Testing and improving simple cryopreservation methods for biotechnological-important cyanobacterial strains from a Culture Collection

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1 Introduction

In the last decade, cyanobacteria have been pointed out as one of the promising group of organisms for the discovery of natural compounds with interest in pharmacology and other biotechnological applications [1]. With almost 400 different cyanobacterial strains, the Blue Biotechnology and Ecotoxicology Culture Collection (acronym LEGE) at CIIMAR (Porto, Portugal) comprises a rich cyanobacteria diversity, most of it isolated from marine environments. The increase of the number of strains in the collection, associated with the ability of cyanobacteria to produce compounds with high biotechnological interest, have demanded the need for suitable preservation of LEGE isolates. So, to avoid the loss of such source of valuable metabolites, we have decided to enhance cryopreservation methods in order to preserve this important biological resource. Several studies have shown that LEGE strains are capable to produce a vast array of bioactive compounds against different biological targets [2,3,4].

Based in previous studies [5,6], we have applied common cryopreservation methods, differing in their cooling step, for a selection of cyanobacteria LEGE strains representative of the overall diversity present in the collection. For each strain, an evaluation of different attributes was made, allowing the grouping of strains through its categorization by cyanobacterial trait. The evaluated features included the (i) cellular arrangement, (ii) presence/absence of vacuoles, (iii) capacity to produce large quantities of exopolysaccharides, and (iv) salt content of culture medium (fig.2). Results from the recovery of cyanobacteria will be presented, while a comparison of the effectiveness of the tested cryopreservation methods and their suitability for each cyanobacterial trait will be discussed.

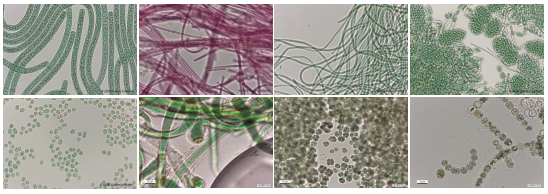


Fig.1- Optical microphotographs from cyanobacteria strains from LEGE Culture Collection.

[1] Nunnery, J.K. et al (2010). Curr Opin Biotechnol, 21: 787-793
[2] Leão, P.N. *et al* (2013). PLoS ONE, 8(7): e69562
[3] Leão, P.N. *et al* (2015). Angewandte Chemie, 4(38), 11063–11067
[4] Costa, M.S. *et al* (2016). Sci. Rep., 6, 23436
[5] Rastol, M.J. *et al* (2013). J Appl Phycol., 25(5), 1483-1493
[6] Day, J.G. et al (2007). Methods in Molecular Biology, 368, 141-151

2 Methodology

Cryopreservation methods and strain selection

Based on

Different cooling step

LEGE strains representative of the overall diversity

(i) cellular arrangement
(ii) presence/absence of vacuoles
(iii) Production of exopolysaccharides (EPS)
(iv) salt content of culture medium

Statistical approach (R Statistical Software)

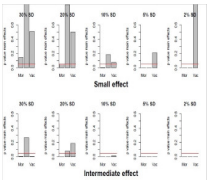


Fig.2- Scheme that represents the aspects taken into account to test cryopreservation methods and to select strains

Tested Cryopreservation Protocols

One-Step Protocol

Cryoprotectant Solution (CPA) Medium + DMSO 10% (v/v)

Uncontrolled cooling rate

Cryoprotected cultures DMSO 5%

Storage at -80ºC

Two-Step Protocol

Cryoprotectant Solution (CPA) Medium + DMSO 10% (v/v)

Uncontrolled cooling rate

Cryoprotected cultures DMSO 5%

Shock freezing step in liquid nitrogen

Storage at -80ºC

Nalgene® Mr. Frosty Protocol

Cryoprotectant Solution (CPA) Medium + DMSO 10% (v/v)

Controlled Cooling rate -1ºC/min

90 minutes

Cryoprotected cultures DMSO 5%

Storage at -80ºC

Rewarming:
Frozen cells are rapidly thaw at 40ºC (until the last ice crystal has just melted)

Cultures Recovery:
Standard culturing protocols, protected from light (24h)

Recovery of cyanobacteria (Qualitative evaluation) (fig.3):
Direct Observation - Presence/Absence of the strain between 5 and 45days

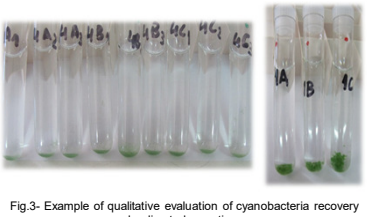


Fig.3- Example of qualitative evaluation of cyanobacteria recovery by direct observation.

3 Results

Cyanobacterial recovery results (Different protocols)

Traits	LEGE Code	Strain ID	Recovery: 5 Days				15 Days				30 Days				45 Days			
#1	LEGE 06225	*Planktothrix mougeotii*	+	+	+		↑	↑	↑		=	=	=		=	=	=	
#2	LEGE 08330	*Microcystis aeruginosa*		+		+	+		↑	↑		↑		↑	↑		=	=
#3	LEGE 06222	*Planktothrix mougeotii*	+	+	+		↑	↑	↑		=	=	=		=	=	=	
#4	LEGE 91093	*Microcystis aeruginosa*	+	+	+		↑	↑	↑		↑	↑	↑		↑	↑	↑	
#5	LEGE 07229	*Planktothrix mougeotii*	+	+	+		↑	↑	↑		↑	↑	↑		↑	↑	↑	
#6	LEGE 00060	unidentified filamentous cyanobacterium	+	+	+		↑	↑	↑		=	=	=		↓	↓	↓	
#7	LEGE 91353	*Microcystis aeruginosa*	+	+	+		=	=	=		↑	↑	↑		↑	↑	↑	
#8	LEGE 12450	*Nostoc* sp.	+	+	+		↑	↑	↑		=	=	=		=	=	=	
#9	LEGE 07211	*Synechocystis* sp.	+	+	+		↑	↑	↑		↑	↑	↑		=	=	=	
#10	LEGE 06077	*Nostoc* sp.	+	+	+		↑	↑	↑		↓	↓	↓		=	=	=	

Mr.Frosty | One-Step Protocol | Two-Step Protocol | Mr.Frosty Presure | Mr.Frosty Centrifugation

Fig.4- Cyanobacterial strains used to test cryopreservation protocols and recovery results

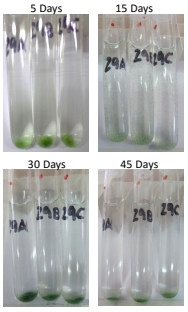
Cyanobacterial recovery results (Mr. Frosty Protocol)

LEGE Code	Strain ID	Recovery: 5 Days	15 Days	30 Days	45 Days
LEGE 06115	*Nodosilinea* sp.	+	↑	↑	=
LEGE 06225	*Planktothrix mougeotii*	+	↑	=	=
LEGE 12451	*Nostoc* sp.	+	↑	=	↓
LEGE 11430	*cf. Phormidesmis* sp.	+	↑	↓	=
LEGE 00060	*unidentified filamentous cyanobacterium*	+	↑	=	↓
LEGE 06135	*Cyanobium* sp.	+	↑	↑	↓
LEGE 91353	*Microcystis aeruginosa*	+	=	↑	=
LEGE 08330	*Microcystis aeruginosa*	-	=	=	=
LEGE 07179	*Hyella* sp.	+	↑	=	=
LEGE 07187	*Chroococcopsis sp.*	+	=	↑	↑
LEGE 06146	*Myxosarcina* sp.	+	=	↑	=
LEGE 06013	cf. Romeria sp.	+	=	↑	=
LEGE 06102	*Nodosilinea nodulosa*	+	↑	=	=
LEGE 06077	*Nostoc* sp.	+	↑	=	↑
LEGE 06018	*unidentified filamentous Synechococcales*	-	↑	=	↓
LEGE 14445	*Tolypothrix* sp.	+	↑	↑	=
LEGE 12450	*Nostoc* sp.	+	↑	↑	=
LEGE 06105	*Plectonema* cf. *Radiosum*	+	↑	↑	=
LEGE 07229	*Planktothrix mougeotii*	-	=	↑	↑
LEGE 00241	*Dolichospermum* sp.	-	=	=	=
LEGE 06226	*Planktothrix mougeotii*	+	↑	=	=
LEGE 06222	*Planktothrix mougeotii*	+	↑	=	=
LEGE 99043	*Cylindrospermopis raciborskii*	-	=	=	=
LEGE 97047	*Cylindrospermopis raciborskii*	-	=	=	=
LEGE 07211	*Synechocystis* sp.	+	↑	=	=
LEGE 91342	*Microcystis aeruginosa*	-	=	↑	↑
LEGE 12431	*Cyanobium gracile*	+	↑	↑	=
LEGE 00041	*Synechocystis salina*	+	↑	↑	↓
LEGE 06026	*Cyanobium* sp.	+	=	↑	↓
LEGE 10387	*unidentified unicellular Synechococcales*	+	↑	=	=
LEGE 06019	*unidentified unicellular Chroococcales*	+	↑	↑	=
LEGE 07171	*Synechococcus nidulans*	+	↑	↑	↓
LEGE 07459	*unidentified unicellular Chroococcales*	+	↑	=	=
LEGE 91093	*Microcystis aeruginosa*	-	=	↑	↑
LEGE 06079	*Synechocystis* sp.	+	↑	↑	↓
LMECYA 179	*Microcystis aeruginosa*	-	=	↑	↑
LMECYA 8	*Microcystis aeruginosa*	-	=	=	=
LEGE 08330	*Microcystis aeruginosa*	-	=	=	↑
LEGE 06222	*Planktothrix mougeotii*	+	=	↓	↑
LEGE 06079	*Synechocystis* sp.	-	=	↑	=
LMECYA 179	*Microcystis aeruginosa*	-	=	=	=
LEGE 07229	*Planktothrix mougeotii*	+	↓	=	=
LEGE 06226	*Planktothrix mougeotii*	+	↑	=	↓

+	Visible presence of the strain
-	No visible presence of the strain
=	No visible difference in comparison to previous date
↓	Visible reduction of strain in comparison to previous date
↑	Visible growth of strain in comparison to previous date

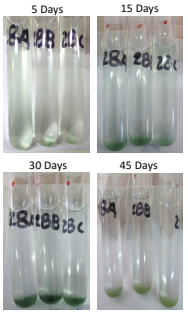
Fig.5- Cyanobacterial strains in Mr. Frosty cryopreservation protocol and recovery results.

LEGE 06026
Cyanobium sp.



A

LEGE 00041
Synechocystis salina



B

Fig.6- Examples of qualitative evaluation of cyanobacteria recovery by direct observation: A- *Cyanobium* sp. LEGE 06026; B- *Synechocystis salina* LEGE 00041.

4 Conclusions and Future Work

Developed cryopreservation methods

Seems to be a good option to cryopreserve cyanobacterial strains (short-term cryopreservation)

Sensitive strains – adapt/adjust cryopreservation protocols

Cyanobacterial strains recovery (quantitative evaluation)

Trypan Blue staining – not appropriate for this purpose (false positives)

Next Steps

Develop a viability assessment protocol for post-thawing cryopreserved strains

Test different Vital stainings:

(i) tetrazolium dye (MTT) and fluorescein diacetate (FDA)
(ii) Plating technique

Test recovery of cyanobacteria cryopreserved samples (replicates), 12, 24 months in -80ºC freezer (long-term cryopreservation)

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