Appendices: Risk Assessment of Fungal Biocontrol Agents Standards

A: Quality control of fungal biological control agents

T. Laengle[1], B. Pernfuss[1], J. Raffalt[2] & H. Strasser[1]

[1] Institut für Mikrobiologie, Leopold-Franzens Universität Innsbruck, Technikerstr. 25, A-6020 Innsbruck, Austria
[2] F. Joh. Kwizda GmbH, Division Agro, Dr. Karl Lueger-Ring 6, A-1011 Wien, Austria

Specific scope

This standard provides protocols for the quality control and the assessment of fungal pesticides as biocontrol agents.

Specific approval and amendment

First approved in February 2004.

Introduction

High and stable quality is essential for any product's performance on the market. Product viability and strain virulence are important for the efficacy of the product and its acceptance by the customer. Contamination control is necessary to ensure the safety of workers during production, operators and consumers.

This publication provides standardised procedures for the assessment of these quality parameters and takes into account the specific requirements that differentiate fungal-based biological pesticides from their chemical counterparts. It also gives guidance for the generation of quality control data and their interpretation and sets minimum standards for key quality parameters.

Key to the use of evaluation protocols

Depending on the intended use, fungal biocontrol products may differ in their formulation. Therefore, different approaches to their

quality control are necessary. In order to create a widely applicable standard protocol for the quality control of products, a dichotomous key is used to differentiate between product groups and varying approaches to their quality control.

1. Place 500–1000 mg of product in sterile test tube. Always wear protective equipment such as safety goggles, gloves and dust mask when handling spore material. Add approximately 5 ml of sterile water. Mix on vortex mixer. Is product homogeneously suspendable in sterile water?

(a) Yes. **go to 2**

(b) No, product not suspendable in water (product associated with carrier material such as barley kernels). **go to 5**

2. Put a drop of approximately 10 µl of the product suspension on a glass slide and cover with cover slip. Observe in a microscope at a minimum of 400 × magnification. Are fungal spores clearly distinguishable from additives?

(a) Yes, spores are clearly visible. **go to 3**

(b) No, spores are not clearly distinguishable from additives and debris. **go to 4**

3. Product is a spore formulation that allows direct and indirect determination of spore density and germinability. Perform the following procedures:

- Contamination control (suspendable products)
- Direct detection of spore density
- Determination of spore viability
- Indirect detection of spore density
- Confirmation of strain identity
- Biotest (see RAFBCA Standard appendix C)

4. Product is a spore or biomass formulation that allows no direct determination of spore density and germinability. Perform the following procedures:

- Contamination control (suspendable products)
- Indirect detection of spore density (CFU method)
- Confirmation of strain identity
- Biotest (see RAFBCA Standard Appendix C)

5. Product is not homogeneously suspendable due to its formulation on a carrier. Perform the following procedures:

- Colonisation test
- Indirect detection of spore density
- Confirmation of strain identity
- Biotest (see RAFBCA Standard Appendix C)

Contamination control (suspendable products)

Introduction

Each lot of fungal spore products has to be analysed for contamination with potentially harmful microorganisms before it can be approved for sale.

It is recognised that it may be very difficult and expensive to keep formulation additives and packaging equipment completely sterile and that the product therefore may contain small quantities of microbial contaminants.

Because the product is used in the production of foodstuffs it must be ensured that the product is compliant with guidelines regulating the hygiene of foodstuffs. This means that the level of unwanted microbiological impurities in the product suspension that is applied to food products must not exceed the allowed limits for microbial contaminants stated in Council Directive 98/83/EC on requirements the quality of water intended for human consumption.

Procedure

The first step in the microbiological evaluation of a fungal spore product is to determine if a microbial impurity can be detected.

For this purpose, spread a small quantity of product on Potato-Dextrose Agar (PDA) plates and CASO Agar plates (6 replicates each) with an inoculation loop. CASO Agar is used for the detection of bacterial contaminants, fungal contaminations can be quantified with Potato-Dextrose Agar. Incubate three replicates of each medium at 25 °C and 60 % rH and three replicates at 37 °C and 60 % rH for one week. If a contamination is detected it is necessary to determine the quantity of the contaminant.

Wearing a face mask, safety goggles and gloves, fill a small amount of the spore powder product into a sterile polypropylene tube with a spatula. Determine the amount of spore powder by weighing the tube before and after adding the product.

Add 10 mL of 0.1 % sterile Tween® 80 solution per 100 mg product and mix on a vortex mixer until a homogeneous suspension is obtained.

Prepare a dilution series in decimal steps (i.e. 1 : 10, 1 : 100, etc). to a dilution of 10^{-9}. Make sure to mix suspension well between dilution steps.

For each dilution, plate six replicates on CASO Agar and six replicates on Potato-Dextrose Agar with 50 µL of the suspension, then incubate three plates each at 25 °C and 60 % rH and three at

37 °C and 60 % rH for up to one week. Check growth every second day to make sure colonies do not overgrow each other.

After incubation count contaminant fungi (PDA-plates) and bacteria (CASO Agar plates) separately for both incubation temperatures on the "best dilution plates" (20–60 colonies per plate).

Calculate amount of bacteria and contaminant fungi growing at 25 °C and 37 °C, respectively, as CFU g^{-1} product using the following formula.

$$[CFU\ g^{-1}\ product] = 2 \times \frac{colonies\ per\ plate}{dilution} \times 10^3$$

The factor 2×10^3 results from the fact that 50 µL = 1/20 mL of each dilution were plated and that the original suspension contained 1/100 of a gram of product per millilitre. Then calculate means and relative standard deviations.

Using the amounts of product per volume of spray suspension recommended on the package, the obtained data can be extrapolated to calculate the amount of potentially hazardous contaminants in the spray suspension applied by the user.

If the contaminants in the calculated spray suspension do not exceed the microbiological requirements stated in Council Directive 98/83/EC the product can be approved for sale.

Direct detection of spore density in the product

For the direct detection of the spore density in a product, a spore suspension with a known amount of product is prepared and examined by microscopic techniques.

For this purpose, weigh sterile polypropylene tubes with an analytical balance and, wearing a face mask, safety goggles and gloves, fill a small amount of spore powder product into the tube with a sterilised spatula.

Determine the amount of spore powder by weighing the tube with the spore powder again, then add 1 mL of sterile 0.1 % (w/v) Tween® 80 solution per 10 mg of the spore powder.

Mix thoroughly, then use a Pasteur pipette to fill a hematocytometer (for instance a Thoma chamber; depth = 0.1 mm, area of small squares = 0.0025 mm^2) with the spore suspension.

Count a minimum of 16 "large squares" and at least 1000 spores per sample. Prepare new solution with more product if less than 50 spores per "large square" are counted.

Calculate the average of all counted small squares and multiply by 4×10^6 to give the number of spores per gram product (volume of

one "small square"-unit = 2.5×10^{-7} mL; amount of product per mL = 10 mg × multiplication factor = 4×10^{8}). Also give relative standard deviation as a measure of variance.

Determination of spore viability

A standard operation procedure for the determination of spore viability, based on Hedgecock *et al.* (1995) and Jenkins (1998), was developed in the BIPESCO FAIR6 CT-98-4105 project. This procedure was adapted for use with different products and fungi.

Unless a standardised growth medium for the tested product is available the following media are recommended for the fungal genera listed below:

Beauveria spp.	Sabouraud-2%-Glucose Agar (S2G)
Metarhizium spp.	Sabouraud-Dextrose Agar (SDA)
Paecilomyces spp.	Oatmeal Agar (OA)
Gliocladium spp.	Malt Extract Agar (MEA)
Trichoderma spp.	Potato-Dextrose Agar (PDA)
Verticillium spp.	Potato-Dextrose Agar
Coniothyrium spp.	Oatmeal Agar
Fusarium spp.	Oatmeal Agar
Sclerotinia spp	Oatmeal Agar
Other genera	MEA, OA, PDA, S2G

If the product is contaminated it is necessary to suppress the growth of the contaminant to rule out any interference with spore germination. For *Beauveria brongniartii* a selective Sabouraud-2 %-Glucose Agar (Strasser *et al.*, 1996) is available which can be modified for *Metarhizium anisopliae* by adding 20 g/L glucose. It may also be suitable for other fungal BCAs like *Paecilomyces* spp., *Verticillium* spp. or *Gliocladium* species. Once a suitable medium has been identified the following procedure is used to determine the germinability of the spores.

1. Prepare fresh agar plates with media suitable for the fungi to be detected in the product. When pouring agar plates the use of a dispenser set to 20 mL (for 94 mm petri-dishes) is recommended, as direct microscopic examination of many plates is much easier when the agar surface is at the same level for all studied plates. Six plates of a 5 mm minimum depth are required per test sample. Dry agar after pouring to reduce condensation of water droplets after refrigeration. Plates should not be stored longer than 2–3 weeks.

2. Prepare a sterile polypropylene tube for each sample and weigh each tube on an analytical balance. Wearing face mask, safety goggles and gloves, transfer a small quantity of spores into a sterile

polypropylene tube, then weigh the amount of spore powder (a quantity of about 10 mg is convenient). Based on the determined spore density in the product (see above) add necessary amount of sterile 0.1 % Tween® 80 solution to produce a suspension of roughly 5×10^6 spores per milliliter. Mix thoroughly and allow the spores to re-hydrate for 60 minutes.

3. Transfer and plate 100 µL of the suspension to at least six agar plates per sample. Incubate plates at 25 °C and 60 % rH.

4. After 24 h, examine plates microscopically at 200 to 320 × magnification. Examine every 6–12 h until germination is observable. Note that the time required for germination is both species-specific and medium dependent.

5. Count germinated and non-germinated conidia and record data with two separate tally counters. A germinated spore is defined as a spore forming a germ tube roughly that length of the spore diameter. It is highly recommended to use a petri dish with ungerminated spores, which is stored at 4 °C, as a reference since the recognition of ungerminated spores can be difficult for inexperienced personnel. A total of 3×100 conidia on each of two plates (6 separate counts) per sample should be counted.

6. For each individual count the percentage of germination is calculated using the following formula.

$$Germination\ [\%] = \frac{a}{(a+b)} \times 100$$

a = number of germinated spores; b = number of non germinated spores

7. Calculate the average germination rate and its standard deviation for the six individual counts.

8. Repeat procedure from point 5 to 7 with fresh plates as long as the germination rate increases (counted plates are discarded). The results from the last count represent the actual germination rate of the product.

The germination rate for a spore-based mycoinsecticide should be above 90 % at packaging (Jenkins *et al.* 1998).

Indirect detection of spore density (CFU method)

This method relies on cultivation techniques to establish the amount of viable propagates in a product.

1. For the selection of culture media the same criteria as in chapter "Determination of spore viability" are applied.

2. After selecting and preparing appropriate media weigh a small amount of the product into sterile polypropylene tubes and suspend in 1 mL of sterile 0.1 % (w/v) Tween® 80 *solution* per 10 mg of product.

3. Prepare a dilution series in decimal steps (i.e. 1 : 10, 1 : 100, etc). to a dilution of 10^{-9}, or less depending on the expected spore density.

4. Plate 50 µl of each dilution on 5 replicates of fresh agar plates and incubate at 25 °C and 60 % rH. After 7–10 days count colonies formed by the BCA.

5. Only use the dilution with the number of colonies ranging between 20–100 as more colonies per plate may result in reciprocal inhibition.

$$[CFU\ g^{-1}\ product] = 2 \times \frac{colonies\ per\ plate}{dilution} \times 10^3$$

Calculate results as CFU g^{-1} product using the above formula and give relative standard deviation as a measure of variance.

Colonisation test

The colonisation test is used to assess the purity and viability of a fungal product grown on a carrier such as barley kernels or similar material.

If the product has been fermented in the packaging, a sterile sample has to be taken prior to the colonisation test.

The product bags are disinfected by wiping the surface with 70 % ethanol. Then cut packaging open with a sterile scalpel and take a sample with a flame-sterilised spoon.

Place 7 kernels on Sabouraud-2 %-Glucose Agar plates, half-concentrated Sabouraud-2 %-Glucose Agar plates and Water Agar plates (5 replicates each). Then incubate at 25 °C, 60 % rH for 14 days.

After incubation, examine kernels for bacterial and fungal contaminants. Count kernels by using the following categories.

Fully colonised barley kernel: at least 90 % of the kernel is colonised by the BCA.

Partially colonised barley kernel: 25 %–90 % of the kernel is covered by mycelium of the BCA.

Weakly colonised kernel: Less than 25 % of the kernel surface covered with mycelium.

Contaminated kernel: Contaminant is clearly visible, but the kernel is still dominated by the desired fungal biocontrol agent.

Heavily contaminated kernel: The kernel is completely overgrown or at least dominated by the contaminant.

For a fresh, packaged product to be approved for sale, a minimum of 80 % of kernels should be partly to fully colonised with the BCA grown on Sabouraud-2 %-Glucose Agar. On water agar, this rate should be above 40 % for kernels or similar carriers.

Less than 10 % of kernels should be in the category "Contaminated kernel", none in the category "Heavily contaminated kernel".

Strain identity

Various molecular methods are available for the identification of specific strains of fungi. The details cannot be discussed in this context, but a method based on microsatellite DNA markers for the identification *B. brongniartii* strains, for example, has been developed during the BIPESCO FAIR6-CT89-4105 EU project (Enkerli *et al.* 2001).

If molecular methods are not available, BCA strains can also be identified by their carbon utilisation patterns using the BIOLOG™-system (Pernfuss *et al.* 2003).

Storage stability and shelf life

The product stability is monitored at –20 °C, +4 °C, +10 °C and +25 °C in order to gain information on the kinetics of product degradation.

Place approximately 5 g of product in inert containers (e.g. polypropylene tubes). Prepare at least 20 containers with the tested product per temperature to allow a prolongation of the experiment beyond the planned time frame when this seems indicated, and to ensure that spare samples are available in case a problem occurs.

Perform quality control procedures listed in this publication at the start of the experiment, after 3 months, 6 months, 9 months and 12 months. Sample one container per date and temperature. Use each container only once.

Plot a degradation curve to determine suggested shelf life of the product and optimal storage conditions.

References

Council of the European Union (1998) Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Communities*. L 330: 32–54.

Commission of the European Communities (2003) Proposal for a Council Directive amending Annex VI to Directive 91/414/EEC as regards plant protection products containing micro-organisms. Document COM(2003) 814 final, Brussels, 22 Dec 2003. http://europa.eu.int/eur-lex/en/com/pdf/2003/com2003_0814en01.pdf

Enkerli J., Widmer F., Gessler C., Keller S. (2001) Strain-specific microsatellite markers in the entomopathogenic fungus *Beauveria brongniartii*. Mycological Research **105**: 1079–1087.

International Biocontrol Manufacturers Association (2003) IBMA position regarding the revision of the European Directive 91/414/EEC. http://www. ibma.ch/pdf/20030905_revision_91414_positionibma.pdf

Jenkins N. E., Heviefo G., Langewald J., Cherry A. J., Lomer C. J. (1998) Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information* **19**: 21–31.

Laengle T., Raffalt J., Pernfuss B., Strasser H. (2005) Appendices: Risk Assessment of Fungal Biocontrol Agents Standards. C: Biotests and field efficacy trials of fungal BCAs against scarabaeid larvae. *Sydowia* **57** (1): 84–93.

Pernfuss B., Schweigkofler W., Strasser, H. (2003) Distinction of the entomopathogenic fungal species *Beauveria brongniartii* and *Beauveria bassiana* by comparing their carbon utilization patterns. *IOBC/wprs Bulletin* **26** (1): 121–124.

Strasser H., Forer A., Schinner F. (1996) Development of media for the selective isolation and maintenance of virulence of *Beauveria brongniartii*. In: Jackson T., Glare T. (Eds.) *Microbial Control of Soil Dwelling Pests*. AgResearch, Lincoln, New Zealand: 125-130.

ANNEX I: Recipes for the preparation of media

Tween® 80 Solution 0.1 % (w/v)

Dissolve 1 g of Tween® 80 in deionised water and and sterilise for 20 min at 121 °C/1.2 bar. The solution has a milky appearance after autoclaving and will become clear when cooling down.

Water Agar

15 g Agar
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar.

Malt Extract Agar

20 g Malt extract
20 g Glucose
1 g Peptone for Soybeans
15 g Agar
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar.

Half-concentrated Sabouraud-2%-Glucose Agar

15 g Sabouraud-2 %-Glucose-Bouillon
(MERCK 108339.0500)
15 g Agar
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar. The medium has a clear yellowish appearance after sterilisation. If the medium turns dark brown it has been overheated and has to be disposed.

Sabouraud-2%-Glucose Agar

30 g Sabouraud-2%-Glucose-Bouillon
(MERCK 108339.0500)
15 g Agar
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar. The medium has a clear yellowish appearance after sterilisation. If the medium turns dark brown it has been overheated and has to be disposed.

Beauveria-selective Sabouraud-2%-Glucose Agar

Prepare Sabouraud-2 %-Glucose Agar supplemented with Cycloheximde (70 mg L^{-1}), Streptomycin (100 mg L^{-1}), Tetracycline (50 mg L^{-1}) and Dodine (100 mg L^{-1}). The antimicrobials are added as an adequate sterile filtered stock solution, except for Dodine, which is dissolved in 1.5 mL ethanol 96 % (v/v) and then added to the medium. Make sure that the temperature of the agar is in the range of 50 °C – 60 °C upon addition of the stock solution.

Sabouraud-Dextrose Agar

30 g Sabouraud-2%-Glucose Bouillon
(MERCK 108339.0500)
20 g Glucose
15 g Agar
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar. The medium has a clear yellowish appearance after sterilisation. If the medium turns dark brown it has been overheated and has to be disposed.

Metarhizium-selective Sabouraud-Dextrose Agar

Prepare Sabouraud-Dextrose Agar supplemented with Cycloheximde (70 mg L^{-1}), Streptomycin (100 mg L^{-1}), Tetracycline (50 mg L^{-1}) and Dodine (CAS-No. 2439-10-3; 100 mg L^{-1}).

The antimicrobials are added to autoclaved (20 min at 121 °C, 1.2 bar) Sabouraud-Dextrose Agar from an adequate sterile filtered stock solution, except for Dodine, which is added dissolved in 1.5 mL. Make sure that the temperature of the agar is in the range of 50 °C – 60 °C upon addition of the stock solution.

CASO Agar

5 g Sodium Chloride
5 g Peptone for Soybeans
15 g Peptone from Casein
15 g Agar
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar.

Oatmeal Agar

30 g Oat flakes
15 g Agar
1 L deionised water

Boil oat flakes in water for 10 min, fill up to 1 L and add agar. Sterilise for 20 min at 121 °C, 1.2 bar.

Potato-Dextrose Agar

39 g Potato-Dextrose Agar
(MERCK 1.10130.0500)
1 L deionised water

Sterilise for 20 min at 121 °C, 1.2 bar.