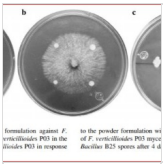

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Granulate formulation protocol

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ABSTRACT

Bioformulation is used in agriculture for various reasons like soil fertility, plant growth promotion, and suppression of phytopathogens. The bacterial inoculants are applied as a formulated product like powder, spray, or pellet. This is a green strategy that is being developed as a less harmful method to protect crops other than pesticides. In the paper published by *Alvarez et al, 2016*, they developed a talc-based powder formulation based on *Bacillus* B25 spores and evaluated some of its characteristics, like shelf life and efficacy against the pathogenic fungus *Fusarium verticillioides*.

Martínez-Álvarez, J. C., Castro-Martínez, C., Sánchez-Peña, P., Gutiérrez-Dorado, R., & Maldonado-Mendoza, I. E. (2016). Development of a powder formulation based on Bacilluscereus sensu lato strain B25 spores for biological control of Fusarium verticillioides in maize plants.. World Journal of Microbiology and Biotechnology, 32(5), 75. https://doi.org/10.1007/s11274-015-2000-5

Colony Forming Units (CFU) is a unit that is used in microbiology to estimate the number of viable bacteria or fungal cells in a sample. It also depends on their ability to multiply under controlled conditions. In the paper published by *El-Hassan and Gowen, 2006*, they analyzed various formulations of *Bacillus subtilis* by counting the CFU of B. subtilis present in every formulated product.

S. A. El-Hassan and S. R. Gowen (2006). Formulation and Delivery of the Bacterial Antagonist Bacillus subtilis for Management of Lentil Vascular Wilt Caused by Fusarium oxysporum f. sp. lentis. Journal of Phytopathology, Volume 154, Issue 3. https://doi.org/10.1111/j.1439-0434.20

Fatty acid analysis is an important means to characterize fats and oils and to determine the total fat content. The resulst can be used to identify the *Bacillus mycoides* strain in the soil and determine its survival with the granulate formulation.

Friedrich von Wintzingerode, Frederick A. Rainey, Reiner M. Kroppenstedt, Erko Stackebrandt (1997). Identification of environmental strains of Bacillus mycoides by fatty acid analysis and species-specific 16S rDNA oligonucleotide probe. FEMS Microbiology Ecology, Volume 24, Issue 3. https://doi.org/10.1111/j.1574-6941.1997.tb00437.x

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Bacterium Inoculum

1 Grow a single colony of bacteria in an assay tube with **5 mL** of Luria Broth (LB) medium

2 Incubate it in an orbital shaker at 200 rev.min^{-1} at **30 °C** for **18:00:00** .

3 After bacterial growth, take a **500 mL** Erlenmeyer flask and add **100 mL** of LB medium in it.

4 Add **1 mL** of the culture (**1 % (v/v)**) in the flask and incubate at **30 °C** and 200 rev.min^{-1} for **24:00:00** , until an optical density of close to 1 is obtained.

Spore Production

5 Add **100 mL** of Difco Sporulation Medium (DSM; 5gl^{-1} peptone, 3gl^{-1} yeast extract, 1gl^{-1} KCl and 0.12 gl^{-1} MgSO47H2O) in a **500 mL** Erlenmeyer flask.

6 Sterilize this DSM medium at **121 °C** and 1.5 psi for **00:15:00** .

7 Add **1 mL** each of: **1 Molarity (M)** $Ca(NO_3)_2$, **10 Milimolar (mM)** $MnCl_2.4H_2O$ and **1 Milimolar (mM)** FeSO4

8 Inoculate with 1X10^6 c.f.u ml^{-1} of the bacterial strain. Keep the culture conditions at **30 °C** and 200 rev.min^{-1} for **72:00:00** .

Powder Formulation

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9 Mix talc (which will be the carrier) with, carboxy-methyl-cellulose (CMC; 1% w/w), $CaCO_3$ (15% w/w) and glucose (0.25% w/w) in powder form.

10 Autoclave the mixture at **121 °C** and 15 psi for **00:15:00** .

11 Mix the same material with the bacterial spore suspension and dry at **55 °C** for **36:00:00** .

12 Using sterile porcelain mortar and pestle pulverize the formulation.

13 Pack it in plastic bags and store it at room temperature.

CFU determination

14 Colony Forming Units (CFU) can be determined by estimating the OD of spore suspension using a tube-reading spectrophotometer adjusted at 1.978 [corresponding to 8.5 · 1010 CFU/ml] at 600nm absorbance wavelength

15 The formulation will be placed on sterile aluminum foil in pans and air-dried for **24:00:00** with occasional stirring in a laminar airflow cabinet.

16 Dried formulations (35% moisture content) of B. mycoides will be passed through a 250µm mesh sieve to attain the desired particle size.

17 Pack in sterilized polypropylene bags, seal and store at **Room temperature** prior to use.

18 Count CFUs to estimate the number of viable propagules of B. mycoides using the standard dilution platin method.

STD dilution method

19 Take three **1 g** aliquots of the dried powder and place in **99 mL** sterile PBST solution (this will include PBS + **0.05 % (v/v)** Tween 20). Stir magnetically at high speed for **00:15:00** . Now dilute this suspension with approximately and take **0.2 mL** of this suspension and plate on Nutrient Agar (NA) media.

Fatty acid analysis

20 By performing saponification, methylation, and then extracting we can obtain fatty acid methylesters from wet biomass.

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21 Next, separate the fatty acid methylester mixtures by using a microbial identification system. Peaks can be automatically integrated, and the Microbial ID will calculate the fatty acid names and percentages.

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