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Research Note

Enumeration of colony forming units of *Trichoderma* in formulations – precautions to be taken to avoid errors during serial dilution

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ABSTRACT: Traditionally serial dilution method is used for the enumeration of viable propagules in a microbial formulation. Conidia of some fungal agents like *Trichoderma* spp. are hydrophobic in nature. They repel water and are not dispersed uniformly in water. To enhance uniform distribution, dispersing agents like Tween 20 have to be used. In many articles in the published literature, high CFUs (>10^{11} CFUs g^{-1}) have been either been claimed or reported to be present in formulations. Further considering the volume (4/3 x 3.14 x r^3) occupied by each conidium (minimum 2 micron diameter), realistically we cannot expect high Colony Forming Units (CFUs) reported or claimed in many research papers. In the present report an attempt was made to estimate the possible 'number of conidia per cc' and 'number of conidia per g' of *Trichoderma harzianum* spores (NBAII Th10–MTCC 5584). The results showed that even at higher dilutions (>10^{-18} to 10^{-20}) colonies of *Trichoderma* appeared on plates which is not theoretically possible. It is proposed that while enumerating the viable propagules in a formulation the serial dilution has to be done with Tween 20 and the tips should be changed for each dilution without fail. The dilution should be restricted to 10^9 or 10^{10}. The limitations of the serial dilution beyond 10^{10} and precautions to be taken are discussed.

KEY WORDS: *Trichoderma* spp., formulation, serial dilution, enumeration, viability

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The number of viable propagules present in a formulation of a fungal antagonist is an important quality parameter. The viable propagule count is determined by plating 1g of representative sample on specific medium after serial dilution and counting the colony forming units (CFUs) of the organism. The Insecticides Act (1968) of the Government of India recommends 2x10^6 CFUs g^{-1} of the formulation for *Trichoderma* spp. and 2x10^8 CFUs g^{-1} for entomopathogenic fungi.

Serial dilution method employed for enumerating microbes in a formulation has some limitations. CFUs count obtained by serial dilution method depends on different factors, *viz*., *i*. uniformity of distribution of spores, *ii*. proportion of propagules in a formulation (v/v) and *iii*. frequency of colonies that appear at different dilutions and plating methods.

Uniform distribution of spores in a suspension is an important criterion that affects enumeration of microbes. Conidia of *Trichoderma* spp. are hydrophobic or lipophilic in nature (Gams and Bisset, 1998). The spores carry hydrophobins in the cell wall that confer the biocontrol potential besides playing an important role in binding with fat or lipid substances present on the host plant surface. When placed in oil or lipid they clog to fat or lipid substances. Hydrophobic spores repel water and do not distribute evenly. Hydrophobicity of spores affects serial dilution. When the spores are not uniformly distributed in water, taking a portion of it as representative sample for further dilution will lead to error in enumeration. Hence to make the dispersal of spores uniform, surfactants like Tween 20, Tween 80, etc. are used.

Number of propagules per unit weight of a formulation depends on the proportion of propagules and the carrier material. Based on the size of the propagule, the number of propagules per unit volume or weight will vary. The smaller the spore size, the higher will be the number of spores per unit volume. For example, bacteria will have more propagules compared to fungi in a given proportion (w/w or v/v). Geometrically when we calculate, the concentration of fungal spores cannot be in the range of 10^{12} or higher as has been reported or claimed in various reports. For example the conidia of *T. harzianum* are sub-globose to ovoidal in shape. The size of conidia of *T. harzianum* is (2.0–2.7) to (3.5–5.0) x (1.8–2.5) to (3.0–4.0) µm with L/W 1.1. to 1.2 (Gams and Bissett, 1998). If we assume

that the minimum diameter of a conidium is 2µm, the volume occupied by each conidium is (4/3 πr^3) is 4.19 μm^3. One ml or 1cc, i.e., (1x1x1cm) or (1000 x 1000 x 1000µm) or ($1x10^{12}$ μm^3) can contain only ($1x10^{12} \div 4.19$) conidia or $2.38x10^{11}$ conidia, assuming that pore space is negligible.

'Colony forming units' is an estimate of number of cells that are capable to form colonies that can grow under the conditions of the test (medium, temperature, time, oxygen, etc.). A colony can arise from one cell or a group of un-separated cells. Sutton (2006) has reviewed the procedures used in counting colonies for fungi, bacteria and yeasts. The earliest report on the allowable number of colonies was made by Breed and Dotterren (1916) who reported that best results were obtained with 50–200 CFUs per plate. Tomasiewics *et al.* (1980) recommended 25–250 CFUs per plate in triplicate as countable range. United States Pharmaceutical Convention 2006 recommended 25–250 colonies for bacteria and *Candida albicans* and 8–80 colonies for *Aspergillus niger* as limits per plate. ASTM (1998) provided the countable range as follows: 20–80 colonies for membrane plating, 20–200 for spread plate, 30–300 for pour plate method in four replicates each. The 'detection level working group DLWG' of Presidential Task Force on Best Practices for Microbiological Methodology formed by USFDA recommended an alternative method called 'dilution to extinction' (AOAC, 2006).

When the microbial population is too high it is treated as too numerous to count (TNTC). Blodgett (2002) suggested an alternative measure for serial dilution experiments with tubes for most probable numbers (MPN). MPN procedures are more suitable for the enumeration of bacteria. Blodgett (2008) discussed the bias in the enumeration of microbes that can happen by exclusion of plate counts outside the acceptable range (25–250 CFUs g^{-1}).

Most of the literature and opinions with respect to advantages and disadvantages of serial dilutions were mainly related to bacteria and very few of them deal with fungi. The brief surfing of literature to know the colony forming units of fungal antagonists in a formulation that have been reported or claimed to be present in a formulation reveals that many researchers have reported formulations with more than $1x10^{11}$ CFUs g^{-1}, interestingly in the range of 10^{12} or more in many cases. Some reports indicate that field application was done at 10^{12} CFUs g^1 or ml^{-1} (Nakkeeran *et al.,* 2005; Jegadambigai *et al*., 2009).

The objective of the present report is to explain why it is not possible to have high CFUs (> 10^{10} per g or ml of a formulation) especially with *Trichoderma* which has hydrophobic spores that do not spread in water uniformly. In spite of the use of a surfactant (Tween 20), high frequency of CFUs of *Trichoderma* can appear at higher dilutions (>10^{14}). The limitations of serial dilution in enumeration of *Trichoderma* CFUs in formulations and precautions to be considered are discussed.

One g as well as one cc (1ml) of conidia of *T. harzianum* (NBAII Th10 – MTCC 5584) separated from solid state fermentation substrate (ragi grains) using Mycoharvester were taken and serially diluted with or without surfactant. Tips were changed for each dilution. For one g of conidia, the dry spores obtained from Mycoharveter was quantitatively measured and taken. For one cc of conidia, the harvested spores were transferred to sterile measuring cylinder under aceptic conditions upto 1ml mark. Three methods of plating *viz.* pour plate, spread plate and running drop method were followed. Tween-20 (sorbitan monolaurate ethoxylate), a non-ionic surfactant found compatible with a number of fungi (Walker, 1981; Boyette and Walker, 1985; Mitchell, 1986), was used at 0.2% concentration wherever required. For pour plate method, 1ml of the spore suspension was used per plate while 100ul of the same was used in spread plate method per plate. In running drop method that is used for enumeration in medical microbiology (Fig. 1d), a drop (50ul) of spore suspension of different dilutions was placed on one side of the plate and allowed to run towards the other end by keeping the plates in slanting position. To cross check the viability haemocytometer count was also recorded. Each plating method was replicated thrice for each dilution and each test was repeated thrice.

When one cc or one ml spores were serially diluted and plated, viable colonies (3–16) appeared even after 10^{-14} dilution (Table 1, Fig. 1a). Few colonies appeared even at 10^{-18} and 10^{-20} dilutions (2–8 and 1–5 respectively) (Fig. 1c). This was observed with or without the addition of surfactant, Tween 20 (Fig. 1b). Though use of Tween 20 facilitated easy distribution of conidia in serial dilution, *Trichoderma* colonies appeared on plates even at higher dilutions. The same trend was observed in spread plate method also (results not shown). In running drop method also the serial dilution did not reflect in the proportional reduction in the spore count at higher dilutions, though fewer colonies only appeared (Fig. 1d). In haemocytometer counts, 1g of spores was found to contain $2.15x10^8$ conidia and 1cc or 1ml of conidia was found to contain $5.36x10^9$ conidia.

The above observations show that the hydrophobic conidia of *Trichoderma* are not uniformly distributed while subjected to serial dilution and they get transferred to higher dilution disproportionately. Though haemocytometer count gives almost real time observation, all the conidia observed need not be viable. Unviable conidia also will form a part of the counting.

Since a formulation contains carrier material (e.g. talc), pore space, water (6–10%) in addition to spore or conidia, the possibility of getting >10^{10} spores per g is

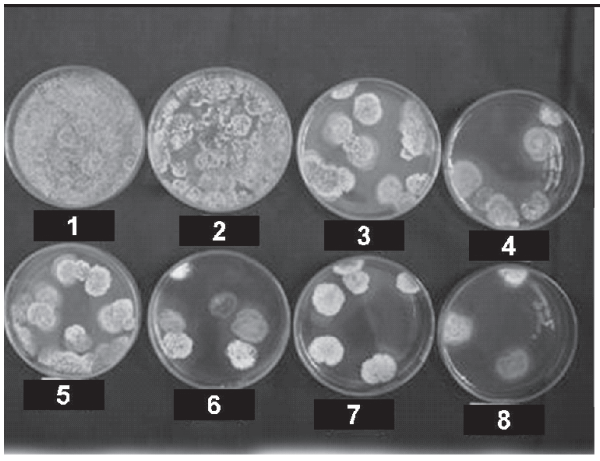
Table 1. Number of colonies at higher dilutions (> 10^{14} dilutions)

Plating method	Without Tween 20				With Tween 20			
	10^{14}	10^{16}	10^{18}	10^{20}	10^{14}	10^{16}	10^{18}	10^{20}
Pour plate method	3-16	3-8	2-8	1-5	3-10	1-5	0-8	0-5
Running drop method	3-8	2-5	1-5	1-4	2-7	0-4	0-4	0-3

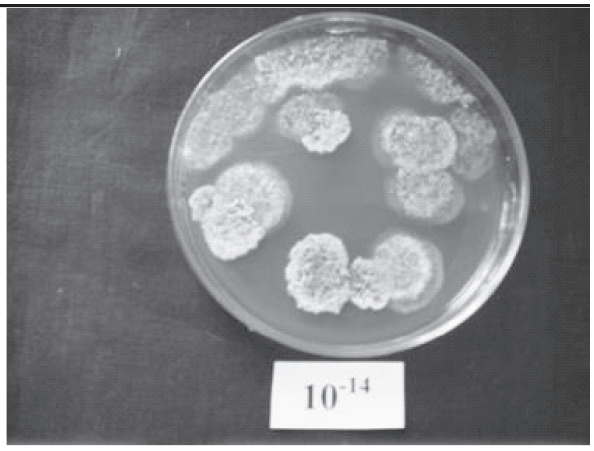
Table 2. Haemocytometer count of *Trichoderma* conidia in spore suspensions prepared with one g or one cc of spores from solid state fermentation

Initial weight or volume of conidia taken	Spore count in haemocytometer	
	Without Tween 20	With Tween 20
One g	$2.15x10^8$	$3.12x10^8$
One cc	$5.36x10^9$	$6.26x10^9$

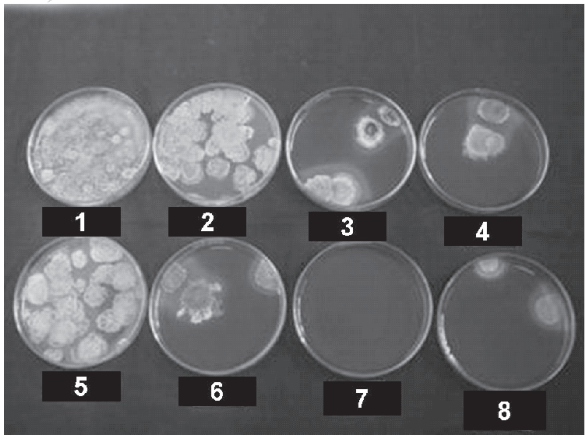
rare. Water content in the spores also will affect the number of spores in one gram. In the above described examples only one g or one cc conidia was taken. In a formulation which is claimed to contain only 2% active ingredient, it is not possible to expect high CFUs (>$1x10^{10}$). However in many researchers have either reported or claimed high CFUs in the formulations. Jegadambigai *et al.* (2009) reported that conidial suspensions of *T. viride* and *T. harzianum* were used at $1x10^{11}$ spores ml^{-1} in field application for the management of *Sclerotium rolfsii* in *Zamioculas zamiifolia* in Sri Lanka. Lee *et al*. (2006) reported that formulations of *T. harzianum* Th459 were developed in cooperation with JGreen Inc. (Changnyung, Gyeongnam province, Korea) in 2000 and concentrated conidia ($1x10^{11}$ g^{-1}), wettable powder ($1x10^9$ g^{-1}), granular formulation ($1x10^8$ g^{-1}) provided by the company was field tested for the management of *Botrytis cinerea* infection on cucumber. US Patent 623912B1 claims that accumulation of *T. harzianum* or *T. reseei* spores at $1x10^{12}$ spores ml^{-1} or greater was used for the waste water



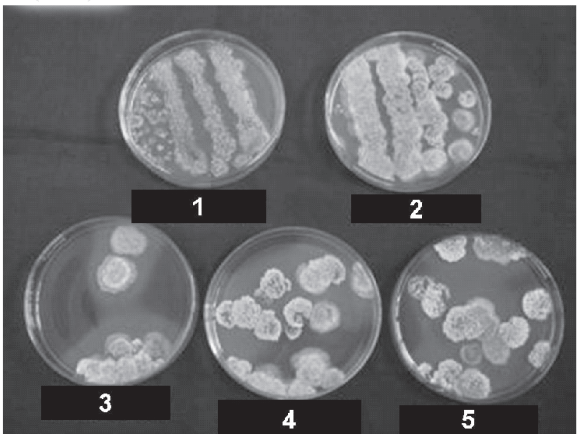
a. Colonies at different dilutions without Tween 20 (pour plate method)



b. Colonies in acceptable range at higher dilutions (10^{14})



c. Colonies at different dilutions with Tween 20 (pour plate method)



d. Colonies at different dilutions – Running drop method

Fig. 1. ***Trichoderma*** **colonies at different dilutions in pour plate and running drop methods (1–8: Dilutions 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}, 10^{-14}, 10^{-16}, 10^{-18} and 10^{-20}, b. Dilution at 10^{-14}, c. 1–8: Dilutions 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12}, 10^{-14}, 10^{-16}, 10^{-18} and 10^{-20}, d. 1: Dilutions 10^{-2}, 10^{-3} and 10^{-4}, 2: dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}, 3: dilutions: 10^{-9}, 10^{-10}, 10^{-11} and 10^{-12}, 4: dilutions 10^{-13}, 10^{-14}, 10^{-15} and 10^{-16}, 5: 10^{-17}, 10^{-18}, 10^{-19} and 10^{-20})**

treatment. Thangavelu *et al*. (2004) used dried banana leaf as substrate for growing *Trichoderma* and reported that $4.6x10^{32}$ CFUs of *T. harzianum* was obtained in gram of dried banana leaf. Zhao (1999) used solid state fermentation technology for the production of *T. viride*. 58% wheat bran was gradually added to one day old liquid medium containing 5% maize starch and the viability of *Trichoderma* in this formulation was $6.4x10^{10}$ conidia g^{-1}. Nakkeeran *et al.* (2005) produced *T. viride* on sorghum grains and used the grains with *T. viride* growth ($1x10^{11}$ CFU g^{-1}) for field application after mixing it with farm yard manure. Sawant *et al.* (1995) used coffee waste and poultry manure for the mass production of *T. harizanum* and they reported that viability was $9x10^{11}$ to $3x10^{12}$ CFUs g^{-1}.

Based on the results presented in this report and logical calculation, it is not possible to obtain high CFUs ($>10^{10}$) in the formulations of any organisms where the propagule size is 2µm or more. Hence it is proposed that while enumerating the viable propagules in a formulation the serial dilution has to be done with Tween 20 and the dilution should be restricted to 10^9 or 10^{10}. The tips should be changed for each dilution without fail. Plates with colony count of 8-80 only should be considered for enumeration. If more than 80 colonies appear at 10^9 dilution, they should be treated as TNTC (too numerous to count); it should be treated as $1x10^{10}$ and only log transformed values (e.g. 10.0 for $1x10^{10}$) should be used for statistical analysis. For enumeration of other hydrophobic spores also, the same suggestions have to be followed to avoid errors in enumeration.

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