THE FERTILIZER INSTITUTE'S

HANDBOOK OF AGRICULTURAL MICROBIOLOGY

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Foreword

Agricultural microbial products, or “microbials,” include biostimulants, biofertilizers, biopesticides, and other products intended for use in agricultural applications which claim living microorganisms on their labels. While these products are not new to the market – the first agricultural microbial product, Nitragin, was patented in 1895 – they have begun to receive increasing attention as growers look for ever more effective options to mitigate yield-limiting factors in a changing world. With this increase in attention has come increased scrutiny by consumers and regulators alike, and much attention has rightly been paid to confirming that microbials deliver their claimed benefits. However, efficacy confirmation is only one piece of the puzzle. In order to be effective and deliver consistent performance, a microbial product must be properly formulated at the time of manufacture and remain stable throughout the supply chain in order to reach the grower’s hands in saleable condition. For this reason, composition verification for microbials is a topic of interest for manufacturers, regulators, and consumers alike.

In 2016, the Oregon Department of Agriculture published a report[1] which appeared to expose widespread label claim disagreement among microbials, calling out examples of agricultural formulations which contained too few microbes – or no microbes at all[1]. While no industry is completely free of unscrupulous actors, many agricultural microbiologists cried foul due to uncertainty surrounding the ODA’s choice of microbiological laboratory methods. The negative results, some argued, could reasonably have been attributable to improper method selection instead of genuinely poor product quality. This situation serves as an example of the disagreement that can arise between the manufacturers of microbial products and the testing laboratories which evaluate their composition. While it will not be the case in every situation, methodological limitations can certainly lead to a disconnect between a microbial product’s true properties and the data presented in a laboratory report. In extreme cases, this can lead to the erroneous rejection of a product lot on the presumption of low microbial activity or mis-formulation when, in fact, it was the inability of the testing method to accurately characterize the product's composition that led to the unflattering result.

Some of this disagreement may be attributable to the absence of standardized and validated methods specifically intended to evaluate agricultural microbials. Many of the standardized assays used to evaluate these products were originally developed for use in other industries, such as environmental monitoring, food safety, pharmaceuticals, and personal care products. These methods are often generic enough to be used with confidence across multiple applications; however, in some cases, tried-and-true lab methods from other industries have limitations which impede their ability to accurately characterize agricultural assemblages. It can be understandably challenging for laboratories to anticipate these complexities, especially fertilizer laboratories that are not accustomed to running microbiological assays.

In order to minimize the occurrence of “bad lab results for good microbials,” the industry must be proactive in our proposal of accurate testing methods that can be used by laboratories to provide fair, accurate, science-based evaluations of quality among microbial products. A conscientious manufacturer will have conducted numerous quality assurance and stability evaluations of their product before commercialization, and this collective

methodological expertise among agricultural microbiologists presents a valuable and often untapped resource for laboratory personnel. In 2022, The Fertilizer Institute launched a Microbiological Laboratory Methods Task Force to examine the suite of laboratory methods that are commonly applied to microbials, and to determine which of these familiar standard methods were appropriate for use with agricultural workhorses such as *Azospirillium, Pseudomonas, Rhizobium, Trichoderma*, and *Bacillus*. Additionally, the group was charged with reviewing novel laboratory methods that were known in published academic literature – but had not yet been validated and standardized in any methodological compendium – for consideration by laboratory personnel. The task force was comprised of subject matter experts from the agricultural microbiology and testing lab industries, respectively, ensuring a wide range of knowledge and a diversity of perspectives. The group's goal was simple: to develop a comprehensive reference document for laboratory scientists that are unfamiliar with agricultural microbiology, reducing the occurrence of "bad lab results for good microbials."

The Fertilizer Institute's Handbook of Agricultural Microbiology, which includes the combined expertise of more than a dozen agricultural microbiologists, is our first step towards achieving this goal. Within this document you will find background information about common agricultural microorganisms and the various methods used to evaluate products containing these species. This volume covers enumeration assays, identification methods for isolated organisms, and contamination screening methods. Cost estimations are issued with regulatory labs in mind: wherever possible, we have tried to set reasonable expectations regarding the cost of analysis based on the price of a single sample submitted to a 3rd party lab for testing, as opposed to relying on bulk pricing or the cost of running a method in-house with access to specialized equipment. We hope that this resource helps fertilizer laboratories select the best tools for the job when they are tasked with characterizing microbials, helping the industry elevate well-formulated products while calling out products that miss the mark – without incurring the kind of methodological disagreements that have often complicated this process in the past.

Best Regards,

John P. Gorsuch
Director of Research & Development: *BiOWiSH Technologies*
Chair: *The Fertilizer Institute's Microbiological Laboratory Methods Task Force*

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Chapter 1
Common Agricultural Mircoorganisms

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Agricultural microbial products, or "microbials," include biostimulants, biofertilizers, biopesticides, and other products intended for use in agricultural applications which claim living microorganisms on their labels. Microbials are populated with a diverse cast of microscopic characters. Some organisms, like *Rhizobium*, have been known to agronomists for over a century and are common in microbial formulations. Other organisms, such as cyanobacteria and archaea, are relatively scarce in the market. In this section we will introduce you to some of the most common organisms found in microbial formulations, as well as a few uncommon organisms. This section will also include guidance regarding how best to evaluate products containing these microorganisms, with more details on the cited methodologies provided in subsequent chapters. In the event that your lab encounters organisms not listed here, please contact the TFI Microbiology Laboratory Methods Task Force at **biologics@tfi.org**.

Actinomycetes

General Information: A group of Gram-positive bacteria which were originally believed to be fungi. They are famous for their production of antibiotics.

Agricultural Significance: Actinomycetes such as *Streptomyces* are common in microbial biostimulant formulations due to their expression of plant-beneficial traits and their contribution to soil ecosystems.

Microbiologist's Notes: *Streptomyces* and other Actinomycetes can be enumerated using ISO 4833 (**page 22**). Other culture media such as Actinomycete Isolation Agar (**page 38**) and Antibiotic Agar may also be used, but methods based on these media are not validated in the ISO or AOAC compendia.

Arbuscular Mycorrhizal Fungi

General Information: Also known as AMF, this group of endosymbiotic fungi forms intimate associations with a plant's cortical root cells. Most are obligate endophytes, meaning they are not culturable independently of their host plants, complicating efforts to enumerate them in the laboratory.

Agronomic Significance: AMF networks are often disrupted by conventional farming practices. Because many crop types perform better in the presence of AMF symbionts, these organisms are common in microbial biostimulant formulations. They are known in the academic literature to improve nutrient uptake and drive soil carbon sequestration in addition to their plant growth promotion properties.

Microbiologist's Notes: Although no standard methods exist in the ISO or AOAC compendia for the enumeration of AMF, at the time of this writing the European Biostimulants Industry Council (EBIC) is in the process of proposing a standardized method for their enumeration in commercial products. While the industry awaits the arrival of a validated, standardized method for AMF enumeration, the academic literature contains bioassays (**page 46**) for the enumeration of these organisms. For more information regarding AMF formulations, please consult Appendix IV – Notes on Units of Potency for AMF Formulations (**page 45**).

Azospirillium

General Information: The genus *Azospirillium* consists of Gram-negative bacteria famous for their ability to fix nitrogen.

Agronomic Significance: Microbial nitrogen fixation, common among *Azospirillium* species, is a key plant growth promoting trait that can help supplement nitrogen deficiencies.

Microbiologist's Notes: Members of the genus *Azospirillium* can be cultured on *Azospirillium* Agar (**page 28**); however, methods based on this medium are not validated in the ISO or AOAC compendia. At the time of this writing, the European Biostimulants Industry Council (EBIC) is in the process of proposing a standardized method for their enumeration in commercial products.

Azotobacter

General Information: The genus *Azotobacter* consists of Gram-negative bacteria famous for their ability to fix nitrogen.

Agronomic Significance: Microbial nitrogen fixation, common among *Azotobacter* species, is a key plant growth promoting trait that can help supplement nitrogen deficiencies.

Microbiologist's Notes: Members of the genus *Azotobacter* can be cultured on *Azotobacter* Agar (**page 39**); however, methods based on this medium are not validated in the ISO or AOAC compendia. At the time of this writing, the European Biostimulants Industry Council (EBIC) is in the process of proposing a standardized method for their enumeration in commercial products. Some members of this genus form colonies on plates of culture medium which produce capsular slime, potentially leading to underestimation of their concentration by plate counting assays due to colony merging (see Appendix II, **page 40**).

Burkholderia

General Information: A widespread group of Gram-negative soil bacteria which includes beneficial species as well as several well-known pathogens. Only the beneficial species are dealt with here.

Agronomic Significance: Plant-beneficial strains of *Burkholderia* can stimulate the production of plant root exudates and are known to produce plant-beneficial compounds including iron-binding siderophores.

Microbiologist's Notes: Some species of *Burkholderia* are culturable on MacConkey's agar and similar formulations, and these may be enumerated using methods such as ISO 21528 (**page 22**). Other culture media formulations are known in the literature, but methods based on these media are not validated in the ISO or AOAC compendia.

Bacillus

General Information: A widespread group of Gram-positive soil bacteria which are famous for their ability to form endospores. These are hardy, dormant cells which are resistant to a wide range of stressors that would kill active bacterial cells.

Agronomic Significance: *Bacillus* species produce a wide range of plant-beneficial metabolites. Some, such as *B. thuringiensis*, can produce insecticidal compounds and are popular in biopesticide formulations. Others can produce fungicidal compounds and are popular in biofungicide preparations.

Microbiologist's Notes: *Bacillus* are culturable on a variety of media and can be enumerated using ISO 4833 (**page 22**) or EN 15784:2021 (**page 19**). Some *Bacillus* strains form colonies which produce alginate and other organic extracellular compounds on laboratory culture medium, while others form "spreading" colonies that can merge together, potentially leading to underestimation of their concentration by plate counting assays (see Appendix II, **page 40**). Culture media formulations which control these variables are known in the literature, but methods based on these media are not validated in the ISO or AOAC compendia.

Methylobacterium

General Information: A group of Gram-negative soil bacteria which sometimes form striking pink colonies on laboratory culture media. Members of this genus are sometimes referred to as pink pigmented facultative methylotrophs, or PPFM.

Agronomic Significance: Plant-beneficial strains of *Methylobacterium* have been shown to produce a variety of plant-beneficial compounds and impart tolerance to abiotic stress in their host plants, a phenomenon known as Induced Systemic Resistance.

Microbiologist's Notes: *Methylobacterium* may be enumerated using ISO 4833 (**page 22**). Other culture media formulations are known in the literature, but methods based on these media are not validated in the ISO or AOAC compendia.

Pseudomonas

General Information: A widespread group of Gram-negative soil bacteria which includes beneficial species as well as several well-known pathogens. Only the beneficial species are dealt with here.

Agronomic Significance: *Pseudomonas* are famous for their plant-beneficial traits, such as the production of iron-chelating siderophores, solubilization of bound phosphorous, production of phytohormones, and induction of systemic resistance.

Microbiologist's Notes: *Pseudomonas* may be enumerated using ISO 4833 (**page 22**) in cases where they are the only formulated G- in the formulation. In cases where their populations must be treated separately from other microbes in an intermediate or complex microbial product (**page 12**), methods such as ISO 13720 (**page 22**) may be more appropriate due to their use of selective culture media.

Rhizobium

General Information: The genus *Rhizobium* consists of Gram-negative bacteria famous for their ability to fix nitrogen, often through endosymbiotic associations with plant roots. This genus was the first to be commercialized as an agricultural microbial, patented under the trade name Nitragin in 1895.

Agronomic Significance: Microbial nitrogen fixation, common among *Rhizobium* species, is a key plant growth promoting trait that can help supplement nitrogen deficiencies

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Microbiologist's Notes: Members of the genus *Rhizobium* can be cultured on *Rhizobium* Agar (**page 39**) and Yeast Extract Mannitol Agar (page 33); however, methods based on these media are not validated in the ISO or AOAC compendia. At the time of this writing, the European Biostimulants Industry Council (EBIC) is in the process of proposing a standardized method for their enumeration in commercial products.

Saccharomyces

General Information: Members of the genus *Saccharomyces* are single-celled saprotrophic yeasts common in a variety of industrial applications.

Agronomic Significance: *Saccharomyces* species are known to produce several plant-beneficial compounds and may also serve as biocontrol agents against nematodes and other pathogens.

Microbiologist's Notes: Members of the genus *Saccharomyces* can be enumerated using ISO 21527-2 (**page 21**). Method EN-15789:2021 (**page 19**) is validated specifically for the enumeration of *S. cerevisiae*.

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Trichoderma

General Information: *Trichoderma* are a widespread group of soil fungi and are often among the most abundant culturable fungi in environmental samples.

Agronomic Significance: Some *Trichoderma* species are used as biocontrol agents against fungal pathogens. Some are also opportunistic plant symbionts that may induce systemic resistance in their hosts.

Microbiologist's Notes: Members of the genus *Trichoderma* can be enumerated using ISO 21527-2 (**page 21**).

Penicillium

General Information: *Penicillium* is a widespread genus of soil fungi which includes producers of the antibiotic penicillin.

Agronomic Significance: Some *Penicillium* species are known to solubilize phosphorous in the soil, and to produce plant-beneficial compounds, including the phytohormones auxin and gibberellin.

Microbiologist's Notes: Members of the genus Penicillium can be enumerated using ISO 21527-2 (**page 21**).

Cyanobacteria

Cyanobacteria are a group of autotrophic Gram-negative bacteria, including some organisms which promote the growth of plants or impart resistance to stress and disease. As autotrophs, these organisms require specialized growth media and techniques to culture in the laboratory. Culture-independent techniques such as flow cytometry (ISO 19344, **page 21**) and PCR-based methods are therefore preferred for enumerating cyanobacteria.

Archaea

Archaea are prokaryotic microorganisms which outwardly resemble bacteria, but which are distinct enough to warrant their own taxonomic Domain. Many archaea express plant-beneficial traits similar to those expressed by bacteria. Some archaea may be cultured on specialized microbiological media, while others are nonculturable. In cases where nonculturable archaea are suspected, culture-independent techniques such as flow cytometry (ISO 19344, **page 21**) and PCR-based methods (**page 34**) may be considered.

Bacteriophages

Bacteriophages, or “phages” for short, are viruses which selectively infect bacteria. They are often strain-specific, making them attractive for biocontrol applications. These claims can be evaluated using plaque assays (**page 36**) which incorporate the phage’s host species as a growth substrate. Clearance zones, or plaques, are then counted by a microbiologist.

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Chapter 2
Types of Microbial Formulations

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Agricultural microbial formulations range from simple to complex in their biological composition. **Simple microbial formulations**, such as the hypothetical examples presented in Figure 1 below, contain only one microbial species or group on their labels. These products are comparatively easy to evaluate, as all of the product's constituent microbes will have the same culture requirements and are thus generally amenable to characterization with a single enumeration method.

Product	Claimed Microorganisms	Claimed Viable Cell Concentration
Simple Microbial #1	*Pseudomonas fluorescens*	2.0E+09 CFU/g
Simple Microbial #2	*Bacillus subtilis*	2.5E+08 CFU/g
	Bacillus pumilus	2.5E+08 CFU/g
	Bacillus velezensis	2.5E+08 CFU/g
	Bacillus licheniformis	2.5E+08 CFU/g

Figure 1 – Examples of simple microbial formulations.

Intermediate microbial formulations, such as the hypothetical examples presented in Figure 2 below, contain multiple microbial groups on their labels; however, all groups within these formulations are compatible with standard, validated laboratory testing methods. In order to evaluate each population within the mixture, it may be necessary to employ multiple target-specific methods (**page 13**) to selectively evaluate each **testable sub-population** within the overall assemblage.

Product	Claimed Microorganisms	Claimed Viable Cell Concentration
Intermediate Microbial #1	*Bacillus subtilis*	1.0E+09 CFU/g
	Pseudomonas fluorescens	2.0E+09 CFU/g
	Rhizobium leguinosarum	2.0E+09 CFU/g
Intermediate Microbial #2	*Azospirillium brasilense*	2.5e+07 CFU/g
	Methylobacterium aquaticum	5.0E+07 CFU/g
	Bradyrhizobium japonicum	5.0E+07 CFU/g
	Saccharomyces cervesiae	1.0E+08 CFU/g
	Trichoderma longibrachiatum	1.0E+08 CFU/g

Figure 2 – Examples of intermediate microbial formulations.

Complex microbial formulations, such as the hypothetical example presented in Figure 3 below, contain multiple microbial groups on their labels; however, they are formulated in ways that make them difficult for a microbiologist to evaluate using standard, validated laboratory methods. These often contain organisms that are not culturable in the laboratory and may include numerous species belonging to diverse taxonomic groups. Note that nonculturable species are often listed with "colony forming units" as their unit of potency even though they are not capable of colony formation on laboratory medium. In cases where these products claim excessive numbers of species per microbial group, efforts to enumerate each sub-population can become complicated. It may be necessary to employ multiple target-specific methods (**page 17**), target-agnostic methods (**page 17**), and next-generation sequencing methods (**page 36**) to selectively evaluate each testable sub-population within the overall assemblage. In cases where the product's manufacturer is unable to provide methodological guidance for the evaluation of their complex assemblage, please contact the TFI Microbiology Laboratory Methods Task Force at **biologics@tfi.org.**

Product	Claimed Microorganisms	Microbial Group	Claimed Viable Cell Concentration
Complex Microbial #1	*Pisolithus tinctorius*	Ectomycorrhizal fungi	1.9e+05 prop/g
	Rhizopogon luteolus		5.2E+03 prop/g
	Rhizopogon fulvigleba		5.2E+03 prop/g
	Rhizopogon villosullus		5.2E+03 prop/g
	Rhizopogon amylopogon		5.2E+03 prop/g
	Scleroderma citrinum		5.2E+03 prop/g
	Scleroderma cepa		5.2E+03 prop/g
	Glomus aggregatum	Endomycorrhizal fungi	83 prop/g
	Glomus intraradices		83 prop/g
	Glomus mosseae		83 prop/g
	Glomus etunicatum		83 prop/g
	Glomus clarum		11 prop/g
	Glomus monosporum		11 prop/g
	Glomus deserticola		11 prop/g
	Gigaspora margerita		11 prop/g
	Trichoderma koningii	Ascomycete fungi	1.9E+05 CFU/g
	Trichoderma harzianum		1.3e+05 CFU/g
	Bacillus subtilis	Gram positive bacteria	5.3E+05 CFU/g
	Bacillus thuringiensis		5.3E+05 CFU/g
	Bacillus licheniformis		5.3E+05 CFU/g
	Bacillus azotoformans		5.3E+05 CFU/g
	Priestia megaterium		5.3E+05 CFU/g
	Bacillus coagulans		5.3E+05 CFU/g
	Bacillus pumilus		5.3E+05 CFU/g
	Bacillus amyloliquefaciens		5.3E+05 CFU/g
	Paenibacillus polymyxa		5.3E+05 CFU/g
	Paenibacillus durum		5.3E+05 CFU/g
	Pseudomonas aureofaciens	Gram negative bacteria	5.3E+05 CFU/g
	Pseudomonas fluorescens		5.3E+05 CFU/g
	Azotobacter chroococcum		5.3E+05 CFU/g
	Saccharomyces cervisiae	Yeasts	5.3E+05 CFU/g

Figure 3 – An example of a complex microbial formulation.

Regardless of the type of microbial product presented to your laboratory for evaluation, the task before you will involve confirming or falsifying the hypothesis that the product is formulated with the microbes claimed on the product label at or above the claimed activity level and is free of non-targeted organisms (NTO). Each of these components of label claim agreement is discussed separately in Chapter 3.

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Chapter 3
Components of Label Claim Agreement

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Agricultural microbials all share a single unifying feature: they contain living microorganisms that are listed on the product label with accompanying units of biological potency. A testing laboratory tasked with evaluating a microbial product must consider three aspects of label claim agreement: enumeration of viable cells, identification of isolated cells, and screening for non-targeted organisms (NTO). Each component of label claim agreement is discussed in further detail below, along with background information regarding the terminology and types of laboratory assays associated with each component.

Enumeration of Viable Cells

Enumeration assays determine the concentration of viable cells in a product sample. Depending on the microbe, biological activity may be expressed as **Colony-Forming Units (CFU)**, commonly used for culturable microorganisms, **Active Fluorescence Units (AFU)**, used to describe viable cells detected via flow cytometry, **propagules**, commonly used for mycorrhizal fungi, and **Plaque-Forming Units (PFU)**, commonly used for bacteriophage viruses (which are not properly "cells," but we will consider them alongside prokaryotic and eukaryotic cells for our purposes here). The concentration of viable cells detected during an enumeration assay must meet or exceed the concentration claimed on the product's label. Enumeration methods are discussed in greater detail in Chapter 4.

Identification of Isolated Cells

Identification techniques ensure that the cells detected during an enumeration assay belong to the microbial species claimed on the product label. These techniques are applied to an isolated colony of bacteria or fungus from a plate of solid medium used during the product's enumeration assay. These assays will vary in their taxonomic resolution, with some achieving confident identification only to the level of genus, while others can achieve confident identification at the level of species. Microbial identification methods are discussed in greater detail in Chapter 5.

Contamination Detection

Contamination detection methods ensure that the product is reasonably free of non-targeted organisms or NTO. NTO may enter microbial formulations at the time of manufacture, during blending and packing, or may enter as spoilage organisms during the supply chain. Contamination Detection Methods are discussed in greater detail in Chapter 6.

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Chapter 4
Microbiological Enumeration Assays

*Please note that the estimated prices included in this chapter reflect expectations at the the time of this Handbook's publication and may not reflect pricing fluctuations in the market.

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Background Information

An “enumeration” is defined as a complete, accurate listing of all items in a collection. In microbiology, enumeration is used to define the act of estimating the concentration of viable cells within a tested sample. Enumeration assays may be broken down into several categories based on the method of cell detection they employ, and their degree of target specificity.

Culture dependent enumeration methods rely upon the ability of the targeted microorganism to grow on synthetic culture medium under laboratory conditions. Usually, this involved culturing a visible colony of the microbe in question on a plate of solid culture medium or in a tube of liquid medium, which is then incubated under conditions (temperature, oxygen availability, etc.) appropriate for the targeted organism. After a suitable incubation period, microbial growth is assessed by a microbiologist. **Aerobic Plate Count (APC)** and **Most Probable Number (MPN)** assays are familiar examples of culture-dependent methods.

Note that culture dependent assays are unable to detect **Viable but Not Culturable (VBNC)** cells. This term refers to bacterial cells that are in a state of very low metabolic activity, generally resulting from stress encountered during manufacturing or during the supply chain. Because they do not divide, VBNC cells are incapable of forming visible colonies on laboratory culture medium and thus cannot be enumerated using culture-based assays. However, VBNC cells remain alive and are capable of resuming normal metabolic activity under optimal environmental conditions. If VBNC cells are suspected in a given product, culture-independent enumeration methods should be considered to confirm their presence.

Culture independent methods rely on factors other than cell growth, such as the abundance of a targeted DNA sequence or the concentration of membrane-intact cells in a sample, to render an enumeration value. Direct microscopic counts (also known as direct-count microscopy), flow cytometry and PCR-based methods, such as Real-Time PCR (RT-PCR) and droplet digital PCR (ddPCR), are examples of culture-independent assays. These methods can be used to enumerate VBNC cells as noted above but are also appropriate for microorganisms that are not culturable under any conditions in the laboratory.

Microbiological enumeration methods may also be categorized based upon their degree of specificity to one or more microbial groups. **Target-specific** enumeration assays will enumerate only a targeted microorganism or microbial group. In culture-dependent assays, this specificity may be achieved through the use of selective microbiological media or the application of a selected stressor (such as a pasteurization step to isolate endospores), or through the use of custom oligonucleotides or antigens in culture-independent assays. **Target-agnostic** enumeration assays will evaluate all viable microbial cells within a tested sample that are compatible with the selected methodology. These assays may be culture-dependent (e.g., APC and Petrifilm® assays) or culture-independent (e.g., flow cytometry).

Aerobic Plate Counting Assays

An **aerobic plate count**, or **APC assay** is a type of microbiological enumeration method that involves the repeated dilution of a tested sample in sterile diluent. This dilution is repeated in series until a targeted concentration of microbial cells is expected to be present per milliliter of diluent. Plate counting assays may be executed using **pour-plate** or **spread-plate** techniques. The former involves suspending a small sample of diluent in molten agar within a Petri dish, which is then allowed to solidify, while the latter involves spreading a small sample of diluent across the surface of a Petri dish containing solidified culture medium. Plates are then incubated at a prescribed temperature and for a prescribed length of time, after which they are removed and examined by a microbiologist. The microbiologist then counts colonies of microorganisms which have formed on or within the culture medium and combines this count with the dilution factor of the sample to report the initial microbial activity of the sample.

The APC assay has been a workhorse of microbial enumeration for over a century and continues to be the most popular enumeration assay across most of the world. Despite its lengthy tenure and widespread use, the APC assay has developed a well-deserved reputation for returning variable data. Unlike the physical and chemical methods commonly used in fertilizer testing laboratories, the APC assay is not a precise analytical tool. The late Dr. Scott Sutton once referred to APC data as an "interpretation of an approximation of microbial activity" due to the method's reliance on composite sampling, serial dilution, and subjective interpretation of colony counts on agar plates[2]. In their seminal 1916 paper on plate counting, researchers Breed and Dotterer[3] allowed estimations of microbial activity derived from three replicate countable plates to deviate from the overall mean by 20%, with individual countable plates being allowed to deviate from the overall mean by 30%. In 1992, Weenk[4] estimated that 5% of this error was attributable to dilution and pipetting error. When working with microorganisms that do not form large, spreading colonies (see below), it is generally held that increasing the number of colonies per countable plate to levels approaching the assay's upper limit of quantification (LOQ) and increasing the number of replicate countable plates per sample can help minimize this variance and produce results in closer agreement with the true activity of the sample[2] [3] [4].

Validated Microbial Enumeration Methods

In this section, we will provide a listing of validated, standardized microbial enumeration assays. Most are variations of the APC assay that utilize different microbiological media to target different agriculturally relevant organisms. **Note**: In each case, a fertilizer testing laboratory may find it convenient to send their samples out to a 3rd party testing laboratory rather than attempting to purchase, onboard, and validate a new and unfamiliar method in their own facility. With this in mind, each of the method summaries below contains an **estimated** cost at a 3rd party laboratory based on feedback from the testing laboratory personnel who sit on the TFI Microbiological Laboratory Methods Task Force. Note that if your lab already has the equipment to run these assays, the cost per sample will likely decrease; however, this manual is written with the non-specialist in mind and, thus, cost estimates are offered with the intent to cover outsourced testing for a small number of samples.

AOAC 990.12 – Aerobic Plate Count in Foods

What is it? A Petrifilm® assay which enumerates populations of culturable microorganisms in tested samples. Rather than forming colonies on a Petri plate, microbes subjected to this assay will trigger a color change via an indicator dye within a thin film of agar when they grow, leading to a scattering of dots on the film which can be counted by a microbiologist.

Appropriate Organisms: Any microorganism that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: Although this is not a traditional plate counting assay, because it involves serial dilution and colony count interpretation it is subject to the same general set of limitations (**page 18**). Because this method does not involve the formation of visible bacterial colonies, this method is only appropriate for the confirmation of total microbial cell counts and not for confirmation that these cells belong to the species claimed on the product label.

EN 15789 - Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of *Saccharomyces cerevisiae* used as feed additive.

What is it? A plate counting assay which enumerates populations of *Saccharomyces cerevisiae* in tested samples. Selective medium (YGC agar) is used to exclude the growth of other microorganisms.

Appropriate Organisms: *Saccharomyces cerevisiae*.

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. Assays which rely on selective microbiological medium carry some degree of risk for returning false positives.

EN 15784 - Detection and Enumeration of *Bacillus* species used as feed additives.

What is it? A plate counting assay which enumerates populations of *Bacillus* endospores (a hardy, dormant cell type formed by *Bacillus* and other endospore-forming bacteria) in tested samples. This method does not rely upon selective microbiological medium to enumerate *Bacillus* cells; rather, it employs a 0.2% NaOH shock to eliminate microbial cells that are not endospores. Any cells which remain culturable after this treatment are considered to be endospores.

Appropriate Organisms: Any mesophilic, aerobic, endospore-forming microorganism (*Bacillus, Paenibacillus, Lysinibacillus,* etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. The NaOH treatment prescribed by this method may be rigorous enough to kill some endospores, resulting in an underestimation of microbial activity; therefore, a heat treatment (80°C for 15-20 minutes) may be preferable where allowed. See **pages 40** for guidance regarding management of spreading type *Bacillus* colonies.

FDA BAM Chapter 3 - Aerobic Plate Count

What is it? A plate counting assay which enumerates populations of culturable bacteria and fungi in tested samples.

Appropriate Organisms: Any microorganism that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly.

FDA BAM Chapter 8 - Enumeration of Yeasts and Molds.

What is it? A plate counting assay which enumerates populations of culturable fungi (yeasts and molds) in tested samples. Selective microbiological media such as acidified Potato Dextrose Agar (PDA) or Dichloran Rose-Bengal Chloramphenicol (DRBC) agar are used to exclude the growth of most bacteria, allowing the targeted enumeration of yeasts and molds.

Appropriate Organisms: Any yeast or mold that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. Assays which rely on selective microbiological medium carry some degree of risk for returning false positives (**page 44**). This method can be used for several purposes: it can be used to enumerate populations of fungi that are claimed on a product label, or to confirm the absence of contamination by culturable yeasts and molds as part of a comprehensive contamination screening strategy.

ISO 13720 - Meat and meat products — Enumeration of presumptive *Pseudomonas* spp.

What is it? A plate counting assay which enumerates populations of *Pseudomonas* in tested samples. Selective CFC/CN medium is used to exclude the growth of other microorganisms, allowing the targeted enumeration of *Pseudomonas* populations.

Appropriate Organisms: Any member of the genus *Pseudomonas*.

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. Assays which rely on selective microbiological medium carry some degree of risk for returning false positives (**page 44**). This method can be used for several purposes: it can be used to enumerate populations of *Pseudomonas* that are claimed on a product label, or to confirm the absence of contamination by *Pseudomonas* as part of a comprehensive NTO screening strategy. Note that sample preparation and setup procedures for food products may differ from those appropriate for microbial products. A testing laboratory with an R&D microbiology team may be best equipped to handle this kind of modification to methodological prescriptions.

ISO 19344 - Enumeration of probiotics using flow cytometry

What is it? A culture-independent (**page 17**) and target-agnostic (**page 17**) biophysical enumeration assay that uses a mixture of membrane permeable and impermeable dyes to treat a tested sample. These dyes create a profile of the sample's cell population, describing whether they are intact ("live"), semi-permeable ("injured") or fully permeable ("dead"). The treated sample is then read by a flow cytometer, providing quantification of each population. Results are returned with units of **Active Fluorescence Units (AFU)** per gram or milliliter of tested sample.

Appropriate Organisms: Any microorganism can be evaluated by flow cytometry.

Estimated Cost at 3rd Party Labs: Up to $250 per sample depending on the testing laboratory and the volume of samples submitted.

Microbiologist's Notes: Because flow cytometry involves neither the formation of visible bacterial colonies nor a sequencing-based approach to identification, this method is only appropriate for the confirmation of total microbial cell counts in a product. Because it is target agnostic, it cannot be used to confirm that these cells belong to the species claimed on the product label.

ISO 21527.2 - Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and molds.

What is it? A plate counting assay which enumerates populations of culturable fungi (yeasts and molds) in tested samples. Selective DG18 agar is used to exclude the growth of bacteria, allowing the targeted enumeration of yeasts and molds. This method is validated only for products with water activity values greater than or equal to 0.60.

Appropriate Organisms: Any yeast or mold that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. Assays which rely on selective microbiological medium carry some degree of risk for returning false positives (**page 44**). This method can be used for several purposes: it can be used to enumerate populations of fungi that are claimed on a product label, or to confirm the absence of contamination by culturable yeasts and molds as part of a comprehensive NTO screening strategy.

ISO 21528 - Microbiology of the food chain — Horizontal method for the detection and enumeration of Enterobacteriaceae.

What is it? A plate counting assay which enumerates populations of Enterobacteriaceae (a family of bacteria which includes *Enterobacter, Citrobacter, Klebsiella,* and *E. coli*) in tested samples. Selective Glucose OF agar is used to exclude the growth of other microorganisms, allowing the targeted enumeration of these populations.

Appropriate Organisms: Any member of the family Enterobacteriaceae.

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. Assays which rely on selective microbiological medium carry some degree of risk for returning false positives (**page 44**). This method can be used for several purposes: it can be used to enumerate populations of Enterobacteriaceae that are claimed on a product label, or to confirm the absence of contamination by these organisms as part of a comprehensive NTO screening strategy.

ISO 4833 - Colony Count at 30°C by the surface plating/pour plating technique.

What is it? A plate counting assay which enumerates populations of culturable bacteria and fungi in tested samples.

Appropriate Organisms: Any microorganism that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. This assay may be conducted either as a spread-plate or a pour-plate assay, meaning that colonies may form both on and within the agar layer (pour-plate) or exclusively at the agar surface (spread-plate). An optional agar overlay step is included in the method which, in some cases, can help control the growth of "spreading" colonies and make them easier to interpret by a counting microbiologist. See **page 40** for guidance regarding management of spreading type *Bacillus* colonies in cases where this mitigation strategy proves unsuccessful.

ISO 6611 - Milk and milk products — Enumeration of colony-forming units of yeasts and/or molds — Colony-count technique at 25°C.

What is it? A plate counting assay which enumerates populations of culturable fungi (yeasts and molds) in tested samples. Selective Yeast extract/Dextrose/Oxytetracycline Agar is used to exclude the growth of most bacteria, allowing the targeted enumeration of yeasts and molds.

Appropriate Organisms: Any yeast or mold that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $50 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. Assays which rely on selective microbiological medium carry some degree of risk for returning false positives (**page 44**). This method can be used for several purposes: it can be used to enumerate populations of fungi that are claimed on a product label, or to confirm the absence of contamination by culturable yeasts and molds as part of a comprehensive NTO screening strategy.

USP 61 - Microbial Enumeration of Nonsterile Products.

What is it? A plate counting assay which enumerates populations of culturable bacteria and fungi in tested samples.

Appropriate Organisms: Any microorganism that is culturable on laboratory growth medium and under standard laboratory conditions (mesophilic, aerobic, etc.).

Estimated Cost at 3rd Party Labs: Generally, less than $75 per sample. Prices will vary from lab to lab and may depend in part upon the number of samples submitted.

Microbiologist's Notes: All plate counting assays share the same general set of limitations (**page 18**), and their results should be interpreted accordingly. This method is commonly used in conjunction with USP 62, which is an enrichment-based method for confirming the presence or absence of specific microorganisms (*Escherichia coli, Salmonella* species, *Staphylococcus aureus, Pseudomonas aeruginosa*, Bile-Tolerant Gram-negative bacteria, *Candida albicans*, and/or *Clostridium* species) within a sample matrix.

5

Chapter 5
Microbial Identification Methods

*Please note that the estimated prices included in this chapter reflect expectations at the the time of this Handbook's publication and may not reflect pricing fluctuations in the market.

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Unlike the animals and plants common in agricultural settings, agricultural microbes cannot be identified at a glance. At the microscopic level, species that differ wildly in terms of phenotype appear to be little more than subtle variations on one or more structural themes such as rods, spirals, and grape-like cocci among bacteria. In the overwhelming majority of cases, even the visible colonies that microbes form on solid medium are not distinct enough to allow an observer to issue a confident identification based on morphology alone. Selective and differential culture media can be used to formulate a hypothesis regarding the identity of grown colonies, but as described in Appendix III (**page 44**), these methods are not infallible and carry a risk of returning false positives. Therefore, specialized techniques are required to identify any microorganism that has been isolated from a product sample. These methods are described in detail below.

When selecting an identification method to confirm that microbes recovered during an enumeration assay belong to the species claimed on the product label, it is important to note the precision, or **taxonomic resolution,** of the method's results. Microbial colonies isolated from a product claiming *Methylobacterium* sp., for example, need only be identified to the taxonomic resolution of genus to confirm label claim agreement; however, if the product claims *Methylobacterium aquaticum* on its label, a method must be selected which is accurate to the taxonomic resolution of species. It is certainly understandable that commonly available and inexpensive methods will be preferable to more specialized and expensive approaches; however, care must be taken when interpreting results in cases where the taxonomic precision of the selected method is lower than the taxonomic resolution of the product's label.

Whole Genome Sequencing

Method Overview: Whole-genome sequencing is a comprehensive method for sequencing entire genomes. It is considered the global "gold standard" for identification of unknown microorganisms.

Benefits: Whole-genome sequencing is the most precise identification tool available for unknown microorganisms. Results are highly accurate and can differentiate even closely related strains of the same microbial species from one another.

Limitations: The method is generally higher cost and has longer turnaround times versus other, less accurate identification methods. This method is not yet standardized in any methodological compendium, though it enjoys widespread use throughout the world.

Cost Range: Up to $500 per sample depending on the testing laboratory and the volume of samples submitted. **Note:** for all methods, in-house testing can dramatically reduce cost; however, capital investment and technical expertise are required to equip a laboratory with this capability.

16S/18S/ITS Sequencing

Method Overview: This method is based on sequencing rRNA genes, which are present in all living organisms. By sequencing the rRNA gene of an organism (16S rRNA for bacteria, 18S rRNA for eukaryotes, the internal transcribed spacer or ITS gene for fungi) and comparing the results to a database of gene sequences, a provisional identification of the organism can be obtained
.
Benefits: This method offers faster turnaround times and lower cost compared to whole-genome sequencing.

Limitations: This method is generally accurate only to the taxonomic level of genus, while most microbial products claim specific species or strains of microorganism. Analysis of these genes using advanced sequencing techniques can allow confident identification to the species level, but these are not widely available in testing laboratories. This method is not yet standardized in any methodological compendium, though it enjoys widespread use throughout the world. Note that for members of the genus *Bacillus*, other gene sequences (such as the *gyrB* gene) may be more accurate than the 16S gene for the purpose of identification.

Cost Range: Up to $250 per sample (but generally less than $50 per sample) depending on the testing laboratory and the volume of samples submitted. **Note**: for all methods, in-house testing can dramatically reduce cost; however, capital investment and technical expertise are required to equip a laboratory with this capability.

MALDI-TOF Mass Spectrometry

Method Overview: This method identifies bacteria and fungi using Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Mass Spectrometry. It works by generating a highly specific “molecular fingerprint” of proteins released from the cells within an unknown colony and comparing this fingerprint to a reference library.

Benefits: This method offers faster turnaround times and lower cost compared to whole-genome sequencing while remaining accurate to the taxonomic level of species. The system has been validated and certified according to the Official Method of Analysis program (OMA) of the AOAC International and according to the new ISO 16140-part 6 standard.

Limitations: MALDI-TOF can only identify an organism that is contained within its reference library. In the event that a novel organism is identified, its identification can be confirmed using whole-genome sequencing and the organism can then be added to the database to support future identifications.

Cost Range: Up to $100 per sample depending on the testing laboratory and the volume of samples submitted. **Note**: for all methods, in-house testing can dramatically reduce cost; however, capital investment and technical expertise are required to equip a laboratory with this capability.

Phenotypic Arrays

Method Overview: This group of methods includes popular platforms such as BioLog, VITEK, and API and involves the inoculation of a microbial culture into the wells of a 96-well plate or testing coupon. Each well contains a specific substrate and an indicator dye that will change color in the presence of growing cells. The result is a "fingerprint" of trait expression patterns that is compared with a reference library to issue an identification of the organism.

Benefits: This method is accurate to the taxonomic level of genus for most non-clinical microbes (such as those found in agronomic microbials) and is recommended by the FDA and USDA for the identification of presumptive pathogens. Validated AOAC methods for identification of certain clinically significant organisms are available.

Limitations: This method has a lengthy turnaround time relative to methods such as rRNA sequencing and MALDI-TOF, and the reference library cannot generally be modified to accommodate a new organism in cases where an unknown microbe does not currently exist within the library. It also cannot accommodate organisms of the same species which differ in their behavior from the trait "fingerprint" loaded into the reference library.

Cost Range: Up to $100 per sample depending on the testing laboratory and the volume of samples submitted. **Note**: for all methods, in-house testing can dramatically reduce cost; however, capital investment and technical expertise are required to equip a laboratory with this capability.

Target-Specific Identification Methods

Methods such as whole-genome sequencing, 16S/18S rRNA sequencing, ITS sequencing, MALDI-TOF, and phenotypic arrays described above are target-agnostic assays designed to identify an unknown microorganism. These methods are useful for identifying a microbe isolated from a commercial product, which could be a label-claimed species or a contaminant. It must be noted that there are several target-specific identification methods available, which are noted below; however, these methods are generally of less interest to a stakeholder who wishes to identify an unknown microbial isolate.

The enzyme-linked immunosorbent assay, also known as **ELISA**, works by detecting specific microbial antigens. If a microbiologist suspects that they may have isolated a pathogen from a tested sample, they can subject the isolate to an ELISA assay specific to that pathogen and use the data to confirm or refute their suspicions.

Polymerase chain reaction, also known as **PCR** (**page 34**), uses target-specific oligonucleotides to detect an organism of interest in a sample. If a microbiologist suspects that their isolate is *Pseudomonas fluorescens*, for instance, they may extract its DNA and treat it with primers specific to *P. fluorescens*. If the primers amplify their target DNA sequence, then the identification is confirmed. This approach requires the microbiologist to formulate a hypothesis regarding the identity of the unknown organism, which is then confirmed or falsified by the ELISA or PCR test. As such, they are not generally considered useful for the identification of an unknown isolate.

6

Chapter 6

Detection of Non-Targeted Organisms

*Please note that the estimated prices included in this chapter reflect expectations at the the time of this Handbook's publication and may not reflect pricing fluctuations in the market.

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Non-targeted organisms (NTO) are defined as any microorganism that is not claimed on the label of a microbial formulation. NTO can sometimes enter microbial formulations during the manufacturing process, either through accidental mis-formulation or environmental contamination. In general, NTO may be broken down into three categories:

1. **Known Pathogens** – These include organisms of clinical significance, such as *Salmonella, E. coli,* and *Listeria*, as well as known phytopathogens such as *Pseudomonas syringae*.
2. **Environmental Spoilage Organisms** – These include organisms such as *Enterobacter, Citrobacter, Serratia*, and *Klebsiella* which can cause spoilage in some product types or contaminate industrial production processes[8]. This group may include opportunistic human pathogens.
3. **"Misplaced Microbials"** – These include organisms that are known to impart agricultural benefits, such as those described in Chapter 1, but which are not claimed on the product's label. Though not necessarily of concern from a product safety or efficacy point of view, these may impact consistency of performance and speak to poor QC processes on the part of the manufacturer.

In theory, compliance with the spirit of label claim agreement would suggest that no detectable NTO should be allowed; however, this level of process control is extremely difficult to achieve in practice. In cases where NTO are detected within agricultural formulations, many industry professionals recommend performing a risk assessment following guidelines from the United States Department of Agriculture (**https://www.usda.gov/codex**). These guidelines are based on the Codex Alimentarius, or "Food Code", a joint food standards program issued by the Food and Agriculture Organization (FAO) and World Health Organization. **Note**: It is beyond the scope of this document to propose limits acceptability for NTO detection, or to inform any action in response to NTO detection or to a Codex risk assessment. The information in this paragraph is presented only in the spirit of providing background information relevant to NTO detection efforts.

NTO detection methods must be selected with the product's formulation in mind. For example, a test for *Pseudomonas* contamination following ISO 13720 would not be selected for use with a *Pseudomonas*-containing product, and a screening for yeasts and molds following ISO 21527 would not be recommended for a product claiming *Trichoderma* or *Saccharomyces* on its label. When selecting a method for detection of NTO in an agricultural formulation, priority is given to standard methods validated for non-sterile applications (food, probiotics, etc.) as opposed to methods validated for sterile applications (pharmaceuticals, etc.).

Validated Pathogen-Specific Detection Methods

There are many validated target-specific assays for the detection of known pathogens in laboratory methodological compendia. When evaluating agricultural microbials, preference should always be given to NTO detection methods that have been validated for use with probiotics and foodstuffs, as opposed to methods validated for sterile matrices such as pharmaceuticals, due to the decreased risk of misleading results when applied to biologically active and heterogeneous agricultural microbials. Therefore, only methods validated for use with nonsterile matrices are presented here for consideration.

It is beyond the scope of this document to propose a comprehensive battery of pathogen-specific tests that must be run with each evaluation of an agronomic microbial product; however, generally speaking, the most robust screening regimen practicable should be applied to minimize the risk of overlooking a clinically significant microorganism.

Salmonella Detection Methods
- ISO 6579-1:2017
- AOAC 2011.03 (VIDAS-based)
- AOAC 2013.02 (PCR-based)
- FDA BAM Chapter 5
- USP 61/62

Generic _E. coli_ Detection Methods
- ISO 7251:2005
- AOAC 991.14
- FDA BAM Chapter 4
- EP 10.5
- USP 61/62

***E. coli* O157-H7 Detection Methods**
- USDA Food Safety Inspection Service (FSIS) MLG 5A.04 (PCR-based)
- AOAC PTM# 031002 (PCR-based)

***Listeria* Detection Methods**
- ISO 11290-1:2017
- AOAC 999.06 (VIDAS-based)
- AOAC PTM#081401 (PCR-based)
- FDA BAM Chapter 10
- USDA FSIS MLG 8.10

***Clostridium* Detection Methods**
- ISO 7937:2004 (targets *Clostridium perfringens*)
- FDA BAM Chapter 16 (targets *Clostridium perfringens*)
- USDA FSIS MLG Chapter 13 (targets *Clostridium perfringens*)
- USP 61/62 (targets *Clostridium*)

***Pseudomonas aeruginosa* Detection Methods**
- ISO 22717:2015
- USP 61/62

***Staphylococcus aureus* Detection Methods**
- ISO 6888-1:2021 (coagulase-positive Staphylococci)
- AOAC 2003.07
- FDA BAM Chapter 12
- USP 61/62

***Bacillus cereus* Detection Methods**
- ISO 7932:2004
- FDA BAM Chapter 14

***Campylobacter* Detection Methods**
- ISO 10272-1:2017
- AOAC PTM #051201
- USDA FSIS MLG 41.04

***Cronobacter sakazakii* Detection Methods**
- ISO 22964:2017
- FDA BAM Chapter 29

***Vibrio cholerae* Detection Methods**
- ISO 21872-1:2017
- AOAC Official Method 988.20
- FDA BAM Chapter 9

***Shigella* Detection Methods**
- ISO 21567:2004
- FDA BAM Chapter 6

***Enterococcus* Detection Methods**
- ISO 7899-2:2000
- AOAC Performance Tested Method 111902

Validated Detection Methods for Broad Microbial Groups

As noted above, organisms such as *Enterobacter, Citrobacter, Serratia*, and *Klebsiella* are known spoilage agents for agricultural microbial formulations[8]. At the time of this writing, there are no validated, target-specific assays to detect these organisms in tested samples; however, many can be detected through the use of validated methods intended to detect microbial groups at the taxonomic resolution of Family or higher. Because these and other spoilage organisms can pose some degree of risk for the end user (in cases where opportunistic pathogens are present) or impact product efficacy, it may be considered advantageous to screen for their presence where practicable.

As before, contamination detection methods must be selected with the product's formulated microorganisms in mind. Products formulated with microorganisms belonging to the taxonomic group targeted by an NTO detection assay would be expected to return positive results when these methods are used; in fact, you may note the presence of each method as an option for the enumeration of such microorganisms in Chapter 6. It is therefore important to carefully select NTO detection methods, and to interpret results with caution, when dealing with agricultural microbial formulations.

Detection of Enterobacteriaceae and Bile-Tolerant, Gram-Negative Bacteria
- ISO 21528
- AOAC 2003.01
- USP 62
- USP <2021>

Detection of Yeasts and Molds
- ISO 21527-1:2008
- AOAC 2014.05
- EP 10.5
- FDA BAM Chapter 18
- USP 61

7

Chapter 7
Enumerating Next-Generation Microbial Formulations

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All of the microbiological methods described for the enumeration of agricultural microbials in Chapter 6 have been validated by organizations such as International Organization for Standardization (ISO), the Association of Official Analytical Collaboration (AOAC), the US Food and Drug Administration (FDA), the United States Pharmacopeia (USP), and the US Department of Agriculture (USDA). Validation and standardization ensure that, all else being equal, methodology remains consistent, and results remain repeatable across different laboratories worldwide; therefore, the evaluation of agricultural microbials should be conducted using validated, compendial laboratory methods whenever possible.

Even so, it is not always possible to limit one's methodological repertoire to standardized, validated methods when characterizing agricultural microbial products. It is well-known among microbiologists that, generally speaking, less than 1% of the microorganisms in a given habitat are culturable on synthetic laboratory growth medium, a phenomenon sometimes referred to as the "Great Plate Count Anomaly." The vast majority of microorganisms on earth fall into the category of "nonculturable," and these organisms are fundamentally incompatible with the standardized, culture-based enumeration assays found in methodological compendia. Although uncommon in the industry, such microorganisms do occasionally make appearances on product labels, including (but not limited to) the following:

- **Fastidious ("picky") heterotrophic microbes.**
- **Autotrophic microbes, including Cyanobacteria.**
- **Obligate endophytes (such as some mycorrhizal fungi).**
- **Bacteriophage viruses.**

While flow cytometry, a standardized culture-independent enumeration method, can be used to quantify total microbial populations in a tested sample, it is not target specific and thus cannot be used to fully verify label claims for mixed-species assemblages. When dealing with assemblages comprised of nonculturable microorganisms, the use of methods drawn from peer-reviewed academic literature may be required to produce fair and accurate enumeration results[5]. In these cases, peer-reviewed methods may be onboarded and executed in the fertilizer testing lab or, often preferably, a microbiology lab with an R&D microbiology department can be identified to carry out the method. Most of these methods fall into one of the categories described below.

Direct Microscopic Counts

This is a target-agnostic method for determination of total cell count that involves loading a sample of microbial suspension onto a specialized slide with Neubauer rulings which facilitate grid counting. The concentration of cells in a tested sample, with units of cells per milliliter, can be calculated by multiplying the number of cells counted per grid by the dilution factor, and then multiplying by a conversion factor based on the volume of the counting grid. To differentiate live cells from dead cells, samples may be subjected to vital staining (also known as viability staining). When *Bacillus* endospores are present, phase contrast microscopy may be employed to differentiate dormant endospores (phase-bright) from metabolically active endospores (phase-dark).

Although this method is rapid and inexpensive, the limitations of direct microscopic counts generally preclude their use for confirmation of agronomic microbial labels claims. Validated and standardized culture-based assessments of cell concentration are available for most relevant microorganisms, and culture-dependent bioassays are available for the enumeration of mycorrhizal fungi (Appendix V). In the case of nonculturable organisms, ISO 19344 provides a validated flow-cytometry method which includes a viability component. These methods would all be considered preferable to a target agnostic, non-validated method like the Direct Microscopic Count.

PCR-Based Methods

Polymerase Chain Reaction (PCR) is a molecular technique for the amplification of selected DNA sequences using target-specific oligonucleotides and the heat-stable DNA polymerase of the bacterium *Thermus aquaticus*. As noted in Chapter 8, some target-specific pathogen detection assays are based on PCR technology. During a PCR assay, a thermal cycler allows for repeated denaturation of double-stranded DNA followed by the annealing of custom oligonucleotides and the extension of a complimentary strand of DNA. In qualitative assays, the reaction is allowed to proceed to completion prior to detection of the amplified strand (or lack thereof) using methods such as gel electrophoresis. Quantitative assays utilize various approaches to correlate PCR results with known standards, allowing for the quantification of specific microbial populations.

PCR-based assays come in three forms:

- **Endpoint PCR** allows the PCR reaction to proceed to completion, reaching an "endpoint" followed by physical detection of the targeted DNA sequence using methods such as gel electrophoresis. This method is used for detection of a targeted DNA sequence only and cannot be used for quantification of microbial populations.

- **Real-Time PCR (RT-PCR)**, also known as **quantitative PCR (qPCR)**, is a quantitative method that combines fluorophore-labeled oligonucleotide probes with the forward and reverse primer sequences that power endpoint PCR assays. During the thermal cycling process, these probes anneal to the template strand and are forced off during replication, releasing their bound fluorophore and causing it to produce light. Quantification is achieved by correlating the lag time between the initiation of thermal cycling and the presence of a detectable fluorescent signal with known cell concentrations, allowing for the interpolation of concentration within the tested sample. When preparing standard curves and interpolating data from RT-PCR assays, it is important to adhere to the **Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE)** guidelines in order to ensure the accuracy of reported enumeration values[6].

- **Droplet Digital PCR (ddPCR)** is a comparatively recent PCR technology which partitions DNA extracted from a product sample into thousands of individual "droplets", which serve as reaction vessels, prior to DNA amplification. While the technical details are beyond the scope of this manual, the ddPCR assay is generally considered superior to RT-PCR for the quantitation of low DNA concentrations from within heterogeneous samples[7].

While preferable to plate counting assays for the enumeration of nonculturable organisms, PCR assays are not without their limitations. Pipetting error, sample heterogeneity, and differing DNA yields across sample types can all impact the accuracy of PCR assays[8]. Additionally, PCR assays are not capable of distinguishing between DNA from living cells and DNA from dead cells, potentially leading to overestimation of the viable microbial population within a microbial product sample. For this reason, it is advantageous to couple PCR assays with a viability component to inactivate extracellular DNA. One such option is **propidium monoazide**, a photoactive and membrane-impermeable dye which cross-links extracellular DNA when exposed to specific light wavelengths in a photolysis unit[9] [10].

When selecting a PCR assay for the characterization of an agronomic microbial product, care must be taken to select an appropriate oligonucleotide scheme for the organisms claimed on the product label. Target specific probe and primer sequences not developed by the product's manufacturer must be validated for their ability to detect the formulated microorganism before their results can be interpreted with confidence. In the absence of appropriate probe and primer sequences in the published literature or provided by the manufacturer, it may be necessary to develop and optimize bespoke oligonucleotide schemes to evaluate the organism in question. These factors must be taken into consideration prior to the selection of PCR as the method of choice for evaluation of an agronomic microbial product.

Finally, care should be taken when applying PCR-based methods to formulations containing bacterial endospores, such as those formed by members of the genera *Bacillus* and *Clostridium*, as the tough protective layers present in these cell types often complicate DNA extraction efforts, leading in some cases to underestimation of endospore populations[11]. While this effect can be mitigated using customized sample preparation protocols[12], these approaches are often time-consuming and must be optimized for use with specific bacterial strains.

Bioassays for Mycorrhizal Fungi

In the context of agricultural microbiology, **bioassays** are methods used to evaluate populations of microorganisms based on their ability to colonize living cells. These methods are most commonly used for obligate **endophytes**, defined as microorganisms which live within the cells or tissues of their host plants for all of their life cycle without causing disease. This category includes the **mycorrhizal fungi**, many of which cannot be cultured on synthetic laboratory culture medium. Note that many of the microorganisms covered in this manual are capable of adopting endophytic lifestyles; however, they retain the ability to form colonies on synthetic culture medium and are therefore able to be evaluated by culture-based techniques without resorting to bioassays.

Bioassays such as the **Most Probable Number (MPN)** assay are most frequently used in agricultural microbiology for the evaluation of viable propagules of mycorrhizal fungi within product formulations. This approach involves preparing serial dilutions of an AMF inoculant and applying the various dilutions to several replicates of an appropriate plant host. This allows the researcher to extrapolate propagule or spore concentration by assessing the extent of root colonization at different dilution levels. At present, these bioassays are limited to regulatory compendia and to academic literature[13]. At the time of this writing, the European standardization committee CEN's Technical Committee 455 – Plant Biostimulants is establishing standard methods for the evaluation of AMF inoculants. These standards will be tested and verified in Europe and, eventually, will be added to the ISO compendium following completion of the requisite validation process. See Appendix IV for an example of a bioassay used to enumerate mycorrhizal fungi in Japanese laboratories, authored by the Japanese Ministry of Agriculture, Forestry and Fisheries.

Because they rely upon the cultivation of living plants, bioassays are time consuming and require specialized equipment to execute properly. Care must be taken to select a facility that is experienced with these methods to avoid the risk of false negative results. It is also critical to ensure that bioassays targeting a specific microorganism are executed using **axenic** – or taxonomically "pure" – product preparations in order to ensure that any measured results are attributable to the organism of interest.

Plaque Assays

Rarely, one may encounter agricultural microbial formulations which contain **bacteriophage** viruses, or "**phages**" for short. These viruses are host-specific and infect only selected species of bacteria, making them attractive for biocontrol applications such as seed coating and foliar sprays. Because a virus is not a self-replicating biological entity, it must be evaluated through culture-independent means or cultured in the presence of a suitable bacterial host. Several of the methods described above, such as RT-PCR, ddPCR, and flow cytometry, can be used to enumerate bacteriophage suspensions; however, they have well-documented limitations when applied to phages. PCR-based assays are more difficult to couple with a viability component where phages are concerned, leading to frequent overestimation of phage titer in tested samples[14 15]. Additionally, these methods are not capable of determining whether a phage remains infective to its targeted pathogen, which is the key measure of interest for the grower.

To ensure the most accurate enumeration possible for phage preparations, most microbiologists rely on **plaque assays** such as the **Double Agar Overlay**. This method estimates phage titer based on the abundance of **plaques** - clearance zones in an otherwise opaque lawn of bacterial growth - on a plate inoculated with a diluted phage preparation. In this manner, the concentration of virulent phage particles may be estimated by the counting technician in terms of **plaque forming units** or **PFU** per milliliter or milligram of sample. The PFU is the viral answer to the bacterial and fungal CFU, or colony-forming unit, which reigns supreme as the unit of measure for the enumeration of most biological products.

ISO 10705 is a standardized and validated plaque assay targeting coliphages, or phages which target coliform bacteria, and is frequently used in environmental monitoring labs. Variations of this method utilizing different host bacteria (with corresponding modifications to incubation time, temperature, and culture medium if needed) are often used to evaluate agricultural bacteriophage formulations. It must be noted that some of the same limitations that impact the accuracy of the APC assay (**page 14**) are relevant to plaque assays as well, and therefore special care must be taken when interpreting plaque assay data sets.

Next-Generation Sequencing

The advent of commercially available **Next-Generation Sequencing (NGS)** technology has allowed microbiologists to profile entire communities of microorganisms. As noted in Chapter 5, bacteria and archaea can be identified using the highly conserved 16S gene, while fungi can be identified using the 18s or ITS genes. NGS technology allows laboratory microbiologists to evaluate the microbial diversity of entire communities, including those found in microbially active products such as probiotics[16]. These tools can be useful for verifying the taxonomic composition of complex microbial formulations (Chapter 2); however, they are only able to determine the identity and relative abundance of these organisms and are generally precise only to the taxonomic resolution of genus. Furthermore, as noted for PCR-based methodologies, NGS-based community profiling assays must be paired with a viability assurance protocol in order to reduce the likelihood of detecting false positive signals from extracellular DNA.

I

Appendix I

Nonstandard Microbiological Culture Media

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Standardized enumeration methods such as ISO 4833, ISO 21527 and ISO 21528 rely upon common, commercially available microbiological media such as Plate Count Agar (PCA), MacConkey's Agar, and Dichloran Rose Bengal Chloramphenicol (DRBC) Agar. However, as described in Chapter 1, it may be necessary to utilize nonstandard culture medium formulation to enumerate certain agricultural microbial products. While it is recommended that these media be purchased from commercial suppliers, the following formulations are presented for reference in the event that preparing small batches of medium from scratch is preferable to purchasing prepared medium.

Actinomycete Isolation Agar

Reagent	Grams per liter
Sodium caseinate	2
L-Asparagine	0.1
Sodium propionate	4
Dipotassium phosphate	0.5
Magnesium sulfate	0.1
Ferrous sulfate	0.001
Agar	15
Final pH (at 25°C)	8.1 ± 0.2

Instructions: Store prepared media at 2-8°C, protected from direct light.

Azospirillium Agar

Reagent	Grams per liter
Yeast extract	0.05
Potassium phosphate dibasic	0.25
Iron (II) sulfate heptahydrate	0.01
Sodium molybdate dihydrate	0.001
Manganese (II) sulfate monohydrate	0.002
Magesium sulfate heptahydrate	0.2
Sodium chloride	0.1
Calcium chloride dihydrate	0.02
Ammonium sulfate	1
Biotin	0.0001
Distilled water	950 mL

Instructions: Adjust pH of the medium to 7.1. Add 1.5% agar if needed. Autoclave for 15 min at121°C. After sterilization add 25 ml each of filter-sterilized 20% glucose and 20% Na-malate.

Azotobacter Agar

Reagent	Grams per liter
Dipotassium phosphate	1.0
Magnesium sulphate	0.2
Sodium chloride	0.2
Ferrous sulphate	TRACE
Soil extract	5.0
Mannitol	20
Agar	15
Final pH (at 25°C)	8.3 ± 0.2

Suspend in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C, 15 PSI for 15 minutes. If slight precipitate occurs after autoclaving, distribute it evenly before pouring into sterile Petri plates.

Rhizobium Agar

Reagent	Grams per liter
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Yeast extract	1.000
Sodium chloride	0.100
Agar	20.000
Final pH (at 25°C)	6.8 ± 0.2

Suspend in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C, 15 PSI for 15 minutes. Mix well and pour into sterile Petri plates.

Yeast Mannitol Agar

Reagent	Grams per liter
Yeast extract	1.000
Mannitol	10.000
Dipotassium phosphate	0.500
Magnesium sulphate	0.200
Sodium chloride	0.100
Calcium carbonate	1.000
Agar	15.000
Final pH (at 25°C)	6.8 ± 0.2

Suspend in 1000 ml of distilled water. Heat just to boiling. Sterilize by autoclaving at 121°C, 15 PSI for 15 minutes. Mix well and pour into sterile Petri plates.

II

Appendix II

Dealing with Spreading Colonies During APC Assays

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Some agricultural microbes, such as *Azotobacter* and *Bacillus*, often form large, spreading colonies on plates of microbiological medium. An example of this effect is presented in Figure 4. This can complicate efforts on the part of microbiology technicians to interpret colony count and, thus, to estimate microbial concentration[17]. This can result in negative outcomes ranging from underestimation of microbial activity in the tested sample to, in extreme cases, an inability to count plates at all due to flooding of the plate with microbial material.

When dealing with spreading colonies, several options are available to laboratories that may facilitate easier and more accurate plate counting. One option involves testing alternative brands of the same commercially available culture medium that is called for in the selected enumeration method. Studies have shown that complex culture media vary in their ability to produce non-spreading colonies as a function of brand, even when their claimed compositions are identical[18]. An example of this effect is presented in Figure 5. Another option involves allowing agar plates to cure on the benchtop after pouring for a period of 48 hours prior to use. This serves to reduce the moisture content of plates and, thus, to inhibit the expression of swarming phenotypes by the microorganism(s) in question. Because these options would not constitute a significant deviation from a standardized enumeration protocol, they are often the most attractive options for testing laboratories faced with enumeration and assemblage containing one or more organisms which form spreading colonies.

For *Bacillus*, another option involves the use of chemical swarming inhibitors to impede the growth of colonies on solid medium. While these methods have been published in academic literature[17 19], they have yet to be standardized, validated, and added to methodological compendia. However, in situations where peer-reviewed but unvalidated methods are considered acceptable, the addition of a swarming inhibitor or other morphological modifier may be evaluated by the laboratory at their discretion. An example of this approach is presented in Figure 6.

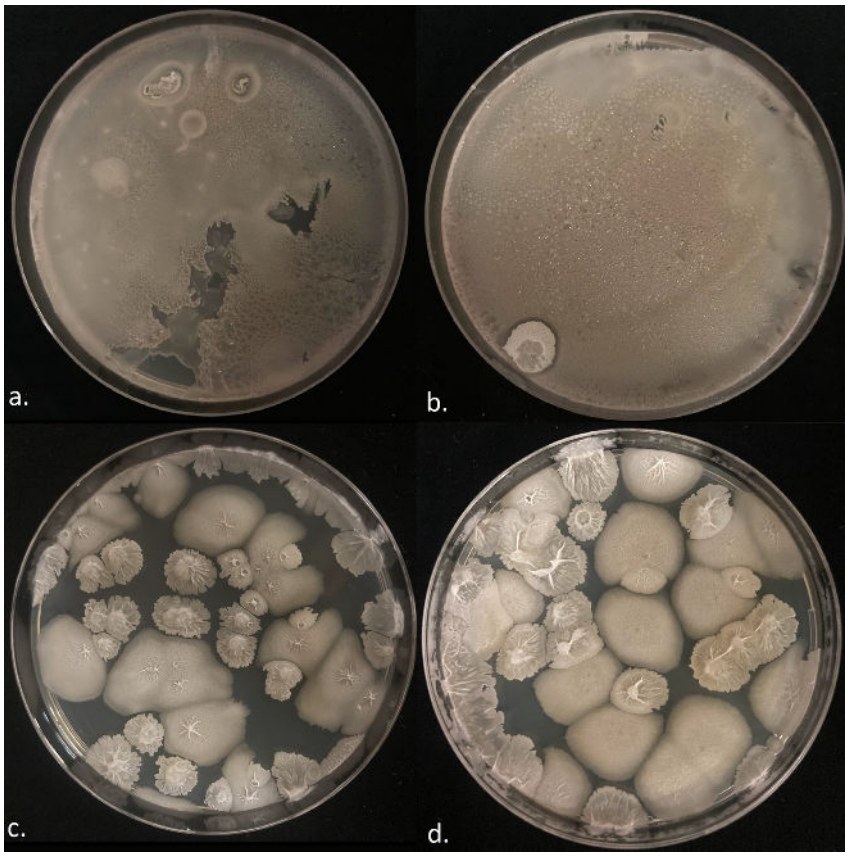

Figure 4 – Examples of spreading colonies formed on Plate Count Agar (PCA) by *Bacillus* spp. During pour-plate (a, b) and spread-plate (c, d) iterations of standardized APC assay ISO 4833.

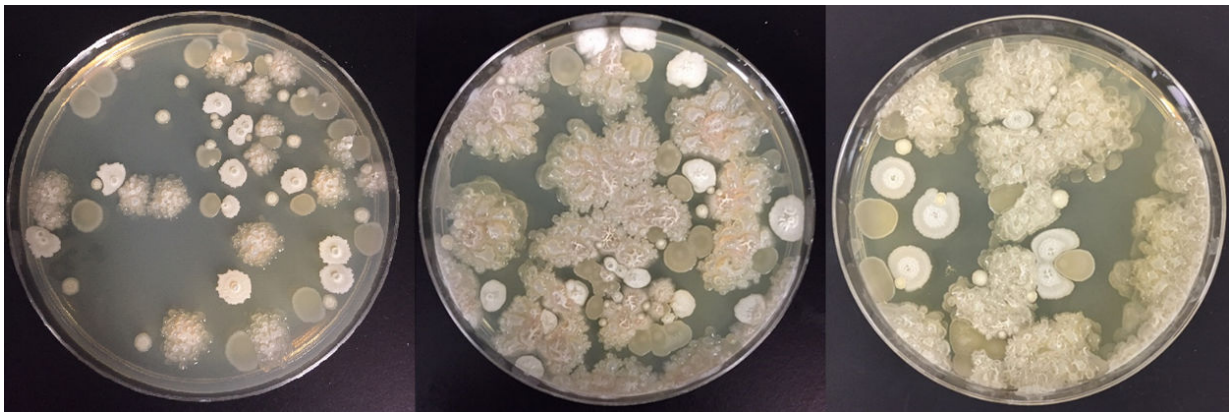

Figure 5 – An assemblage of *Bacillus* species from an agricultural microbial product incubated for 24 hours at 37°C on three different brands of Tryptic Soy Agar (TSA). In cases where colony swarming impedes colony counting by laboratory technicians, it may be advantageous to examine alternative brands of the culture medium prescribed by the method in question.

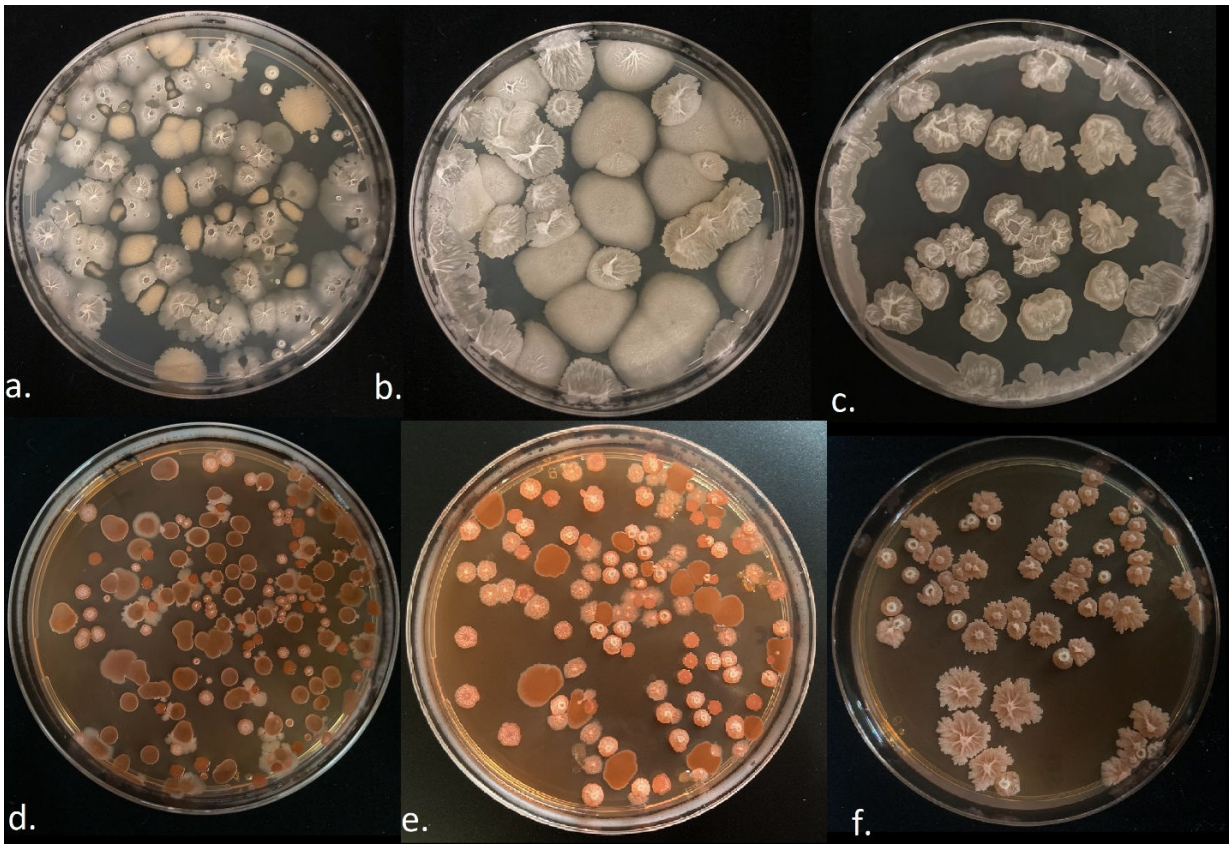

Figure 6 – Assemblages of agricultural *Bacillus* species plated on Plate Count Agar, which does not contain a chemical spreading inhibitor (a, b, c) and on Meat Extract Casein Peptone Agar, which does include a chemical spreading inhibitor (d, e, f). Methods prescribing the inclusion of spreading inhibitors are known in academic literature, but at the time of this writing have not yet been added to methodological compendia.

If your laboratory encounters formulations of *Azotobacter*, *Bacillus*, or any other microorganism which forms spreading colonies that are not controlled by any of the mitigation strategies above, please contact the TFI Microbiology Laboratory Methods Task Force at **biologics@tfi.org.**

III

Appendix III

Notes on Selective Microbiological Media

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Many of the target-specific methods described in this manual owe their specificity to selective microbiological media, which are media formulated to permit the growth of targeted organisms while inhibiting or excluding the growth of non-targeted organisms (NTO). While selective media work as expected in the vast majority of situations, in some cases NTO which are resistant to a specific selection agent may grow on selective medium (Figure 7). If such results are not scrutinized, they can lead to false positives and reporting of misleading data. In cases where the results of an enumeration assay based on selective microbiological media are considered dubious for any reason, it is recommended that the detected organism(s) be isolated and identified using a suitable identification method (Chapter 5).

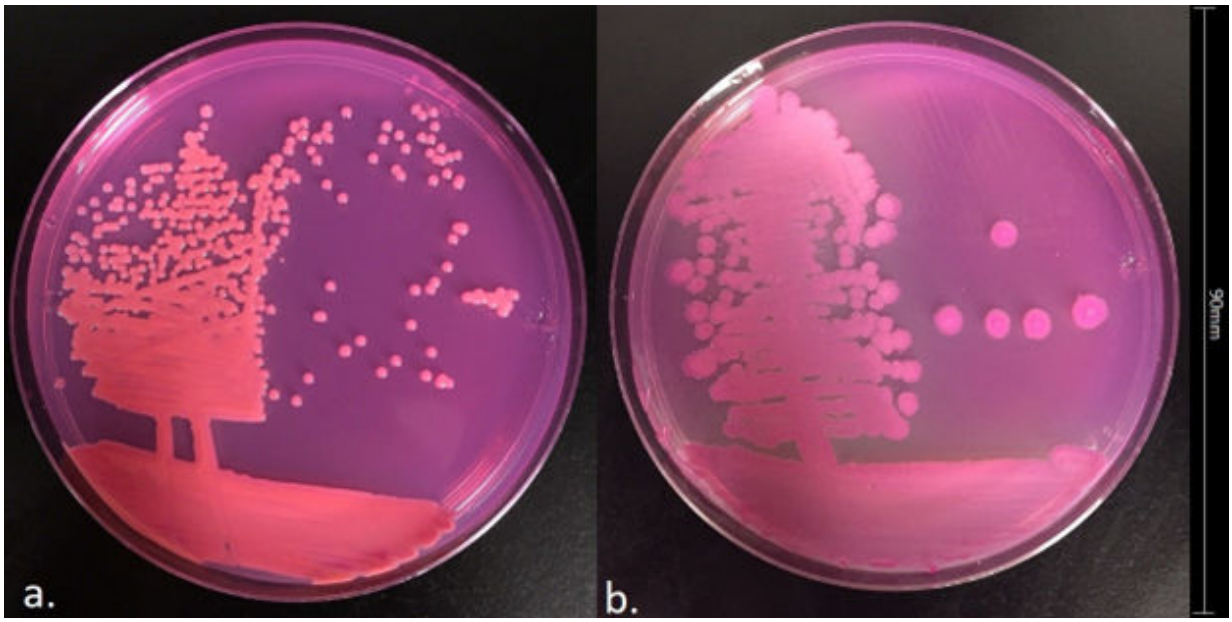

Figure 7 – A yeast (a) and a strain of *Pseudomonas taiwanensis* (b) growing on DRBC agar pursuant to FDA BAM Chapter 8 (**page 20**). In this case, the *P. taiwanensis* is resistant to one or more of the inhibitory compounds intended to exclude colony formation by bacteria on DRBC plates.

IV

Appendix IV

Notes on Units of Potency for AMF Formulations

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As noted in Chapter 1, AMF formulations may list either propagules or spores as their units of biological potency. Each of these units denotes a different type of biological material. The term **propagules** may refer to any one of many biologically viable materials, including hyphal fragments, colonized roots, and spores. The term **spores**, in contrast, specifically denotes robust and metabolically dormant reproductive structures produced by AMF. Spores may be free or may be contained intra-radically within propagules.

The type of biological material incorporated into AMF formulations can have implications for the enumeration, shelf-life, and efficacy of these products. Though common in the industry, propagules such as hyphal fragments may have a shelf-life as short as two weeks[20] owing to the inability of coenocytic hyphae to remain viable propagation units should they be broken during the manufacturing process. The homogeneity of propagule distribution within and across production lots may also vary with the efficiency and efficacy of the blending process, a situation which can introduce more variation into enumeration data sets than would otherwise be expected. In contrast, AMF spore preparations, both free and intra-radical, are known to be more robust than propagules and often exhibit extended stability through the supply chain; thus, they are considered by some researchers to be superior to propagules for use in commercial products[20]. Unlike propagules, spores are also easier to enumerate using laboratory methods. Enumeration techniques involving staining with semi-vital dyes (such as trypan blue) can be used to differentiate intact and dead spores, providing a viability-linked assessment of spore concentration.

If your laboratory encounters formulations of AMF and would like guidance regarding the best approach for confirming the product's composition, please contact the TFI Microbiology Laboratory Methods Task Force at **biologics@tfi.org**.

V

Appendix V

Standard Bioassay Protocol
for AMF Inoculants in Japan

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The protocol below is taken from Salomon et al 2022[13] and is accessible in this publication's supplemental information. At the time of this writing, the European standardization committee CEN's Technical Committee 455 – Plant Biostimulants is establishing standard methods for the evaluation of AMF inoculants. These standards will be tested and verified in Europe and, eventually, will be added to the ISO compendium following completion of the requisite validation process.

Standard bioassay protocol for AMF inoculants in Japan

Excerpt from Soil Productivity Improvement Act (Law No.34 of 1979, amended in 1996)

Ministry of Agriculture, Forestry and Fisheries, Japan

1) Preparation of growth medium: Apply a standard amount of product (inoculum) to 50 cm^3 vermiculite and sow seeds of an assay plant.
2) Growth conditions: Grow the plants at 25˚C under a lighting condition of 15,000 – 20,000 lx (16 h light / dark cycle) for 4 weeks.
3) Assessment of mycorrhizal colonization:
   The roots are detached from the shoots, washed and cleared in 10% (w/v) KOH at 90˚C. Roots are then soaked in 5% (w/v) HCl for 10 min at room temperature and stained with 0.1% (w/v) aniline blue or trypan blue at 90˚C for 30 min.

   The stained roots are spread to a Petri dish with 1 cm grid lines, and the presence and absence of colonization are counted using the intersect gridline method (McGonigle et al., 1990). More than 100 intersections per sample are to be counted in three replication samples.

   Percentage of colonization is calculated as follows:

$$Colonization\ [\%] = \frac{No.\ colonized\ intersections}{No.\ total\ intersections} \times 100$$

4) Quality criteria for AMF inoculants: Root colonization ≥ 5%
5) Mandatory information on the product label
   - Colonization [%] in bioassay and used host plant
   - Used carrier material
   - Applicable and non-applicable plants
   - Expiration date

Standard *in vivo* bioassay

Here, we describe a standardized *in vivo* bioassay for the evaluation of AMF inoculum viability and its effect on plant growth. Further specifications for this protocol are given in Table 3. Detailed instructions are available at: https://dx.doi.org/10.17605/OSF.IO/R9WGN.

1) The soil for this bioassay contains low concentrations of plant-available P which are sufficient to allow healthy plant growth, without suppressing mycorrhizal root colonization. One practical solution is the reduction of soil P through mixing soil with an inert substrate like sand or vermiculite and the re-introduction of P in the form of slow-releasing monocalcium phosphate (CaH_2PO_4). Other essential plant nutrients, such as nitrogen, potassium and micronutrients, are added throughout the bioassay in the form of nutrient solutions that are lacking P (modified Long Ashton -P, see Suppl. S3). The soil is sterilized to inactivate any native AMF propagules.
2) The inoculum is tested against a non-inoculated control group to quantify the MGR. Each treatment has a minimum of 6 biological replicates to allow the statistical testing of effects. For the inoculated group, the inoculum is applied as recommended by the manufacturer. Suitable host plants are added, either as seeds or seedlings. The soil is regularly watered, to near field capacity and the nutrient solution is applied weekly or biweekly. All groups are treated identically in terms of dry soil weights, water and fertilizer applications, and homogenous seedling materials.
3) At the end of the bioassay, plants are destructively harvested by carefully removing the plants from the pots and washing the soil off the roots. A subsample of about 300 mg fresh roots is taken and stored in 50% EtOH. The shoots and roots are separated, dried at 65 °C for at least 48 hours and the dry weights recorded. The MGR can be calculated as followed:

$$\frac{[\text{Biomass (inoculated)} - \text{Biomass (control)}]}{\text{Biomass (control)}}$$

The subsampled roots are stained following the ink-vinegar method as described by Vierheilig et al. (1998) and visualized in the book by Brundrett et al. (1996) First, the roots are washed with water and cleared in 10% KOH, either at room temperature for 3-4 days or for 10-15 minutes at 80 °C. The exact time depends on the plant species, root thickness and root pigmentation. Roots are fully cleared when only the cell wall and cell membrane remain visible under a dissecting microscope. Roots are washed again with water and stained in a 10% ink and 90% vinegar solution for 15 minutes at 65 °C. After staining, roots are washed under water and de-stained for one day in an acidified water solution, containing 2% household vinegar (approx. 5% acetic acid). Roots are now ready for examination or can be stored in a 50% glycerol solution. The colonized root length can be determined following the grid-line intersect technique described by (McGonigle et al., 1990).

Formulation for modified Long Ashton nutrient solution lacking P (Cavagnaro et al., 2001). **Concentrations are referring to the chemical compound rather than the element.**

Macronutrients		Micronutrients	
Potassium sulphate (K_2SO_4)	2 mM	Boric acid (H_3BO_3)	46.3 µM
Magnesium sulphate $MgSO_4$)	1.5 mM	Manganese chloride ($MnCl_2$)	14.4 µM
Calcium Chloride ($CaCl_2$)	3 mM	Zinc sulphate ($ZnSO_4$)	1.4 µM
Iron (Fe) EDTA	0.1 mM	Cupric sulphate ($CuSO_4$)	0.5 µM
Ammonium sulphate $(NH_4)_2SO_4$	4 mM	Sodium Molybdate ($NaMoO_4$)	0.1 µM
Sodium Nitrate ($NaNO_3$)	8 mM		

Preparation of 5 L of modified Long Ashton nutrient solution lacking P

Stock solution	mL in 5 L	Final concentration
250 mM Potassium sulphate	40	2 mM
375 mM Magnesium sulphate	20	1.5 mM
1 M Calcium chloride	20	4 mM
110 mM Iron (Fe) EDTA	5	0.1 mM
2 M Ammonium sulphate	10	4 mM
1 M Sodium nitrate	40	8 mM
1 L Micronutrient solution	5	

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