

LONG-TERM PRESERVATION OF VITALITY AND LIGHT EMISSION OF LUMINOUS BACTERIAL STRAINS

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Summary

It was established that lyophilization and cryogenic method were the most appropriate ones for preservation of luminous bacteria without changes in their morphology and properties. The strains kept their viability during a two years observation period. The reactivation of luminous bacteria, which were preserved under liquid paraffin, led to the restoration of their vitality and ability for light emission only in the course of 1 to 5 months.

Introduction

It is well known that lyophilization and cryogenic method are applied for the long-term preservation of bacteria without significant changes in their morphology and properties [2, 10]. The strains maintain their viability for decades [9, 10]. There are no literature data about the application of these methods for luminous bacteria. The long-term preservation of luminous cultures without a loss of their basic property (light emission) remains still a problem with no optimum solution. The investigation of different strains of luminous bacteria isolated from the Pacific Ocean show that their retaining is possible if they are periodically resolved at every 7 days [11]. Luminous strain isolated from the Straits cultured on the optimal medium has lost its viability for 10 to 15 days [6].

The continuous cultivation of luminous

bacteria created conditions for accumulation of dark mutants with selective advantages [3]. The luciferase synthesis is delayed during the early exponential phase and is sharply increased during the late exponential growth phase [5, 7, 8]. There are two factors related with this process. The first one is the luciferase inhibitor, contained in the culture media. The second is the low concentration of accumulated in the medium autoinductor, which stimulates the synthesis of the luminous system at transcriptase level [1, 4, 7].

The aim of the present study was to settle the conditions for the continuous preservation of viability and light emission of luminous bacteria by the application of lyophilization and cryogenic methods, as well as placing them under liquid paraffin.

Materials and Methods

Bacterial strains. The standard strains were a gift from Prof. Makemson (International University, Florida): *Vibrio harveyi* MAW, *V. harveyi* B-392, *Photobacterium fischeri* WH69, *Shewanella* MS32. Strains isolated by us from the Black Sea aquatoria, cape Galata and resort St. Konstantin, were *V. harveyi* G and *V. harveyi* SK.

Culture media. Glycerol Marine Agar (GMA) containing (g/l): glycerol - 3 ml, peptone - 5, yeast extract - 1, NaCl - 0.25 M, MgSO₄ - 0.025 M, MgCl₂ - 0.025 M, KCl - 0.010 M, agar - 15, was used for strains *V. harveyi* and *Ph. fischeri*. The medium Marine Agar (MA) consisted of (g/l): peptone - 5, yeast extract - 1, NaCl - 0.25 M, MgSO₄ - 0.025 M, MgCl₂ - 0.025 M, KCl - 0.010 M, agar - 15, was used for the strain *Shewanella* MS32.

Protective media. The liquid paraffin was sterilized at 120°C, 1 atm., for 20 min. The medium – 10% sucrose, 1.5% gelatin, 0.1% agar or 5.5% dried skim-milk, was applied when the cultures were suspended for lyophilization. For cryogenic treatment, 10% glycerin (final concentration) was used.

Cultivation and preservation methods.

The investigated strains were cultivated at 25°C. In one of the variants, the cultures developed on straight agar were conserved by covering with sterile liquid paraffin (1 cm layer height). The samples were stored at 4 - 8°C.

Parameters for the vacuum sublimation drying were as follows. The freezing of the samples was performed at a cooling speed of 1°C.min⁻¹. In order to keep the sublimation temperature in the range from minus 28° to 32°C the pressure in the lyophilization chamber should be adjusted to 15 Pa and the sublimation plates should be warmed at a speed of 0.3°C.min⁻¹ up to 40°C. Secondary drying was realised at sample's temperature 22°C, vacuum 1 - 2 Pa for 5 - 6 hours. The ampoules were closed under vacuum and stored at 4 - 8°C.

For the strains kept in liquid nitrogen, the freezing was carried out at a speed of 10°C.min⁻¹ until minus 196°C.

The strains preservation was recorded every two hours by the intensity of light emission visually observed in a dark room, after eye adaptation for approximately 10 min [6].

Results and Discussion

Taking into account the peculiarities needed for continuous cultivation we selected the optimal period (late exponential phase maximal accumulation of autoinductor and elimination of the inhibitory action) for cryogenic preservation and lyophilization of the strains, as well as for their placing under liquid paraffin. In this period - from 10th to 15th hour, the highest emission was established. The strains preserved under paraffin in the course of 1, 2, 3, 4, 5 months, fully restored

their ability to emit light after cultivation on appropriate medium. On the 6th month restoration of viability and light emission was observed only for *Shewanella*, while *Ph. fischeri* M32, *V. harveyi* MAW and *V. harveyi* B392 grew well but were not able to light, most probably due to the higher survival rate of the accumulated dark mutants. On the 10th, 14th and 18th month the result were not reproducible, concerning also *Shewanella* 32 strain (Table 1).

Table 1. Preservation of the investigated strains by different conservation methods.

Strain	Methods	Preservation (months)								
		1	2	3	4	5	6	12	18	24
<i>V. harveyi</i> MAW	liquid paraffin	+	+	+	+	-	-	-	-	-
	lyophilization						+	+	+	+
	liquid nitrogen						+	+	+	+
<i>V. harveyi</i> B-392	liquid paraffin	+	+	+	+	-	-	-	-	-
	lyophilization						+	+	+	+
	liquid nitrogen						+	+	+	+
<i>Ph. fischeri</i> WH69	liquid paraffin	+	+	+	+	-	-	-	-	-
	lyophilization						+	+	+	+
	liquid nitrogen						+	+	+	+
<i>Shewanella</i> MS32	liquid paraffin	+	+	+	+	+	+	-	-	-
	lyophilization						+	+	+	+
	liquid nitrogen						+	+	+	+
<i>V. harveyi</i> SK	liquid paraffin	+	+	+						
	lyophilization									
	liquid nitrogen									
<i>V. harveyi</i> G	liquid paraffin	+	+	+	+	-	-			
	lyophilization						+			
	liquid nitrogen						+			

For the period of 6 months, 1, 1.5 and 2 years all the strains preserved by freezing or lyophilization restored their ability to emit light from 7th to 10th hour after cultivation on appropriate medium (Table 1).

Morphological changes in the shape of the colonies, as well as in temperature characteristics, were not detected. Similar results were obtained when luminous microorganisms were preserved under vaseline [11].

The present results allow us to recommend the preservation of luminous bacteria on straight agar under liquid paraffin for a short-term period (up to 5 months). For a long-term preservation lyophilization and cryogenic method are recommended. The experiments for determination of the most continuous time for keeping the viability and light emission of luminous bacteria using these two methods are still in progress.

Acknowledgements: This work was supported by a grant K-309/93 from the National Fund for Scientific Research.

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