

Development and validation of a reference material for food microbiology using *Bacillus cereus* spores

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INTRODUCTION

Food microbiology laboratories have an increasing need for Reference Materials (RM) to evaluate their analytical performances. The ideal food microbiology RM is a material that contains a precise, homogenous and stable number of microorganisms. Commercially available RM generally present important drawbacks: short shelf-life, low assigned value, high uncertainty, heterogeneity problems or important costs for transport at frozen temperatures. REQUASUD is a Walloon organization that includes a network of six food microbiology laboratories, taking part in internal and external proficiency testing and willing to develop stable reference materials for its ring-tests.



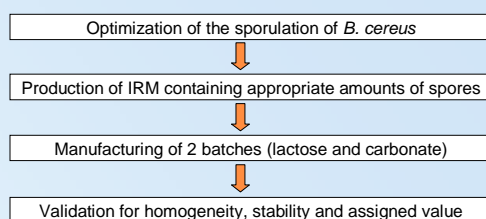
Figure 1 – *Bacillus* spore (ESA/DLR)

OBJECTIVES

The purpose of this research was to develop an Internal Reference Material (IRM) for the enumeration of total flora and the setup of control charts. The new IRM should have the following characteristics: homogeneity, stability, representativity of food contaminations, precision of the assigned value, cost-effectiveness, easy storage and shipment (ideally at room temperature). The IRM should be validated following ISO guidelines. The main innovation of this research was the use of *B. cereus* spores to build an IRM, because of their natural resistance and stability at room temperature.

DEVELOPMENT OF THE IRM

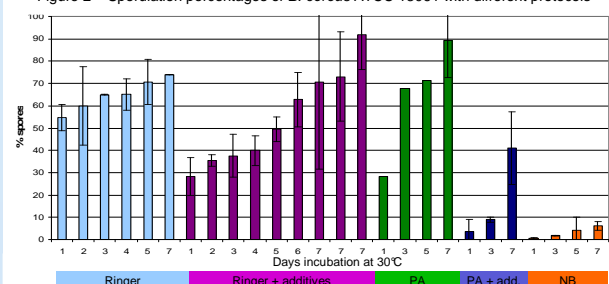
STEPS



OUTPUTS

- A modified PA solution yielded, in 7 days, a suspension containing 90% spores (Figure 2).
- Inoculation of several supports with 10⁴ spores. Selection of 2 solid supports (lactose and CaCO₃).
- 500 inoculated lactose pellets and 500 inoculated calcium carbonate pellets.
- Following the ISO 13528, ISO 5725 and ISO 19036 guidelines.

Figure 2 – Sporulation percentages of *B. cereus* ATCC 13061 with different protocols



VALIDATION OF THE IRM

- Following the ISO standards, the general principle for the validation of reference materials used in proficiency testing is the following: The variance between IRM units (s^2_{IRM}) must not contribute for more than 10% to the global variance of the proficiency testing (s^2_{PT})
- The global variance of the proficiency testing (s^2_{PT}) is obtained on the basis of the past proficiency testing results. For REQUASUD, it was calculated as the robust mean of the variance of the last 20 proficiency testing.
- This principle is used for the validation of the batch **homogeneity**, **stability** and **assigned value**.

$$\Rightarrow s^2_{IRM} < 0,1 s^2_{PT}$$

$$\Rightarrow s^2_{PT} = 0,122 \log$$

Homogeneity

- 20 units of the IRM batches were enumerated for the calculation of s^2_{IRM} . The batch is homogenous if $s^2_{IRM} < 0,1 s^2_{PT}$

	IRM CaCO ₃	IRM lactose
s^2_{IRM}	0,0027	0,0016
$0,1 \times s^2_{PT}$	0,0122	
Homogenous ?	YES	YES

Stability

- Three units of both IRM batches were enumerated weekly
- The data were compiled into control charts and analyzed:
 - The lactose IRM was stable for 14 weeks and then a rapid drop was observed
 - The calcium carbonate IRM displayed a very good stability during 38 weeks at room temperature (Figure 3)

Assigned value

The IRM's assigned value and uncertainty were calibrated using a Certified Reference Material (CRM)

- Calculation of the IRM's **assigned value** (X_{IRM}) using the CRM's assigned value (X_{CRM}) and the global difference D:

$$\log X_{IRM} = \log (X_{CRM} + D)$$

Results (in log CFU/unit):

X_{CRM}	3,756
D	3,826
X_{IRM}	4,094

- Calculation of the IRM **uncertainty** (U_{IRM}) using the CRM uncertainty (U_{CRM}) and the uncertainty on the difference (U_D):

$$U_{IRM} = \sqrt{U^2_{CRM} + U^2_D}$$

Results (in log CFU/unit):

U_{CRM}	0,104
U_D	0,041
U_{IRM}	0,112

The assigned value of the IRM is: **4,094 log CFU/unit**
 The uncertainty of the IRM is: **0,112 log CFU/unit**
 Each IRM unit thus contained: **12400 ± 5100 CFU.**

CONCLUSIONS

- This work revealed new paths for the development and validation of reference materials for food microbiology. Spores of *B. cereus* set on a calcium carbonate support (Figure 4) displayed an excellent stability during at least eight months at room temperature, which is a clear advantage compared to the existing reference materials.
- The choice of a food pathogen for the inoculation of the material and the use of contamination levels close to those encountered in foods (10⁴ CFU/unit) make this new reference material even more valuable for food microbiology laboratories.
- The manufacturing and validation process was compiled in a procedure that will be useful to laboratories willing to develop and validate stable reference materials.

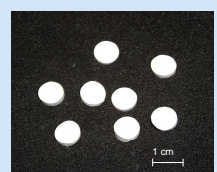


Figure 4 – REQUASUD's internal reference material.

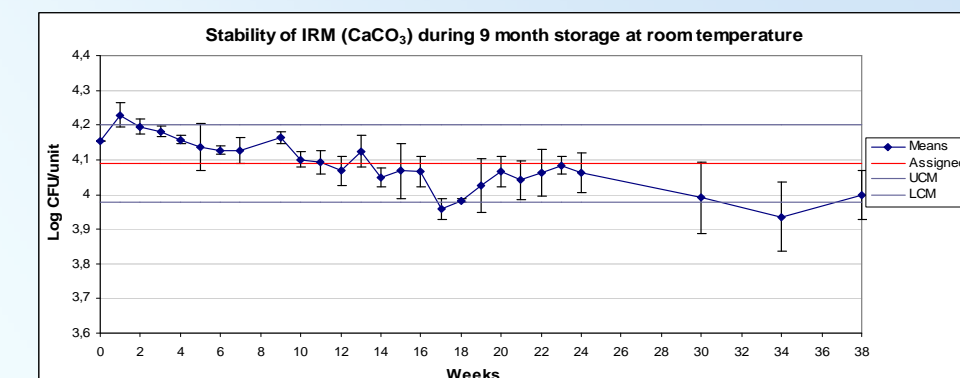


Figure 3 – Control chart of the calcium carbonate reference material for enumeration of total flora (ISO 4833). The assigned value is 4,094 log CFU/unit and the Upper and Lower Confidence Limits (UCM and LCM) are 4,094 ± 0,112, as calculated during the validation process.