Using Particle Size Analysis to Determine the Hydrophobicity and Suspension of Fungal Conidia with particular relevance to formulation of biopesticide

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Abstract

Fungal formulations are vital for effective biopesticide development. Good formulations help to optimise field efficacy while poor formulations result in product failure. This study aimed to produce a hydrophobicity test that would be appropriate for fungal conidia produced to a commercial quality and determine relative hydrophobicity of fungi from four different genera by using laser diffraction. A particle size analyser was used to determine the hydrophobicity of: three *Metarhizium acridum* samples, *M. anisopliae*, *Beauveria bassiana*, *Trichoderma stromaticum*, *T. harzianum*, *T. viride* and *Alternaria eichhorniae* conidia, by suspending the conidia in three different liquids: Shellsol T (a mineral oil), water and 0.05 % Tween 80. Hydrophobicity was determined by the size of the particles formed in each of the liquids. All the *Metarhizium* samples were the most hydrophobic followed by *B. bassiana* and *A. eichhorniae*. The *Trichoderma* samples were the least hydrophobic. As a comparison a phase exclusion assay and a salt-mediated aggregation and sedimentation (SAS) test were performed. It was not possible to get a reliable reading for the *B. bassiana, A. eichhorniae* and *T. viride* samples using the phase exclusion assay. The addition of salt in the SAS test did not affect the rate of sedimentation. It was hypothesised that conidia size affected the results of the SAS test that made *A. eichhorniae* the most hydrophobic conidia. Particle size analysis was a more accurate test for comparing fungi from difference genera compared to the SAS test and phase exclusion assay. PSA was also used to test three emulsions and demonstrated that different formulations had an effect on particle size.

Keywords: formulation; microbial biopesticides; hydrophobicity; particle size analysis; *Metarhizium*; *Beauveria*

1. Introduction

When formulating fungal conidia it is crucial to understand how they disperse in formulating media in order to maintain stability. Large particles, including aggregations of fungal conidia are undesirable for two main reasons. Firstly if the conidia are clumped the likely hit rate and hence control of the pest, whether the pest is an insect, plant or fungus, is reduced due to uneven loading of the conidia in the spray droplets (Bateman, 2004). Secondly large clumps in the formulation are more likely to lead to blockages of the sprayer (Chapple *et al.*, 2007).

There are numerous methods to determine the hydrophobicity of fungi including: contact angle measurements, salt aggregation tests, phase distribution assays (Mozes and Rouxhet, 1987), polystyrene microsphere assays (Clement *et al.,* 1994) and salt aggregation and sedimentation tests (Jeffs and Khachatourians, 1997); but depending on the method used different results can be obtained (Mozes and Rouxhet, 1987). For fungal conidia that are obviously at different ends of the hydrophobicity scale, most methods can separate out the fungi into an order of the most hydrophilic to the most hydrophobic. However, when dealing with fungi of different sizes and with similar hydrophobicities it is sometimes hard to obtain a definitive rank. The rank of hydrophobicity of a group of fungi may be an important factor as to which fungus is chosen to formulate into a product (Talbot *et al.*, 1996) or how much surfactant may be required to obtain a homogenous suspension.

In this study a simple technique has been investigated to determine the relative hydrophobicity of fungal conidia by using laser diffraction. A particle size analyser was used to determine the relative hydrophobicity of fungal spores suspended in

different polarity liquids. Not only does this method determine how easy it is for conidia to be suspended in a liquid but also how the conidia, on its own or formulated, interact with each other in a particular suspension. Two other hydrophobicity tests were performed on the same fungal samples. A phase exclusion assay, where an organic layer is added to an aqueous fungal suspension and the rate of migration of the conidia into the aqueous phase is determined by using optical density (OD) as a measure of the polar layer. The more hydrophobic the fungus the quicker it will migrate into the non-aqueous layer and hence OD will decrease. Secondly, a salt-mediated aggregation and sedimentation assay which also uses OD to measure the rate at which conidia aggregate and sediment out of suspension (Jeffs and Khachatourians, 1997). Those conidia that aggregate out fastest have a greater hydrophobic nature than conidia left in suspension.

2. Materials and methods

2.1. Fungal isolates

A range of fungi from four genera was used in this study (Table 1). Three different batches of *Metarhizium acridum,* IMI 330189, were tested to see if there were any differences in hydrophobicity of fungal conidia, when they were produced in different ways. Of these, sample DM2 was produced in a laboratory and samples ME 006 and ME 008 were produced by a commercial company. *Beauveria bassiana* and the *Trichoderma* species were mass-produced by a two-stage process based on the method used by Cherry, Jenkins, Heviefo, Bateman and Lomer (1999). The first stage was a liquid culture followed by conidiation on a sterile solid substrate, Basmati (Tilda) rice. However, *Alternaria eichhorniae* produced low conidial numbers when mass produced on rice. Therefore, the second stage of mass production for *A. eichhoeniae* was adapted by pouring the liquid culture onto foiled trays and allowing the culture to dry slowly for the liquid to evaporate and conidiation to occur. The fungi were harvested from the solid substrate using a 'MycoHarvester v.1' (Bateman, 2003, www.mycoharvester.info), which enables aerial conidia to be extracted from a solid substrate while removing virtually all large fragments of mycelium or solid substrate, so leaving mainly single conidia (Bateman *et al.*, 2002). The 'MycoHarvester' was adapted for extraction of *A. eichhorniae* conidia by replacing the substrate column (under negative pressure from the air intake at its base thus creating a fluidized bed mechanism) with a suction tube to directly remove the conidia from the trays. The conidia were dried to below 5 % moisture content by placing the conidia in an airtight container with non-indicating silica gel beads for 5 days. Once the desired moisture content was achieved the conidia were packed in hermetically sealed tri-laminate sachets and stored at 5 °C until required.

2.2. Salt-mediated aggregation and sedimentation (SAS) test

This method was based on the one used by Jeffs and Khachatorians (1997). For each fungal treatment, conidia were suspended in two buffer solutions; 2.0 mM di-sodium hydrogen orthophospate buffer (pH. 6.8) and a 1:1 ratio of 2.0 mM di-sodium hydrogen orthophospate buffer and 10 mM ammonium sulfate buffer. The resulting conidial suspensions were vortexed for 10 seconds and their optical density (OD) was measured using a spectrophotometer (Pharmacia, Pharmacia LKB, Novaspec II) set at 610 nm in 3.5 ml polystyrene cuvettes. Conidia were added until OD readings of 0.6 were achieved, the suspensions were incubated at 25 °C. After 30, 60 and 120 minutes the samples ODs were re-measured. The rate of sedimentation was determined by calculating the percentage differences in OD between the original OD reading and the subsequent readings. This experiment was replicated on three separate occasions.

2.3. Phase exclusion assay

The phase exclusion assay was based on a method used by Mozes and Rouxhet (1987). Conidia were suspended in 0.2 M tris buffer (pH. 7.0) and agitated in a vortex blender for 10 seconds before measuring the OD at 610 nm. The suspensions were adjusted for each sample to give an OD reading of 0.6. Once this was achieved 5 ml toluene (anhydrous, 99.8%, Sigma-Aldrich) was added to each sample in a 1:1 ratio, the samples were blended further for 20 seconds and incubated at 25 °C. After 30 minutes the aqueous layer was removed, being very careful not to remove any of the toluene, and the OD of the aqueous layer was re-measured. The percentage of conidia left in the aqueous layer was calculated. Those conidia that migrated to the organic

layer at a faster rate were more hydrophobic than those spores left in the aqueous layer. This experiment was replicated on three separate occasions.

2.4. Particle size analysis (PSA)

Conidia of each sample were suspended in 10 ml of three different liquids with varying polarity: distilled water, 0.05 % Tween 80 in distilled water and Shellsol T (Alcohols Ltd., Hertfordshire, UK) a paraffinic oil. Particle size spectra of the resulting suspensions were measured with a Malvern 2600 particle size analyser (Malvern Instruments Ltd., Spring Lane South, Malvern, Worcs., WR14 1AT, UK). The instrument was fitted with a 63 mm lens using model independent analysis and a PS1 sample cell that contained a small magnetic stirrer. Each reading consisted of a background measurement with the blank formulating liquid, followed by the gradual introduction of concentrated suspensions using a pipette. Each reading comprised of 1000 scans (equivalent to sub samples). Each sample was run twice through the Malvern.

2.5. PSA of emulsion concentrates of IMI 330189

From the PSA testing *M. acridum* was shown to be very hydrophobic indicating that these conidia prefer to be suspended in oil. However, a large majority of sprayers are water-based. Hence *M. acridum* conidia were used to demonstrate the effects of emulsion formulations in water on particle size. Commercially produced conidia of IMI 330189 (see above) were prepared as emulsion concentrates using three different emulsifiers (Table 2). First a stock suspension of conidia was prepared to mix with the different emulsifiers in the following way:

Dry conidia	110 g/kg

Structuring agent	10 g/kg
Ondina EL	880g/kg

The structuring agent was first dispersed in Ondina EL oil (a paraffinic oil) using a Silverson L4RT mixer at approximately 6000 rpm for 2 minutes (Silverson Machines Ltd., Chesham, Bucks, UK). This was followed by the addition of the dry conidia. The conidia were mixed into the formulation for two minutes at approximately 6000 rpm.

The conidia stock suspension and the various emulsifiers were combined at the 5 % level to make 3 x 250 g blends (Table 2). The resulting conidia emulsion concentrate (EC) suspensions were mixed using the Silverson L4RT mixer for no more than two minutes at approximately 6000 rpm. BotaniGard® ES, an emulsifiable suspension mycoinsecticide (active ingredient *Beauveria bassiana*) used to control whitefly, aphids, thrips and mealybugs in ornamentals and vegetables, was used as a standard control to compare against the physical properties of the new formulations.

Emulsion particle/oil droplet size was measured with a Malvern 2600 particle size analyser used in the same way as mentioned above. Each reading consisted of a background measurement of distilled water followed by the gradual introduction of emulsion concentrate using a pipette. A reading was taken when the obscuration of the laser was optimal in the 'illustrate live' command. Measurements were repeated to check for consistency and are presented here as means.

2.6. Statistics

All percentage data was arcsine transformed prior to any statistical tests being carried out. To determine if the type of buffer had a significant effect on the rate of

sedimentation, a one-way ANOVA was performed. Kruskal-Wallis non-parametric tests were carried out on the transformed data to determine if the fungal isolate had an effect on the rate of sedimentation, phase exclusion or particle size. All statistical tests were carried out using SPSS for Windows version 17.0.0. to the 95% significance level.

3. Results

3.1. Salt-mediated aggregation and sedimentation (SAS) test

The SAS test is based on the principle that the more hydrophobic conidia will aggregate together and hence sediment out of suspension at a faster rate than those conidia which are more hydrophilic. The addition of salt had no significant ($P>0.05$) effect on the sedimentation of any of the samples tested. Thus data for the buffer and buffer and salt samples were amalgamated. There were very highly significant differences (Chi-squared = 64.3; df = 8; $p < 0.001$) in the rate of sedimentation of conidia (Figure 1). The *Trichoderma* isolates were rated as the least hydrophobic fungal conidia with the least amount of sedimentation. The *Metarhizium* and *Beauveria* isolates were in the next group of fungi ranging from 53-71 % conidia left in suspension after 120 minutes. The *Alternaria* isolate was ranked the most hydrophobic conidia when using the SAS method with only 46 % of conidia left in suspension after 120 minutes.

3.2. Phase exclusion assay

The phase exclusion assay works on the principle that more hydophobic conidia will migrate from an aqueous phase to a solvent phase at a faster rate than those fungi which are more hydrophilic. Figure 2 shows the percentage of conidia left in the aqueous phase after 30 minutes of being combined with toluene. Highly significant differences showed that the *Metarhizium* samples were rated as the most hydrophobic, followed by the *Trichoderma* sample FA 64 and the DIS 219f *Trichoderma* sample was ranked as the least hydrophobic (Chi-squared = 15.251; df = 5; p = 0.009). Practical difficulties with the *B. bassiana,* SP2 002, *A. eichhorniae*, WH3a and *Trichoderma* sp., T22 samples resulted in large standard errors. Each time the

experiment was replicated a different percentage of conidia left in aqueous suspension was achieved, varying from 50 % to 250 % for SP2 002, 26 % to 193 % for WH3a and 17 % to 102 % for T22. The number of conidia in the samples did not increase, so the increase in OD was due to another, undetermined factor. For this reason these results were omitted from the graph and statistical tests.

3.3. Particle size analysis (PSA)

PSA examined how conidia clumped or dispersed when suspended in liquids with different polar properties. Hydrophilic conidia suspend better in water as singular conidium giving a smaller size in the PSA test i.e. less clumping of conidia. Whereas, hydrophobic conidia are more likely to suspend singly in Shellsol T giving a smaller particle size under these test conditions. *M. anisopliae* isolate TNS 10 (Figure 3i), *M. acridum* isolates ME 006 (Figure 3ii) and ME 008 (Figure 3iii), *B. bassiana* isolate SP2 002 (Figure 3iv) and *A. eichhorniae* isolate WH3a (Figure 3v) all suspended better in Shellsol T than water. When this is plotted on a graph, the curve for the Shellsol T sample is relatively near the Y axis and relatively steep. For example, on Figure 3i. 90 % of all particles were less than 10 µm in size when suspended in Shellsol T. In contrast, the curves for the samples suspended in water are further away from the Y axis and less steep. For example, on Figure 3i. 90 % of the particles suspended in water were up to 95 µm in size. Isolates ME 006 and ME 008 suspended equally well in 0.05 % Tween 80 as compared to Shellsol T but, when examined under a microscope, differences could be observed (Figure 4). Conidia suspended in Shellsol T mainly showed single conidium evenly dispersed (Figure 4.i), whereas conidia suspended in 0.05 % Tween 80 showed single conidium interspersed with a few clumps (Figure 4.ii). The conidia suspended in only water showed no

individual conidium but many clumps of varying sizes (Figure 4.iii). *B. bassiana*, SP2 002 initially suspended very well in 0.05 % Tween 80 but, after 60 % of the particles were suspended, particle size increased and at 90 % particle size was up to 49 µm compared to 21 µm when suspended in Shellsol T.

Three of the *Trichoderma* isolates, DIS 219f (Figure 5i), FA64 plate 0 (Figure 5ii) and FA64 plate 5 (Figure 5iii) suspended better in water than in Shellsol T, suggesting that these conidia are more hydrophilic than ME 006, ME 008, TNS 10, SP2 002 and WH3a. When examined under a microscope DIS 219f (Figure 6) showed the opposite of the *M. acridum* sample (Figure 4). There was clumping of conidia when suspended in Shellsol T (Figure 6.i) but none when suspended in 0.05 % Tween 80 (Figure 6.ii) or water (Figure 6.iii). The fourth *Trichoderma* isolate T22 behaved in a very similar manner, regardless of suspending liquid, until around 50 – 60 % cumulative particles were suspended, when 0.05 % Tween 80 had the smallest particle size, followed by Shellsol T and then water (Figure 7).

M. acridum isolate DM 2 had very similar results for suspension in water and in Shellsol T. The conidia suspended better in 0.05 % Tween 80 (Figure 8). However, care has to be taken when interpreting these results as there were difficulties in suspending conidia in the water phase as most of the conidia floated on the surface, consequently unusual results occurred and this may account for the difference between DM 2 and the other *Metarhizium* isolates.

3.4. PSA of emulsion concentrates of IMI 330189

The PSA method was used to determine the effect of emulsion formulation on particle/droplet size. The particle/droplet size of BotaniGard, the standard, was 14 µm. Formulations 1 and 2 were similar in particle size with a mean particle size of 22 µm and 15 µm, respectively. However, formulation 3 had a mean particle/droplet size of 43 µm. That is over 300 % larger than BotaniGard.

4. Discussion

The study of hydrophobicity of fungal conidia can be approached using many different methods but none seem to give a consistent answer. A summary of the rankings of hydrophobicity for the phase exclusion assay, the SAS test and the PSA method are shown in Table 3. Of the three tests the PSA rankings were the most accurate when samples were checked by microscopic examination of the conidial suspension in the different suspending liquids (Figure 4). The SAS test was the next most reliable test, excluding *Alternaria* due to size differences. The phase exclusion test did not give reliable results for the *Beauveria* and *Alternaria* samples.

The conidia used in this study were mass produced using a two phase process which produced very hydrophobic conidia. The conidia were dried to a moisture content (MC) of approximately 5 %. This is known to be a suitable moisture content for storage of certain fungi such as *Metarhizium* and *Beauveria* (Hong *et al.*, 1997; Hong *et al.*, 2000; Hong *et al.*, 2002). The fungi used by other researchers were probably not dried to a MC of 5 % and in most cases the fungal spores were stored in a liquid suspension (Jeffs *et al*., 1999; Jeffs and Khachatourians, 1997; Mozes and Rouxhet, 1987). Shan *et al.* (2010) produce and dried conidia in a similar manner to the current study, i.e. aerial conidia dried to 5 % MC. To overcome problems in assessing conidial hydrophobicity, using an aqueous-solvent partitioning method, they added 0.02 % Tween 80 to help suspend the conidia. However, no mention was made of the possible effect of Tween 80 on the hydrophobicity on conidia.

The results from the emulsion particle/oil droplet size indicate that different formulating oils and emulsifiers can give very different particle sizes when suspended

in water. Droplet size is directly proportional to its size (Gan-Mor and Matthews, 2003). This means that relatively fewer, larger, emulsion droplets will have a higher number of conidia present in them compared to smaller emulsion droplets. When sprayed the larger emulsion droplets will give a less even coverage as the conidia are clumped into relatively fewer droplets compared to the smaller emulsion droplets resulting in decreased efficacy of the application (Gan-Mor and Matthews, 2003). This study highlighted the importance of checking formulations as formulation 3 had a very high mean particle size compared to the standard control and other formulations. Ettmueller *et al.* (1995) concluded similar findings when they evaluated the distribution and sedimentation of dispersion chemical formulations, such as suspension concentrates, in spray tanks and found that different emulsifiers suspended and resuspended with different 'ease'.

For operational reasons, high quality formulations consisting of stable suspensions, require stringent particle size specifications (*i.e*. consisting mostly of single conidia with mycopesticides) that would usually be monitored with a PSA (Cherry *et al.*, 1999). Use of such instruments is therefore practical and a good method of not only determining hydrophobicity of the conidia but also of examining how conidia interact within a particular liquid. Conidia may suspend in a liquid but within that suspension the conidia may be clumped and/or unevenly distributed (Bateman, 2004). The PSA method not only indicated which was the more suitable liquid but also allowed clumping to be detected. This is vitally important for formulation of conidia as formulations need to be homogeneous to ensure a stable formulation, an even spray, and hence a greater hit rate after application (Burges, 1998).

The difficulties encountered when some conidia did not suspend easily, in the PSA method, i.e. *B. bassiana* in water, resulted in 'false' readings. Only a subsample of the conidia, those that were relatively hydrophilic compared to the majority, were recorded. An improvement in the method would have been to rank samples that did not suspend well in Shellsol T, or water as either highly hydrophilic or highly hydrophobic, respectively.

The SAS method ranked *A. eichhorniae,* isolate WH3a, as the most hydrophobic sample, in contrast to the other two tests, which ranked *A. eichhorniae* as the second least hydrophobic fungus. These contrasting results may be due to the size of *A. eichhorniae* conidia which can vary in size from 20 to 69 µm depending on the age of the conidia (David 1991), compared to less than 10 µm for the other fungal conidia examined in this study (Kirk *et al.*, 2008). As *A. eichhorniae* conidia are up to an order of magnitude greater in size than the other conidial samples, in compliance with Stokes' law, the conidia are going to settle at a faster rate. Hence, some caution has to be taken when using the SAS method to rank the hydrophobicity of different sized fungal conidia.

In the phase exclusion assay *B. bassiana*, SP2 002, *A. eichhorniae*, WH3a and *Trichoderma* sp., T22, conidia, when mixed with the toluene phase, did not separate out fully after 30 minutes. This was most evident in the *B. bassiana* sample, with small bubbles of the aqueous phase captured in the toluene layer. Hence, it was very difficult to get enough of the aqueous layer to take an accurate reading. The strong interactions of the conidia with the water interfered with the OD readings. There were also some instances where the OD reading for *B. bassiana, A. eichhoriae* and

Trichoderma sp. conidia actually increased. Two possible explanations for this is that firstly conidia had an emulsifier affect when the water and toluene layers were mixed causing the layers to form an emulsion of sorts. Secondly conidia may have imbibed water and increased in size. Imbibition would result in larger particles being detected and less light able to pass through the sample, resulting in an OD reading greater than the original reading. However, 30 minutes was probably not long enough for the conidia to imbibe sufficient water to swell up. With work on *Aspergillus fumigatus*, Renwick *et al.* (2006) demonstrated that it took 2 hours before conidia were observed to be swollen. Further studies would need to be carried out to determine why the OD reading increased.

The biggest limitation encountered with all the methods was suspending very hydrophobic conidia in an aqueous liquid. This resulted in difficulty in reading particle size as the conidia floated on surface of the water. For the phase-exclusion assay and the SAS tests it was difficult to get an initial OD reading of 0.6 as conidia would float on the surface of the water. This problem does not seem to be mentioned in other studies on hydrophobicity (Jeffs and Khachatourians, 1997; Mozes and Rouxhet, 1987). Some conidia will suspend into an aqueous suspension as each conidium is slightly different and hence hydrophobicity will vary between conidia and between isolates of the same species. Thus while a small percentage of the relatively less hydrophobic conidia are suspended in the aqueous phase, the majority of the conidia will be floating on the surface of the liquid or stuck to the side of the container (personal observation). This is not a true reading but a small sub-sample of the population.

In conclusion, the PSA method was a quick and simple way to test the relative hydrophobicity of fungal conidia. Size of the conidia did not affect the results as encountered when using the SAS method and no extraction was required as in the phase exclusion assay, where difficulties occurred. In addition to the PSA method determining relative hydrophobicity, it also helped to explain how the conidia react with each other, i.e. clumping, when suspended in different liquids, and how non-inert formulation ingredients affected particle size, which would be of interest to a formulation scientist.

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Legends:

Table 1. Fungi used in this study. The isolate number represents conidia stored in the CABI genetic resource collection (commonly known as the IMI collection), which is part of the UK culture collection. The code represents identification codes for this experiment. * = not all isolates were logged in the CABI collection as they were held locally.

Table 2. Emulsifier type and quantity used in the formulation.

Table 3 Comparison of the ranks of hydrophobicity obtained for the different fungal groups using three different hydrophobicity determining methods. PE = phase exclusion, SAS = salt and sedimentation assay and PSA = particle size analysis. Rank is 1= the most hydrophobic and 4 = the least hydrophobic.

Figure 1. Percentage of conidia left in suspension after 120 minutes of being mixed with buffer solutions, using the salt-mediated aggregation and sedimentation method. Error bars = standard error.

Figure 2. Percentage of conidia in the aqueous phase after 30 minutes of being combined with toluene using the phase exclusion assay. Error bars = standard error.

Figure 3. Cumulative particle size of different fungi suspended in three different liquids. Key: ♦ = suspended in water, ▲ = suspended in Shellol T and ■ = suspended in 0.05 % Tween 80.

Figure 4. Microscopic examination of *M. acridum*, IMI 330189, conidia suspended in Shellsol T (i), 0.05 % Tween 80 (ii) and water (iii). Photograph by Roberto Alves.

Figure 5. Cumulative particle size of *Trichoderma* sp. (three different samples, i. *T. harzianum,* DIS 219f, ii. *T. stromaticum* plate 0 and iii. *T. stromaticum* plate 5) suspended in three different liquids. Key: ♦ = suspended in water, ▲ = suspended in Shellol T and ■ = suspended in 0.05 % Tween 80.

Figure 6. Microscopic examination of *T. harzianum*, DIS 219f, conidia suspended in Shellsol T (i), 0.05 % Tween 80 (ii) and water (iii). Photograph by Roberto Alves.

Figure 7. Cumulative particle size of *Trichoderma viride* isolate T22 suspended in three different liquids. Key: ♦ = suspended in water, ▲ = suspended in Shellol T and ■ = suspended in 0.05 % Tween 80.

Figure 8. Cumulative particle size of *M. acridum* suspended in three different liquids. Key: ♦ = suspended in water, ▲ = suspended in Shellol T and ■ = suspended in 0.05 % Tween 80.

Table 1.

Fungus	**Isolate No.**	**Code**
Metarhizium acridum	IMI 330189	DM2
Metarhizium acridum	IMI 330189	ME 006
Metarhizium acridum	IMI 330189	ME 008
Metarhizium anisopliae	IMI 385045	TNS 10
Beauveria bassiana	IMI 390162	SP2 002
Trichoderma stromaticum	*	FA 64
Trichoderma harzianum	IMI 385767	DIS 219f
Trichoderma viride	*	T22
Alternaria eichhorniae	*	WH3a

Table 2.

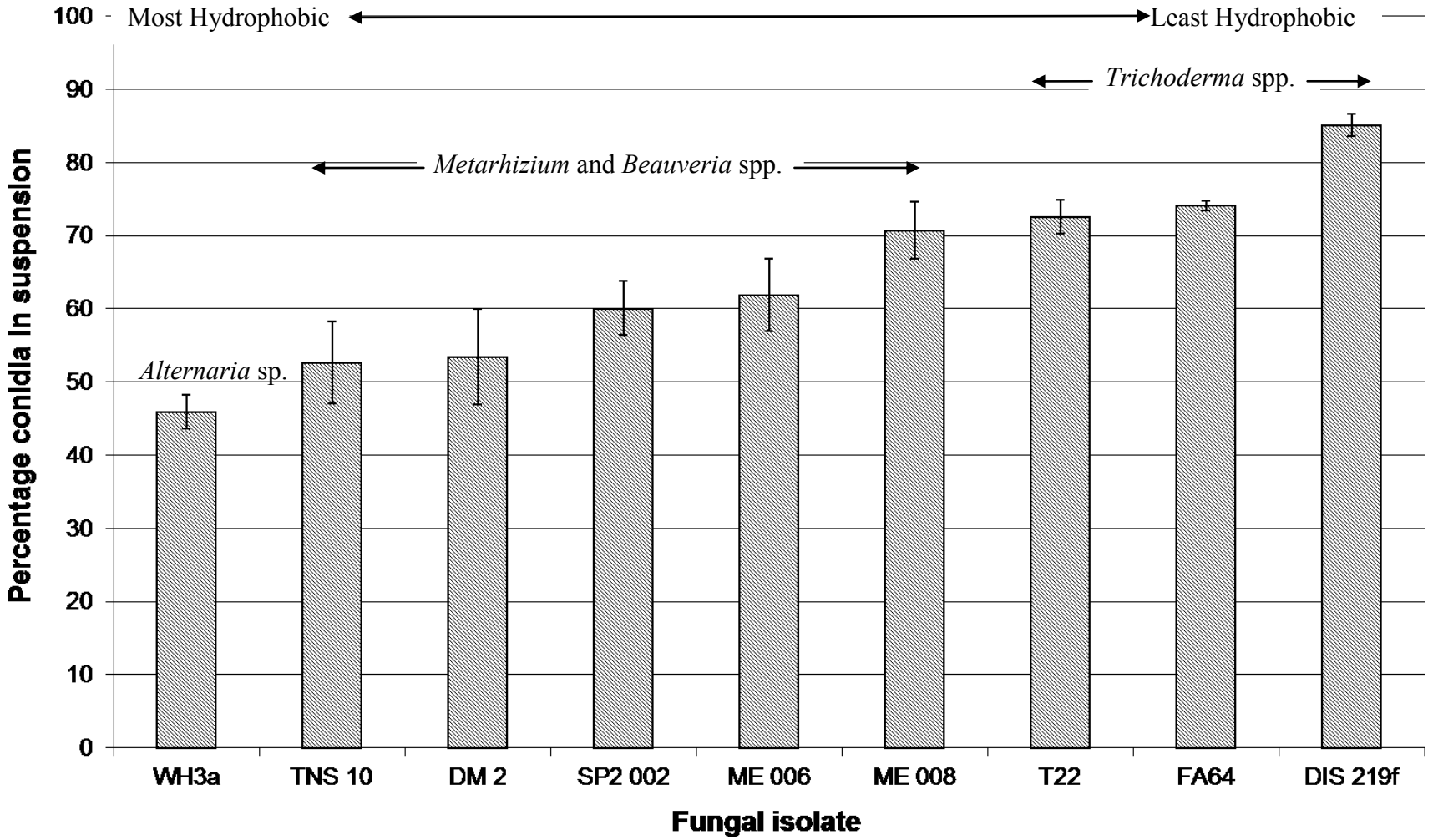
Formulation	**Emulsifier type**	**Percentage of emulsier in formulation**
1	Non-ionic	5 %
2	2 parts: a. Anionic/nonionic blend b. non-ionic	a. 2.8 % and b. 2.2 %
3	Anionic/nonionic blend	5 %

Table 3.

Fungus	**Rank**		
	PE	**SAS**	**PSA**
Metarhizium sp.	1	3	1
Beauveria	4	2	2
Alternaria	3	1	3
Trichoderma sp.	2	4	4

.

Figure 1.


100
Most Hydrophobic
Least Hydrophobic
Trichoderma spp.
Metarhizium and Beauveria spp.
Alternaria sp.
90
80
70
60
50
40
30
20
10
0
Percentage conidia in suspension
WH3a
TNS 10
DM 2
SP2 002
ME 006
ME 008
T22
FA64
DIS 219f
Fungal isolate

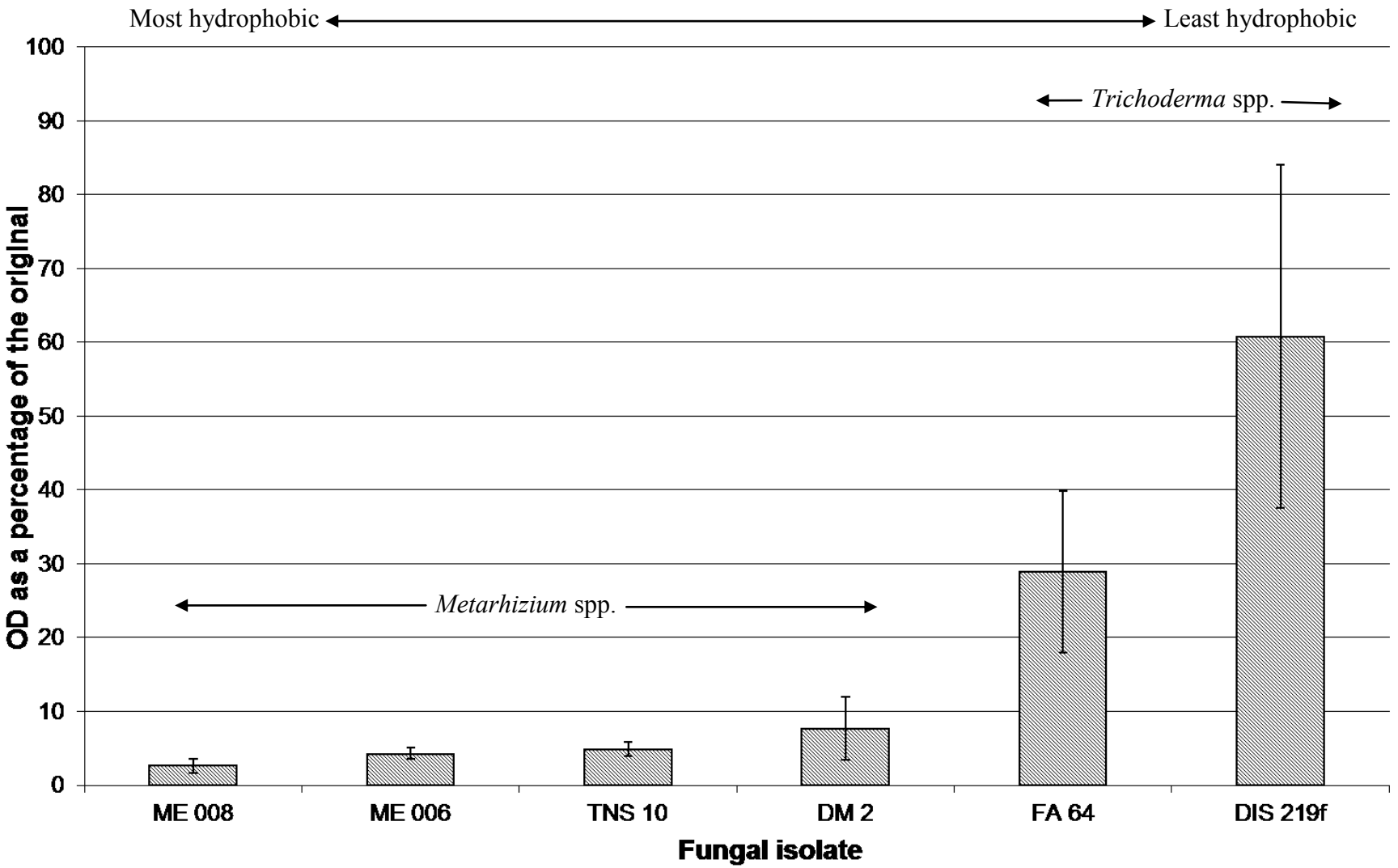
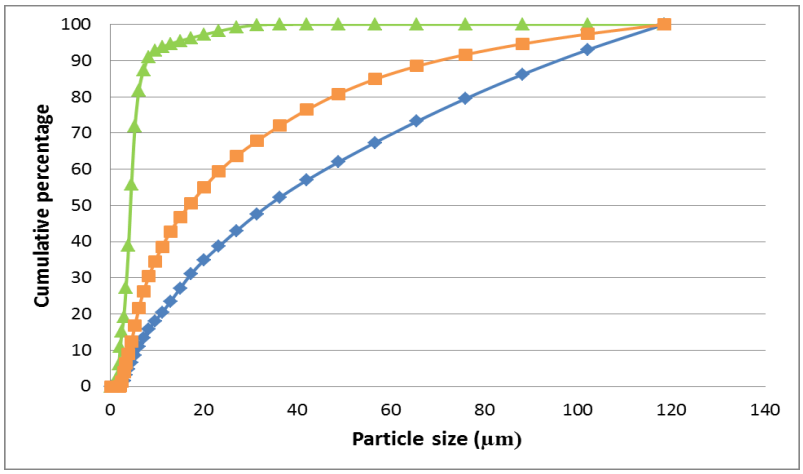
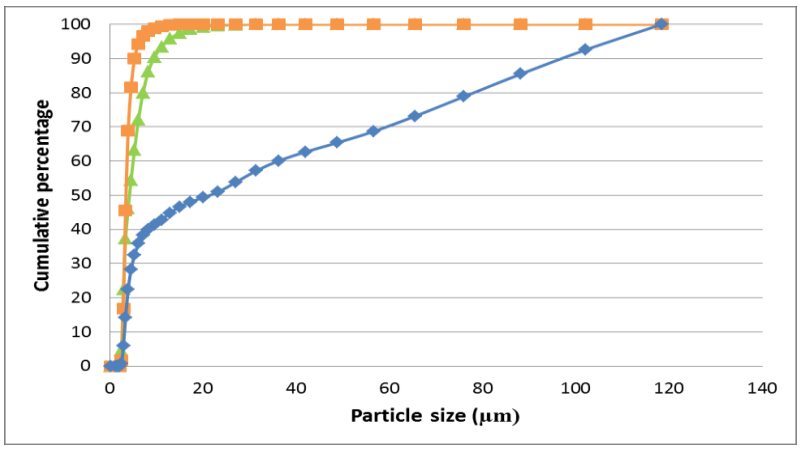

Most hydrophobic
Least hydrophobic
Trichoderma spp.
Metarhizium spp.
OD as a percentage of the original
100
90
80
70
60
50
40
30
20
10
0
ME 008
ME 006
TNS 10
DM 2
FA 64
DIS 219f
Fungal isolate

Figure 2

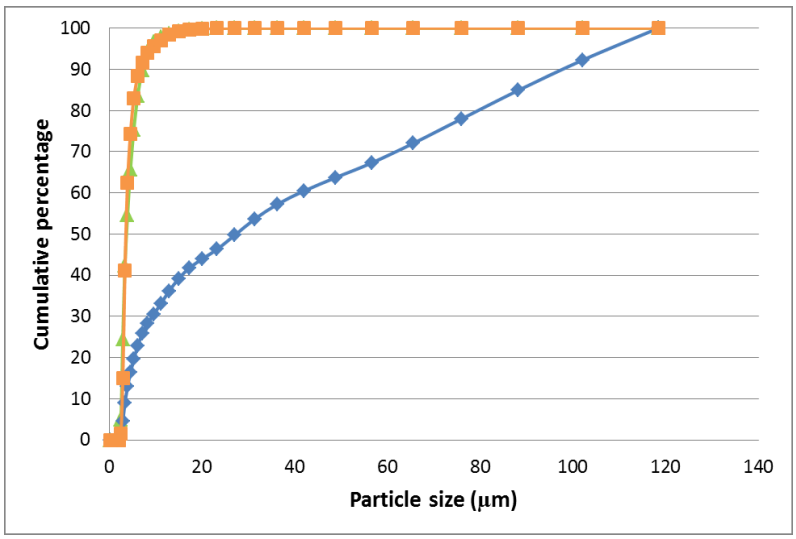
i. *M. anisopliae* TNS 10



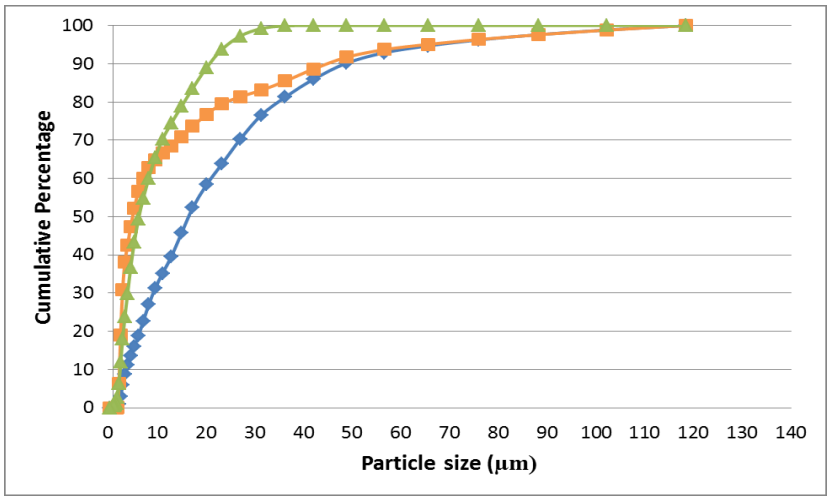
ii. *M. acridum* ME 006



iii. *M. acridum* ME 008



iv. *B. bassiana*SP2 002



v. *A. eichhorniae* WH3a

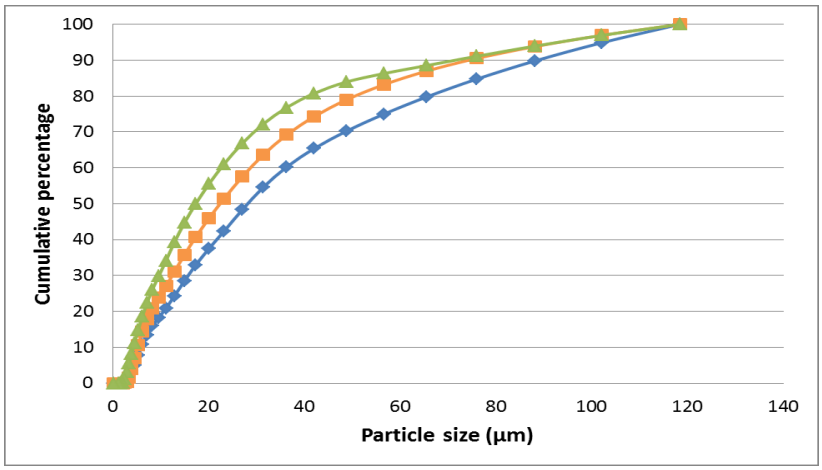


Figure 3.

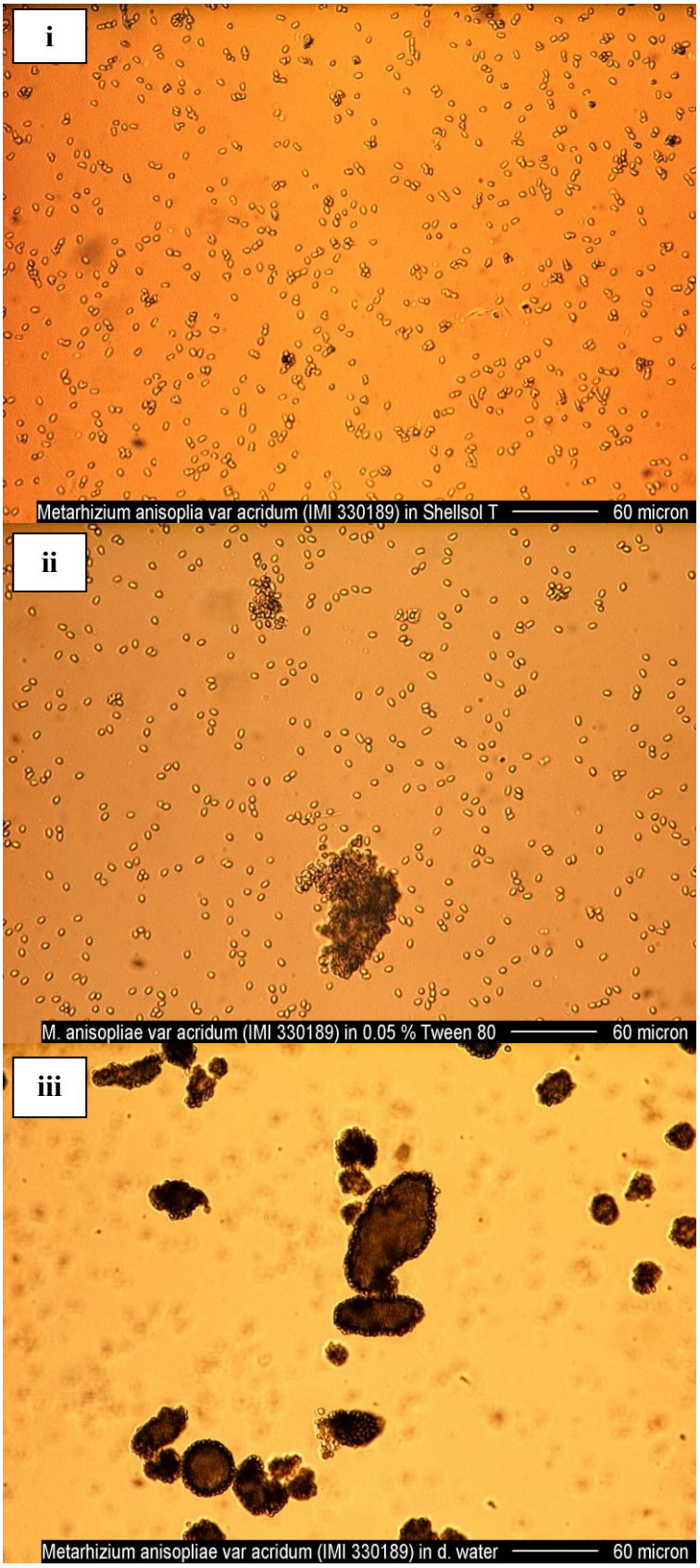
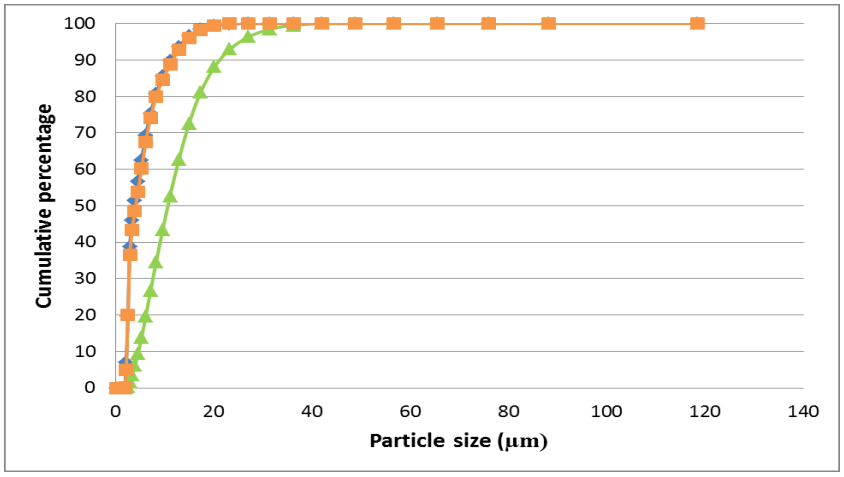
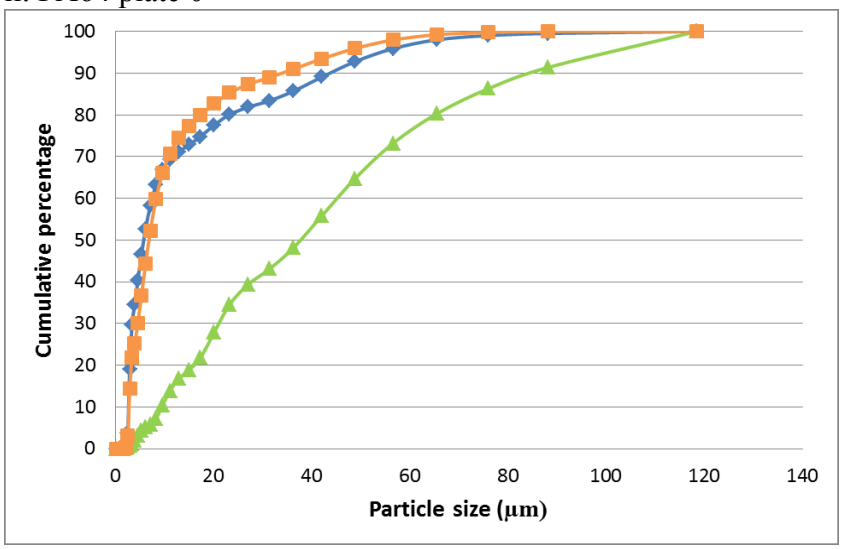

i
Metarhizium anisoplia var acridum (IMI 330189) in Shellsol T
60 micron
ii
M. anisopliae var acridum (IMI 330189) in 0.05 % Tween 80
60 micron
iii
Metarhizium anisopliae var acridum (IMI 330189) in d. water
60 micron

Figure 4.

i. Dis 219f



ii. FA64 plate 0



iii. FA64 Plate 5

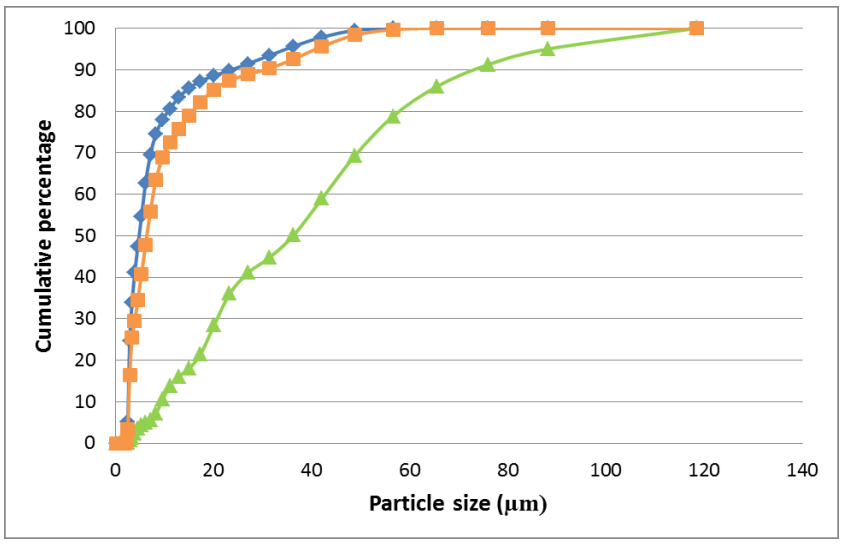


Figure 5.

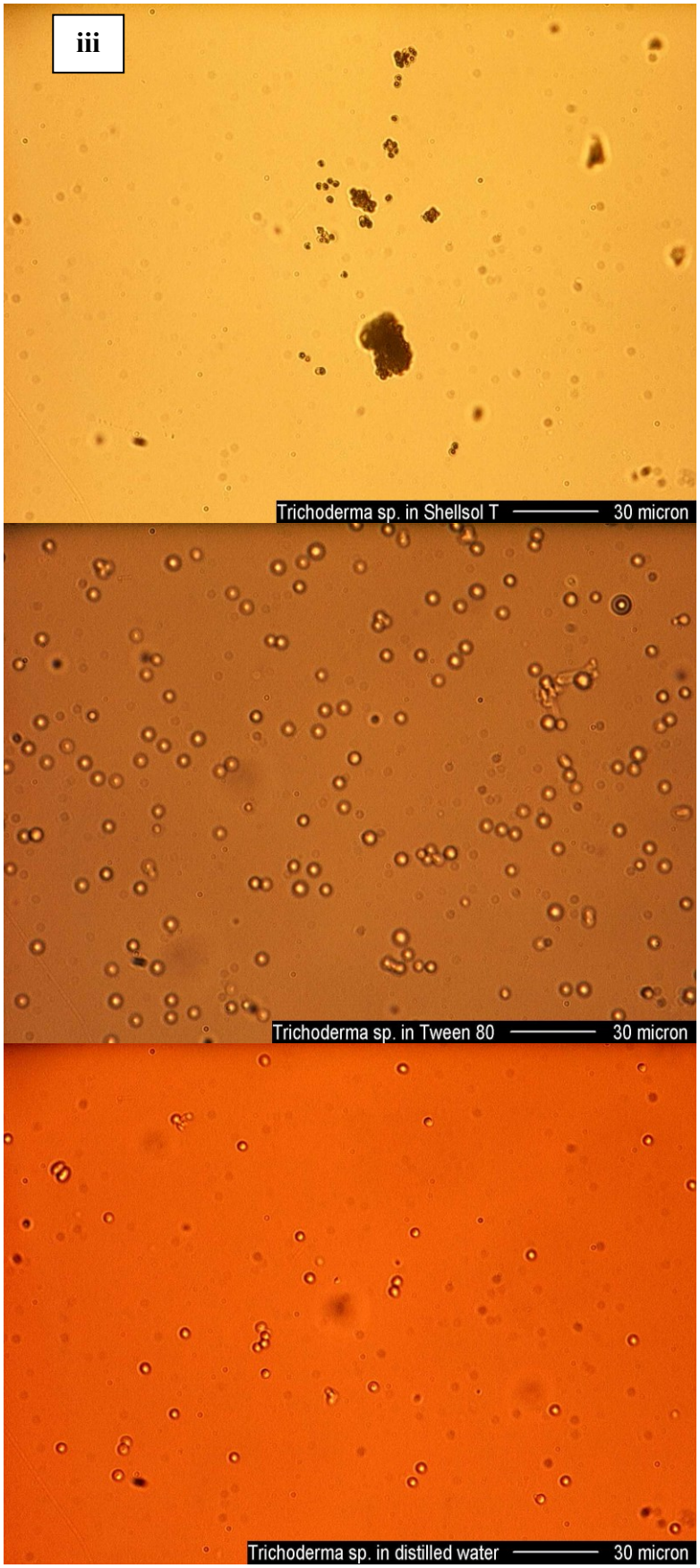

iii
Trichoderma sp. in Shellsol T 30 micron
Trichoderma sp. in Tween 80 30 micron
Trichoderma sp. in distilled water 30 micron

Figure 6.

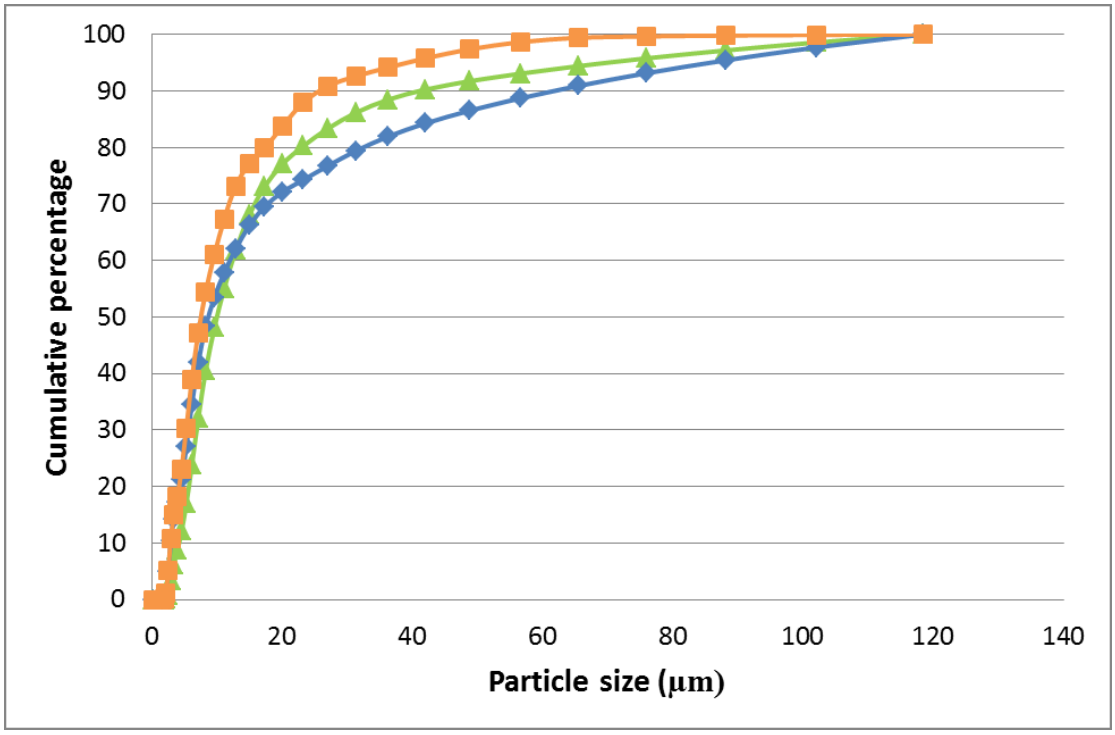

Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Particle size (µm)

Figure 7.

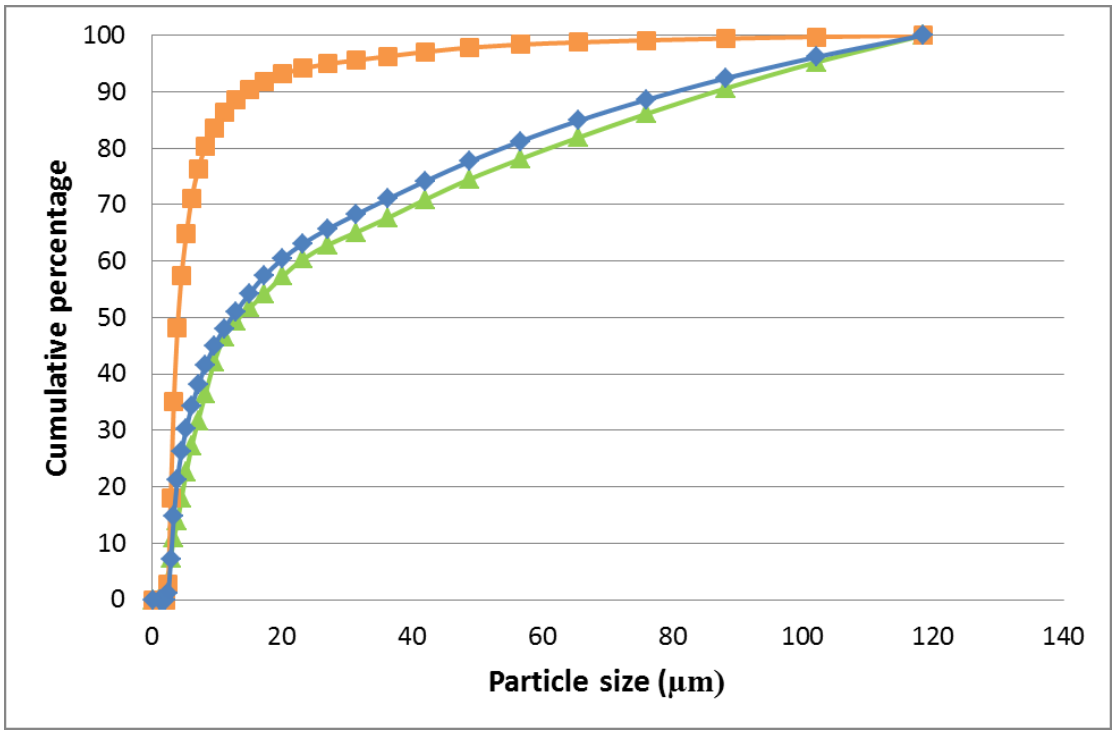

Cumulative percentage
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Particle size (µm)

Figure 8.