EMbaRC

European Consortium of Microbial Resource Centres

Grant agreement number: **228310**

Seventh Framework Programme

Capacities

Research Infrastructures

Combination of Collaborative Project and Coordination and Support Actions

Deliverable D14.30 (formerly D.JRA 1.1.3)

Title: **Recommendation of best protocols for preservation**

Due date of deliverable: M40

Actual date of submission: M44

Start date of the project: 1st February 2009 **Duration:** 44 months

Organisation name of the lead beneficiary: UVEG-CECT

Version of this document: V0.2

Dissemination level:

PU	**Public**	**X**
PP	Restricted to **other programme participants** (including the Commission)	
RE	Restricted to a **group defined by the Consortium** (including the Commission)	

EMbaRC is financially supported by the Seventh Framework Programme (2007-2013) of the European Communities, Research Infrastructures action

Document properties	
Project	EMbaRC
Workpackage	JRA1
Deliverable	D.JRA 1.1.3
Title	Recommendation of best protocols for preservation
Version number	V0.2
Authors	Amparo Ruvira & David Ruiz Arahal (UVEG-CECT)
Abstract	The objective of Task JRA1.1 was to improve current protocols or develop new ones to increase the recovery rates and extend the shelf-life of preserved material for which conventional methods give low results. The first step was to identify by means of lists what each partner considers delicate or recalcitrant among its own holdings. Those working with prokaryotes have placed more emphasis on freeze-drying whereas those working with eukaryotes focus mainly on deep-freezing of cultures. This document provides valuable information that surpasses even the grounds for the next actions to be taken within the project.
Validation process	Document prepared by UVEG-CECT in collaboration with all task contributors and submitted to the Executive Committee for agreement.

Revision table			
Date	***Version***	***Revised by***	***Main changes***
17.09.2012	0.1	A. Ruvira (UVEG-CECT)	First draft
26.09.2012	0.2	D.R. Arahal (UVEG-CECT)	Minor corrections

Contents

1 Background and Objectives

The *ex situ* preservation and long term storage of microbial strains without apparent loss of their properties led to the establishment of culture collections operating as services more than one century ago. Culture collections have been acting since as repositories and providers of biological material and many of them are evolving to Biological Resource Centres (BRCs) according to the definition given by the OECD (2001) for those that meet the modern demands for the advancement of Biotechnology and Life Sciences. Although the experience gained on preservation and maintenance of microbial strains and other biological materials is large after those many years, it would be wrong to assume that technically it is a resolved issue. First, BRCs must meet the high standards of quality demanded by the international community of scientists and industry. Second, because we are still exploring the vast diversity of the microbial world, and new species, genera, families and so on, are being discovered every year, many of which represent a challenge for the BRCs that try to hold them.

The objective of task JRA 1.1 was to improve current protocols or develop new ones to increase the recovery rates and extend the shelf-life of preserved material for which conventional methods give low results. The first step was to identify by means of lists what each partner considers delicate or recalcitrant among its own holdings (D.JRA1.1.1). The next step was the design and testing of the experimental work (D.JRA1.1.2). Finally, in this deliverable the results are analyzed to make recommendations of protocols that enhance preservation survival.

2 Prokaryotes

2.1 Strains selected

The compiled list of delicate or recalcitrant strains provided by CRBIP, DSMZ, CECT and LMG was too vast, so a selection was proposed attending to several criteria:

Strain	Medium	Temperature	Atmosphere
Aeromonas salmonicida CECT 894^T	(1) Nutrient Broth/Agar I	24 ºC	Aerobic
Aliivibrio fischeri LMG 4414^T	(12) Marine Broth	20 ºC	Aerobic
Helicobacter pylori CIP 103995^T	(6) Columbia agar with 10 % horse blood	37 ºC	Microaerobic
Flavobacterium columnare LMG 10406^T	(215) Modified Shieh agar	25 ºC	Aerobic
Vibrio agarivorans CECT 5085^T	(30) Marine Broth/Agar	26 ºC	Aerobic
Xanthomonas fragariae DSM 3587^T	(830) R2A Medium	28 ºC	Aerobic

2.2 Protocol

In the first part of the experimental process, the partners measured the effect of nine variations on the method on the selected bacterial strains sensitive to drying, each partner analyzed their own strains. For each strain the following batches were prepared:

Batch #1. Is the Reference batch. Normal cultivation conditions to late exponential phase are applied and skimmed milk is used as lyoprotectant.

Batch #2. Normal cultivation conditions to late exponential phase and broth medium + 10% trehalose as growth medium.

Batch #3. Low temperature treatment (2h at 7ºC) before the preservation step

Batch #4. Shorter incubation time (1/3 less), using skimmed milk as lyoprotectant

Batch #5. Longer incubation time (1/3 more), using skimmed milk as lyoprotectant

Batch #6. Normal cultivation conditions to late exponential phase and skimmed milk + 10% trehalose as protectant

Batch #7. Normal cultivation conditions to late exponential phase and skimmed milk + cultivation broth (1:1) as protectant

Batch #8. Normal cultivation conditions to late exponencial phase and horse serum + 10% trehalose as protectant

Batch #9. L-drying on skimmed milk plug (if partner can perform it)

The viability (cfu per ml) was checked, always in triplicate, on the first week post treatmentat months 6 and 12, and after accelerated storage (14 days at 37ºC, corresponds to 20 years storage at 4ºC). Residual moisture content was also determined.

In the second part, the strains were exchanged among partners and only the best and the worst batches were analyzed: *A. salmonicida* CECT 894^T was analyzed by DSMZ and LMG testing batch1, 8 and 9 (LMG); *F. columnare* LMG 10406^T was analyzed by CIP testing batch1, 5 and 6; *C. fetus* CIP 53.96^T was analyzed by CECT 1 and 6; *A. fisheri* LMG 4414^T was analyzed by CIP 1 and 7; and CECT analyzed the equivalent strain of *X. fragariae* DSM 3587^T (*X. fragariae* CECT 549T) testing batch 1 and 7. In this case, the viability was checked on the first week post treatment and after accelerated storage.

2.3 Results

The results obtained at the first part of the experimental process showed the choice of the lyoprotectant had the biggest impact on viability after freeze-drying and during storage. Thus, skim milk alone as lyoprotectant (batch1) resulted in lowest process viability in all cases (except *X. fragariae* DSM 3587^T), whereas the best freeze-drying and storage conditions depended from strain to strain. For *A. salmonicida* CECT 894^T, the best process and storage survival was obtained when horse serum supplemented with trehalose was used as lyoprotectant (batch8). *A. fisheri* LMG 4414^T should be freeze-dried in skim milk supplemented with marine broth in a 1:1 ratio (batch7) to obtain highest process and storage survival. Freeze-drying *C. fetus* CIP 53.96^T using skim milk supplemented with trehalose as lyoprotectant (batch6) resulted in best process and storage survival. The plant pathogenic *X. fragariae* DSM 3587^T expressed high viability after freeze-drying and storage for all tested lyoprotectants and could be considered as not recalcitrant.

On the contrary, *V. agarivorans* CECT 5085^T and *F. columnare* LMG 10406^T did not survive the freeze-drying process under all tested conditions.

The results obtained at the second part of the experimental process showed for *A. salmonicida* CECT 894^T the best and the worst method could not be reproduced by three partners (CECT, DSMZ and LMG), although skim milk alone as lyoprotectant (batch1) resulted in lowest process viability in all cases, but only CECT results after accelerated storage showed no survival. However, the best method (batch8) is the same in the three cases but with different survival factors. *A. fisheri* LMG 4414^T, batch1 resulted in lowest process viability in two cases (CIP and LMG), but only CIP results after accelerated storage showed no survival, and the best method (batch7) had different

survival factors. For *F. columnare* LMG 10406^T the results showed no reproducible process survival by two partners (CIP and LMG) and no further conclusions could be made. For *C. fetus* CIP 53.96^T, the best (batch6) and the worst (batch1) condition could not be reproduced by three partners (CECT, CIP and LMG) it showed different survival factors, and only CIP experiment showed survival after accelerated storage in the batch5. Thus, for all exchanged strains the freeze-drying process (device dependent) had major impact on process and storage survival.

3 Eukaryotes

3.1 Strains

The organism lists provided by CABI, CBS, MUM, CECT and UCL were extensive. In order to provide a representative list for further analysis and protocol optimisation the list was reduced to a group of key organisms:

Name	Collection	Strain #	Taxonomy
Conidiobolus rhysosporus	CBS	141.57	Zygo
Ramularia variabilis	CBS	434.67	Asco
Botrytis elliptica	CBS	108966	Asco
Penicillium expansum	MUM	0.01	Asco
Aspergillus ibericus	MUM	3.49	Asco
Trichophyton rubrum	MUM	10.132	Asco
Hebeloma crustuliniforme	MUCL	52208	Basidio
Laccaria bicolor	MUCL	52210	Basidio
Paxillus involutus	MUCL	52217	Basidio
Mortierella alpina	CECT	2977	Zygo
Coniophora olivacea	CECT	20145	Basidio
Suillus luteus	CECT	20236	Basidio
Diplocarpon rosae	CABI	381057	Asco
Saprolegnia diclina	CABI	308259	Chromist
Phytophthora citropthora	CABI	396200	Chromist

3.2 Protocol

In a first stage, strains were distributed among concerned partners to undertake baseline assessments before preservation:

- **Moleculars methods:**

Three different methods were used:

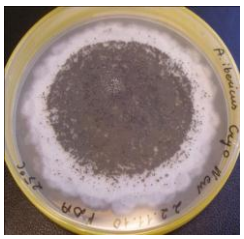
- ISSR
- SSR-PCR
- AFLP

- **Analysis of culture characteristics:**

Growth rates as indicator of post preservation recovery and changes in culture morphology before and after preservation were analysed.

Phytophthora citrophthora, 29 d MEA

Aspergillus ibericus, 3d PDA



- **Other:**

Other methods as MALDI-TOF and FDA viavility were tested pre and post preservation

Cultures were preserved by 3 methods and again analyzed by the same tests after resuscitation. Depending on the strain the preservation method selected was water (W), freezing -80ºC (F), liquid nitrogen (LN) and/or lyophilization (L):

Strain nº	Taxon name	Treatment		
CECT 2977	*Mortierella alpine*	W	F	LN
CECT 20145	*Coniophora olivácea*	W	F	LN
CECT 20236	*Suillus luteus*	W	F	LN
IMI 396200	*Phytophthora citrophthora*	W	F	LN
IMI 381057	*Diplocarpon rosae*	W	F	LN
IMI 308259	*Saprolegnia diclina*	W	F	LN
CBS 141.57	*Conidiobolus rhysosporus*	W	F	LN
CBS 108966	*Botrytis elliptica*	W	F	LN
CBS 434.67	*Ramularia variabilis*	-	-	-
MUM 0001	*Penicillium expansum*	W	F	L
MUM 0349	*Aspergillus ibericus*	W	F	L
MUM 10132	*Trichophyton rubrum*	W	F	L
MUCL 52208	*Hebeloma crustiliniforme*	-	-	-
MUCL 52210	*Laccaria bicolor*	-	-	-
MUCL 52217	*Paxillus involutus*	W	F	LN
Water **(W)** Freezing -80ºC **(F)** Liquid Nitrogen **(LN)** Lyophilization **(L)**				

Besides, new preservation methods have been developed: Encapsulation Vitrification Cryopreservation and “LN2 method” involving pre-growth in ampoules (protocol for the long-term storage of ectomycorrhizal fungi), where cultures were grown directly in the cryovials avoiding damages to cultures caused by handling.

3.3 Results

The results showed that when viable cultures were obtained after preservation, they appeared to retain their genomic integrity, but there was evidence of delayed growth and attenuation in some cultures. Not all fungi were successfully preserved by all methods. It was found that a cryopreservation protocol used by the MUCL collection in Belgium, that limited manipulation of the fungus before preservation, was particularly effective in preserving some of the more delicate fungi and this method was evaluated by the project partners.

Regarding to other preservation methods, MUCL cryopreservation method was robust with encouraging results. For example, with cultures of *Serpula lacrymans* / *Phytophthora citrophora* 100% viability was evident. Much improved results over original testing. For encapsulation dehydration method, initial results were very encouraging, especially for *basidiomycetes* and *chromists*. For example, 93% recovery achieved with *Serpula lacrymans* and 52% with *Suillus luteus*. Finally, the “LN2 method” result showed that is a suitable and easy-to-apply procedure for the long-term maintenance of a large set of ECM fungi

Conclusion

We have indeed increased the recovery and survival rates by improving existing protocols and even two new ones were developed. Moreover, to the best of our knowledge this is the first time such an approach has been tacked collectively. An additional achievement is the experience gained on devising a rationale for future studies on viability.

Significance of this deliverable

One of the challenges of long term microbial storage is to preserve viability.

Existing methods were improved collectively for recalcitrant species, in particular fungi, and two new preservation approaches were explored, giving encouraging results.

Regarding bacteria, the impact of the device used for freeze-drying on viability recovery was demonstrated through interlaboratory assays.