation

Before and after lyophilisation was checked the cultures behavior by assessing the capacity of multiplication and formation of biomass on Gauze agar medium, by semi quantitative evaluating of the average surface area of colonial development after 120 hours on the stationary cultivation at temperature of 28 °C. To appreciate the growth potential, considering a standard area of possible colonies expansion of 4469 mm^2, the following system of assessment was used:

+ + + colonies occupied the entire surface (4469 mm^2) - score 4;
+ + - colonies occupied 2/3 of surface (2979 mm^2) - score 3;
+ - - colonies occupied 1/2 of surface (2235 mm^2) - score 2;
+/- - - less than 1-2 colony have grown (< 500 mm^2) - score 1;
- - - absence of colonial development - score 0.

RESULTS AND DISCUSSION

Analyzing the influence of the type of protective agents on streptomycetes viability and vitality preserved by lyophilisation were obtained the results presented in Figure 1.
As can be seen in Figure 1, using 20% skim milk as cryoprotective mixture ensures the best level of protection of cells in case of all three selected strains streptomycetes, followed by 10% glycerol and 10% polyethylene glycol.
Protective effect varies depending on the strain and type of cryoprotective agent, fact which leads the idea that for each strain treatment conditions should be optimized to ensure the best conditions for high viability and biotechnological conservation of the cultures preserved by lyophilisation.

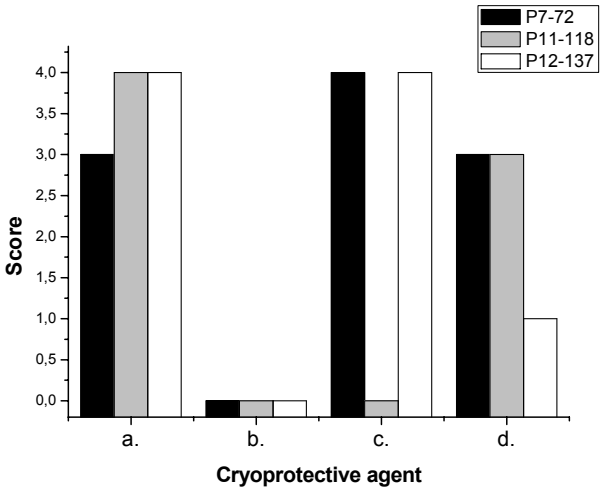

Figure 1. *Protective effect of various cryoprotective agents on the streptomycetes viability and vitality*
a. 20% skim milk; b. 10% glycerol; 10% gelatine; 10% polyethylen glycol (PEG)

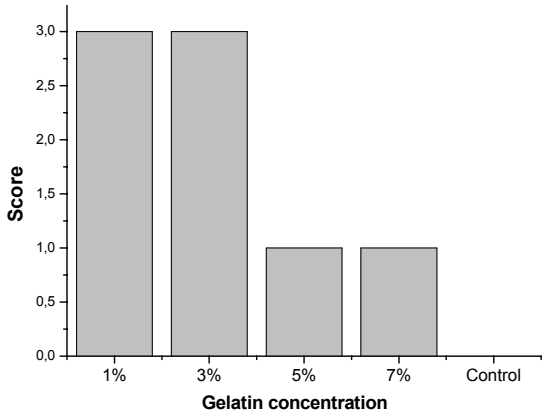

Figure 2. *Effect of the gelatin on lyophilisates streptomycetes (strain Streptomyces sp. P12-137)*

Streptomyces sp. P11-118 has not survived to lyophilisation process when gelatin was used as a cryoprotective agent. An increased percentage of viable cells has been obtained when using 10% polyethylene glycol (Figure 1c).
The best results were obtained in the case of the strain *Streptomyces sp.* P12-137 when used in 1% and 3% gelatin concentration (Figure 2).
When 5% skim milk was used, has proven effective in maintaining viability after lyophilisation for the strain tested compared with control (Figure 3).

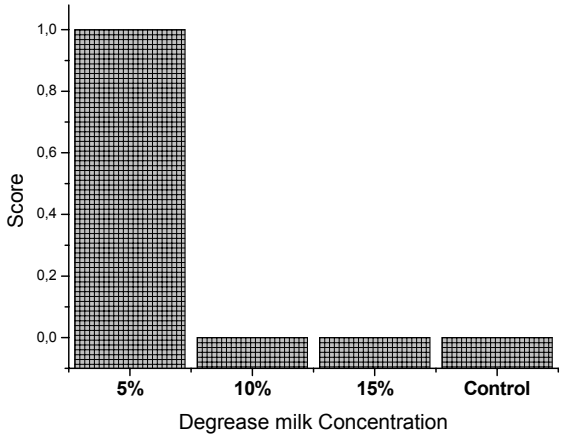

Figure 3. *Influence of the concentration of skim milk as cryoprotective agent on strain Streptomyces sp.P12-137*

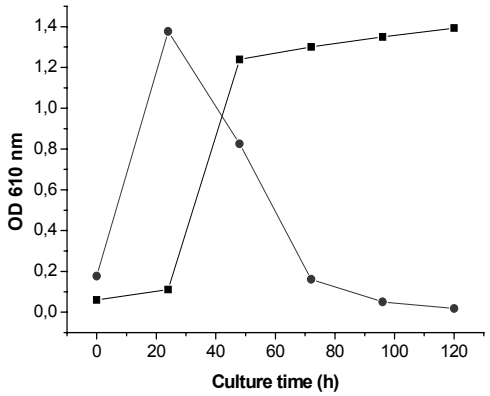

Figure 4. *Dynamic grow in submerged cultures of the strain Streptomyces sp. P12-137 before (■) and after lyophilisation (●)*

The comparative study regarding dynamics of multiplication before and after lyophilisation shows obvious changes in the culture behavior (Figure 4). These results confirm that microorganisms are subjected to the heat stress during preservation procedures by freeze drying and require a reactivation on rich medium.

CONCLUSIONS

Streptomycetes conservation by freeze drying is an appropriate method recommended to preserve genetic and metabolic characteristics of commercial starter cultures.
In the present study, cryoprotective agents such as skim milk, gelatin and PEG have proven effective in conserving strain of some *Streptomyces*, while glycerol has not proved effective in protecting streptomyces.
Treatment which the cells are subject to during lyophilisation changed physiological behavior, event what is explained by the stress of thermal and baric exerted on the process during the operation.
The study offers some useful preliminary data for future research that will pursue the optimization of lyophilisation parameters and assess behavior and freeze-dried stability cultures during storage.

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