



D2.2. Assessment of shelf-life properties of microbe-biomass formulates

Lead Beneficiary: Université de Bourgogne Franche-Comté (UBFC)

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DOCUMENT INFORMATION

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Related task(s)	Task 2.2. Design of microbe-biomass formulates.
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EXECUTIVE SUMMARY

The present Deliverable (D2.2) contains the results obtained in the context of Task 2.1 (**Design of microbe-biomass formulates**). It is dedicated to the **assessment of formulate shelf-life properties**. Viable cell concentrations for each formulation are determined directly by colony counting on agar plates after various lengths of times. All microbial resources foreseen in SIRAM will derive from Task 1.1 activities and detailed in D1.2.

1. ON-GOING EXPERIMENTS

The assessment of shelf-life is ongoing for formulations described in D2.1. Table 1 and paragraph 2 detail experiments and results obtained up to now for partners involved in microorganisms delivery.

Table 1: Summary of ongoing experiments

Partner	CS	Experiment #
UCSC	CS1	2.1 and 2.2
ARC	CS4	2.3
CRRA	CS7	2.4
ENAM	CS6	
USMBA	CS6	

2. DETAILED EXPERIMENTS AND RESULTS

2.1. Freeze drying of bacteria to be applied in CS1 and shelf life

Strains listed in D1.2 and M1.2 were freeze dried after growth in Tryptone Soya Broth medium for 24 hours. Before freeze-drying, pelleted cultures of PV4, PV38, BS25 and BS27 were resuspended in a solution of 5% sucrose and 5% lactose, according to Stephan et al.¹, while PV9 was resuspended in a solution of 4.7% trehalose, 1% glucose and 1.45% xylitol (Han et al.²). Freeze dried cultures were stored at -20°. Shelf-life was assessed after one month by plate count. Results are shown in Table 1. As it can be observed, after one month the same number of CFU/g as order of magnitude could be obtained as lively colony on agar plate.

Table 1. Plate count results (CFU/g) of freeze dried strains just after freeze-drying and after 1 month at -20°C.

Strain	CFU/g just after freeze-drying	CFU/g after 1 month
BS 25	$6 \cdot 10^{10}$	$1.6 \cdot 10^{10}$
BS 27	$1.3 \cdot 10^{12}$	$9.9 \cdot 10^{11}$
PV 4	$5.1 \cdot 10^{10}$	$2.7 \cdot 10^{10}$
PV 9	$3.5 \cdot 10^{11}$	$1.5 \cdot 10^{11}$

¹ Stephan, Dietrich, Ana-Paula Matos Da Silva, and Isabella L. Bisutti. "Optimization of a freeze-drying process for the biocontrol agent *Pseudomonas* spp. and its influence on viability, storability and efficacy." *Biological control* 94 (2016): 74-81.

² Han, Lingjuan, et al. "Optimization of a protective medium for enhancing the viability of freeze-dried *Bacillus amyloliquefaciens* B1408 based on response surface methodology." *Cryobiology* 81 (2018): 101-106.

PV 38	$4.7 \cdot 10^{11}$	$1.6 \cdot 10^{11}$
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2.2. Survival of inoculated strains in vermicompost (UCSC)

To test survival of strains inoculated in vermicompost, elutriate was prepared as described in M1.3, *i.e.* vermicompost was diluted according to a ratio 1:4 weight/volume in distilled water. This solution was shaken for 1 hour and the biomass was let sediment for 24 hours. The supernatant of this solution constitutes the elutriate. In this experiment, elutriate was inoculated with freeze-dried strains or fungal spore suspension at a concentration of 10^8 CFU/ml or 10^8 spores/ml, as described in D2.1. Survival of inoculated strains was monitored in time by plate count agar. Results are shown in Figure 1. As it can be observed, differently from what observed for manure in M1.2, strains are able to survive in this substrate and, in the case of strains BS25 and BS27, they grow after 5 days of the inoculum. In the condition of the experiment, strains stop growing after 5 days from the inoculum, but this can be due also to lack of oxygenation or depletion of nutrients from the elutriate. Therefore, data will be further validated on samples collected from pot and field experiments by qPCR with strain-specific primers designed thanks to genome sequencing of the various strains.

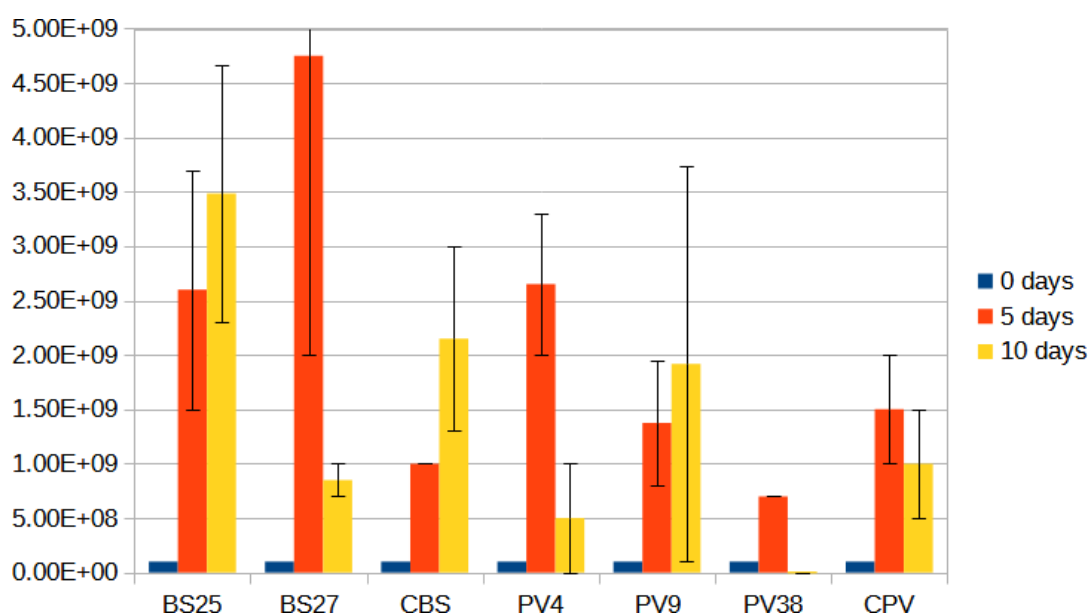


Figure 1: results (CFU/ml vermicompost elutriate) of plate count from samples of vermicompost elutriate inoculated with the strains and consortia selected in previous months, at start and after 5 and 10 days from inoculum.

2.3. Shelf life of rice bran formulations of fungi to be applied in CS4 (ARC):

The formulations of the antagonistic isolates was stored at 4°C. The effect of storage period on the biological activity of formulated antagonists was tested in the laboratory from 1 up to 6 month 1 gof each different type of formulation and suspended in 200 mL of sterile distilled water. One hundred micro liters of the respective dilution of formulations was plated onto *Trichoderma*-selective medium (TSM) containing carbedazim ($25 \mu\text{g ml}^{-1}$) in Petri dishes (Elad and

Chet, 1983³) with 3 replicates per treatment. The Petri plates incubated at 28°C for five days and the colonies and spores formed counted according to Jayaraj et al., 2006⁴.

Table 2: Effect of storage period on viability of rice bran formulations

Formulation/time	CFU /ML after storage period (months)					
	Zero	One	Two	Three	Four	Five
<i>Trichoderma longibrachiatum</i>	2x10 ⁸	1x10 ⁷	7.5x10 ⁶	5.65x10 ⁶	4.7x10 ⁶	4.45x10 ⁶
<i>Trichoderma asperellum</i>	2x10 ⁸	5x10 ⁶	4x10 ⁶	3.5x10 ⁶	2.5x10 ⁶	2x10 ⁶

Table 2 show the results (CFU/ml rice bran formulation) of plate count from samples inoculated with the strains and consortia selected in previous months, at start and after 0, 1,2,3,4 and 5 months from inoculum. Moreover, data further validated on samples collected from pot and field experiments.

2.4. Shelf life of compost formulations of fungi and bacteria (CRRA):

The formulations of the antagonistic isolates were stored at 4°C. The effect of the storage period on the biological activity of formulated antagonists was tested in the laboratory from 1 up to 6 months 1 g of each different type of formulation and suspended in 200 mL of sterile distilled water. 100 µl of the respective dilution of formulations were plated onto *Trichoderma* and *Pseudomonas*-selective medium in Petri dishes. Each treatment consisted of 9 plates per replicate (3 replicates), and the experiment was performed twice. The Petri plates were incubated at 28°C (*T. longibrachiatum*) and 30°C (*Pseudomonas* sp.) for 5 days and the colonies and spores formed were counted. The number of colony forming units (CFU) was calculated according to Mouria et al. (2012)⁵.

Table 3: Effect of storage period on viability of fungi and bacteria formulations

Strains	CFU/g after 1 month	CFU/g after 3 months	CFU/g after 6 months
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³Elad, Y. and Chet, I. (1983) Improved Selective Media for Isolation of *Trichoderma* spp. or *Fusarium* spp. *Phytoparasitica*, 11, 55-58

⁴Jayaraj, J.; Radhakrishnan, N.V.; Velazhahan, R. (2006). Development of formulations of *Trichoderma harzianum* strain M1 for control of damping-off of tomato caused by *Pythium aphanidermatum*, *Archives of Phytopathology and Plant Protection* 39(1):1-8.

⁵Mouria B, Ouazzani-Touhami A, Douira A, 2012. Isolement et identification de la mycoflore du compost des déchets urbains solides. *Nature & Technologie* 9: 13–28

<i>T. longibrachiatum</i>	$5.27 \cdot 10^{11}$	$0.43 \cdot 10^{11}$	$7.71 \cdot 10^9$
<i>Pseudomonas</i> sp.	$4.52 \cdot 10^{12}$	$1.47 \cdot 10^{12}$	$9.16 \cdot 10^{11}$

3. CONCLUSIONS AND NEXT STEPS

In general, shelf-life assessment for formulations developed for CS1, CS4 and CS7 had positive results, with no loss of vitality determined for formulations 1 and 5 described in D2.1. Next steps involve:

- For CS1, formulation 2, *i.e.* inoculum on vermicompost, needs further validation by pot experiment. The pot experiment has just been terminated and results are under analysis. Survival of microorganisms will be checked in pot and in field by strain-specific primers qPCR on DNA extracted from rhizosphere.
- For CS4, assessment of shelf-life formulations described in D2.1.