MASS PRODUCTION OF FUNGAL PATHOGENES FOR INSECT CONTROL

INSECT PATHOLOGY MANUAL

Section VII

CONTENTS

INTRODUCTION

The use of pathogens has been proposed for the control of many different insect and mite pests. The use of pathogens as control agents is termed 'microbial control' and the pathogen is made up as a biological pesticide which can be applied to control a pest. The product based on a pathogen is called a 'biopesticide' or, if the active agent is a fungus, it is called a 'mycopesticide' or 'mycoinsecticide'.

The first step in developing the use of a pathogen as a control agent, is to find an effective strain of a pathogen. This is usually done by developing a bioassay procedure as described in Section 3. For laboratory tests, we can usually obtain enough pathogen by using standard techniques such as growing fungi on agar in Petri dishes, or in bottles. Such laboratory based techniques can usually supply enough infective propagules to carry out bioassays and preliminary small scale application tests on the research station. Once a suitable isolate has been identified from the bioassay procedure, the isolate should then be tested on a larger scale, in the field. However, the amount of pathogen needed for replicated field trials can be hundreds or thousands of times greater than for small-scale work and it becomes impractical to simply use more Petri dishes or more insects. At this stage, more efficient methods for mass production must be developed.

This section describes a method for mass production of fungal spores which has been developed from methods in use in Brazil and China and which is particularly well adapted to the production of *Metarhizium flavoviride* conidia for use against grasshoppers and locusts. The method has been successfully used at IITA in Cotonou to produce spores for field trials. It is robust and simple but should only be attempted by people with at least some preliminary training in pathology or sterile (aseptic) technique. The production of conidia is carried out in two stages, a liquid culture stage, followed by a solid culture stage. The method is still experimental and further changes and improvements can be expected.

Rice is used as a solid substrate in this experimental system. The fungus grows equally well on maize or other grains, and research is in progress to find non-food substrates for fungal growth. Current research at IITA is working towards an 'intermediate technology' production. For this work, we are trying, as far as possible, to phase out the use of expensive capital items and any imported ingredients. We also hope to substitute a cheaper ingredient such as maize, bran, cotton seed or brewery waste (mash) for the rice substrate. We are working towards a system which could be put into place in any town in Africa, either under private or government control. IITA and IIBC are interested in helping any such enterprise to get started by providing training and quality control certification. Please contact the directors of the IITA biological control programme or IIBC for further information.

A general description of mass production procedures is given in the following section. This gives an overview of the LUBILOSA mass production system and provides explanations for the use of various techniques and procedures. A full description of the methods and procedures for fungal culture maintenance is given.

1. GENERAL PROCEDURES FOR TWO STAGE PRODUCTION OF FUNGAL PATHOGENS

CULTURE MAINTENANCE

When mass producing a fungal pathogen, it is essential that the strain is well maintained. It must be free from all contamination and carefully conserved so that it not only remains viable in agar culture, but also retains its virulence. If fungal strains are sub-cultured too often on artificial media, they can lose their virulence. This can be prevented by maintaining isolates for storage on Potato carrot agar (PCA) slopes, which may be kept in the refrigerator for between six months and one year. The spores from these cultures can then be used to inoculate working cultures on Sabouraud dextrose agar (SDA) slopes (the recipes for these media can be found below). In the longer term, fungal strains should be cycled through the original host or closely related species periodically.

A full description of how to run and maintain a working fungal culture collection.

LIQUID MEDIA

STAGE 1:

A liquid stage in the mass production system encourages rapid mycelial growth of the fungal culture which can then be used to inoculate the second, solid stage in the production process. The advantages of this procedure are that liquid cultures of fungi are fast growing and are therefore the most economical method of production. Where possible, industrial production of fungus is carried out in liquid culture in large fermentation vessels which have electronic controls and monitoring. Unfortunately, aerial conidia are not produced in liquid culture but require a surface where conidia can be formed at the interface between the substrate and the air.

There are a number of advantages in using a two stage production system which uses actively growing liquid culture as inoculum for the solid substrate:

1 The competitiveness of the fungus is enhanced. This reduces the risk of colonisation of the solid substrate by contaminating microorganisms.

2 Colonisation and conidiation on the solid substrate by the actively growing liquid culture is more rapid, thus economising on space.

3 The liquid culture stage can act as a check for contamination originating from the slope culture.

4 It ensures even coverage of the substrate, resulting in homogeneous growth and maximum conidiation.

The liquid medium used in the first stage of production should contain a supply of carbohydrate (for energy) and nitrogen (in the form of proteins or amino acids or as inorganic nitrogen such as KNO_3, from which proteins can be synthesised) which are essential for growth. Any microbiological medium must supply these two components in one form or another.

A cheap and effective liquid medium for the mass production of fungi can be prepared using sugar (sucrose) as the carbon source and dried, waste brewers' yeast as the nitrogen source (a method for the preparation of brewers' yeast for use in mass production is given below).

An alternative source of carbon is glucose (also called D-glucose or dextrose). Other carbon sources such as fructose and maltose may also be used but these are less commonly found in general microbiological media and are likely to be more expensive than sucrose or glucose. Malt extract can also be a good source of carbon.

Other nitrogen sources include yeast extract, peptones and other meat digests. Inorganic nitrogen sources may also be used but these tend to be less easily utilised by fungi and can result in only limited growth.

SOLID SUBSTRATE

STAGE 2:

The solid substrate phase of mass production provides a physical support for the fungus to produce aerial conidia (the infective propagules which are best suited to storage and formulation in oil. Usually, the substrate is a cereal or cereal by-product such as rice, millet, maize or wheat bran. Because these are natural products, their nutrient status is undefined. The fungus will use a certain proportion of the nutrients supplied by these cereal products during growth and sporulation, but the majority of the calorific value will remain unused. In some ways, the structure of the substrate is more important than the nutrients supplied. An ideal substrate will provide a high surface area to volume ratio with the individual particles remaining separate to provide inter-particle spaces for aeration and formation of conidia. For this reason, broken white rice is often the preferred substrate as the individual rice particles are small, providing a large surface area and if prepared carefully, remain separate from each other after autoclaving and inoculation.

DRYING AND EXTRACTION

Once the conidiation process is complete and conidia have been formed over the whole surface of the solid substrate the next stage in the process will depend largely on the intended use of the end product. At this point it is important to ensure that the method of extraction is compatible with the intended formulation. If, for example, the conidia are to be formulated in oil for ULV application, a very fine powder with uniform particle size is required (see product specifications in this bulletin). Therefore, the extraction process must be both efficient and selective for conidia but not rice dust. If, however, the intended use is for soil pests such as white grubs, the conidia may be left on the substrate and the whole product of the fermentation may be used for application directly into the soil.

Whatever formulation is required the product must first be dried in order to ensure that the conidia will remain viable during storage. Once conidiation is complete, some degree of drying can be achieved simply by opening up the containers with the substrate and allowing the contents to air dry. This process can be speeded up by dehumidifying or air conditioning the room or accelerating ventilation with a fan but this will depend on local environmental conditions.

Once the moisture content of the conidia and substrate has reached between 20 and 30%, the conidia may be extracted from the substrate. The conidia should then be further dried to approximately 5% before storage. Drying the conidia powder after separation from the substrate reduces the amount of space required for the drying process and is more economical. There is little point in drying the substrate down to such a low level if it is only to be discarded afterwards.

QUALITY CONTROL

Quality control should be carried out both during the production process and ultimately, on the end product. Both of these aspects of quality control are essential for the successful production of good quality viable conidia which are free of potentially dangerous contaminants. Quality control procedures carried out during the production process and monitoring for the entrance of contaminants into the system have been incorporated into the LUBILOSA production routine in order to help in locating the source of these contaminants when detected (Contamination Control, CC). Quality control carried out on the final product (the spore powder) is designed to ensure that the product meets pre-determined specifications. These include viability, virulence, moisture content (for long shelf-life), the number of infective propagules per g product, particle size spectrum (suitable for the application equipment) etc. (Product Quality Control).

SUMMARY OF MASS PRODUCTION PROCEDURES

Conservation and management of fungal cultures
Preparation of liquid medium
Preparation of sporesuspension
Inoculation of liquid medium
Incubation on shaker
Preparation of rice *or* other solid substrate
Inoculation of solid substrate
Incubation in plastic bowls
Extraction

2. CULTURE MAINTENANCE

ORGANISING A CULTURE COLLECTION

Details of how to isolate fungi from infected insects are given in Section 3. This section covers the basic procedures for managing fungal isolates in a culture collection. Good management of an isolate collection is essential for successful mass production. Records should be kept of all isolations as they are performed.

1 Buy a hard backed note book and label it on the outside with a title e.g. 'Fungal Isolates'.

2 Each time a fungal isolate is obtained either from an insect, a fellow scientist or from a culture collection, enter the details in the book. Use a separate page for each isolate and fill in the following information:

a) Date.

b)An 'accession' number. You should give each new accession a sequential number. Your numbering system may be very simple e.g. 1, 2, 3 and so on. Alternatively, you can include a little more information such as the name of your research lab e.g. IIBC 1, IIBC 2 etc...

c)Name of the collector.

d) The species of fungus e.g. *Metarhizium flavoviride* or if the isolate has not been fully identified give as much information as you can e.g. *Beauveria* sp.

e)The host insect from which the fungus was isolated e.g. *Zonocerus variegatus*.

f) The location at which the infected insect was collected e.g. Malanville, Benin and the date of collection.

g) Any previously assigned accession number.

h) Any other information. Keep the book up to date with information about each isolate, such as references to bioassays or field trials and whether the isolate has been supplied to other laboratories.

3 Place the insect cadaver in a paper bag with some dry silica gel and label with the laboratory accession number. In hot and/or humid climates, these should be stored in a refrigerator or freezer. Then, if problems occur in the isolate collection, it is possible that a re-isolation can be made from the original specimen.

Sometimes, not all information for the isolate records book will be available at the same time. However, you should always enter as much information as you can. If an isolate has been obtained from another culture collection, you should make a note of the number given by that culture collection for future cross reference. If you send the isolate to another culture collection such as the International Mycological Institute (IMI) for safe keeping, make a note of it's new accession number so that you can re-locate it later. Make a space for additional notes such as when the isolate is sub-cultured or re-isolated. An example of a typical entry in a fungal isolates records book is given on the next page.

A records book is essential to any culture collection, it should be kept safe and updated as shown above. If you have access to a computer, the same information may also be stored in a database or similar programme. However, although computerised information can be both useful and time saving, problems can still occur and computers should never be relied on as the only source of information. Always keep a records book as a back-up to computerised information.

Example:

page from a 'Fungal Isolates Records Book'

Date and Accession No	Accession No. Collector	Species, Host Insect, Origin, Date of Collection
11.9.92 IIBC 34	C. Lomer	*Metarhizium* sp. ex. *Kraussaria angulifera* Senegal. 16.7.92

Notes:

Isolated onto PCA + antibiotics (11.9.92), second sub onto 3 PCA slopes (22.9.92) also working cultures on Malt extract and SDA.One PCA slope (22.9.92) sent to IMI culture collection (IMI accession No. IMI 312659), stored as freeze dried culture and in liquid nitrogen.

Laboratory culture on PCA in refrigerator.

N.B. This isolate seems to grow best on Malt extract agar, no sporulation on SDA.

Single spore (ss) isolation made from 22.9.92 culture labelled as IIBC 34ss. Isolation tested for virulence in standard bioassay and re-isolated from infected insect onto PCA+antibiotics (1.11.92). Second sub of ss culture onto 3 PCA slopes and working cultures on Malt extract.

One PCA slope (IIBC 34ss, 1.11.92) sent to IMI culture collection (IMI accession No. IMI 312659ss), stored as freeze dried culture and in liquid nitrogen.

PCA slope from IIBC 34ss (1.11.92) subbed onto PCA for further storage on 1.6.1993 (3rd sub from original isolation).

Isolate re-isolated through *S. gregaria* using spores from 1.6.1993. Isolated onto PCA + antibiotics on 14.1.1994. Second sub onto 3 PCA slopes (2.2.1994) and stored in refrigerator, also working cultures on malt extract agar.

ss isolate used in UV experiment by Dave Moore and student - showed good UV resistance under sunlight simulator.

ss isolate used for mass production.

STORAGE OF ISOLATES

Isolates should be grown on PCA and kept refrigerated for medium-term storage, other methods should be used for long-term storage of all isolates of potential future interest. Such long-term methods include freeze drying and storage in liquid nitrogen. However, these methods require expensive capital equipment and in many laboratories this is not feasible. An alternative method is to store the isolates on PCA slopes (agar slants in Universal bottles) covered with sterile mineral oil. If kept refrigerated, these should remain viable for as long as 30 years, but this will vary according to the isolate.

At least 2 methods of storage should be used to guard against accidental loss. Some examples of simple long term storage techniques are given below:

N.B. Only use isolates which have been sub cultured less than 4 times from an insect isolation for long term storage.

Storage under mineral oil

1 Grow the fungal isolate on a shallow PCA slope until fully sporulated.

2 Pour approximately 10 ml of sterile mineral oil (pure white paraffin oil) into a universal bottle, allow one bottle of mineral oil for each slope to be stored. Sterilise the mineral oil for 15 min at 121°C on two separate occasions to ensure sterility.

3 Pour cold sterile oil over the top of the agar slope so that the culture is completely submerged. Screw the lid of the culture bottle down tightly and place in a refrigerator. This method of storage will keep isolates viable for between 2 and 30 years depending on the fungal isolate.

Silica gel storage

Non-indicating silica gel must be used in this technique as the indicating type contains copper at levels which can be toxic to fungi. It should also be noted that silica gel heats up when water is added to it. Therefore, in this technique the silica is pre-cooled by freezing to prevent excess heat from killing the spores on addition to the gel.

1 Grow the fungal isolate on a PCA slope until fully sporulated.

2 Half fill a number of glass universal bottles with silica gel beads and screw on the caps loosely. Prepare at least 3 bottles of silica for each isolate to be stored.

3 Dry heat sterilise the bottles containing the silica gel by placing them in an oven at 180°C for 3 hours.

4 Stand the sterilised bottles in a tray containing water to a depth of about 2.5 cm (1 inch), put the whole lot in a freezer and leave overnight.

5 Make up a 5% solution of skimmed milk for each isolate to be stored and sterilise by autoclaving for 15 min at 121°C. Once sterilised, cool the milk solution in a refrigerator, this can also be left overnight.

6 The next day, remove the frozen tray of bottles from the freezer and allow to stand at room temperature whilst preparing the spore suspensions. During this time, the ice in the tray will begin to melt, this helps to dissipate the heat created when the spore suspension is added.

7 Make up a spore suspension of each isolate for storage in the cooled milk solution. This is best done by adding a little of the milk solution to an agar plate or slope containing the spores and rubbing the surface gently to dislodge the spores. Pour the resulting spore suspension back into the remaining milk solution and shake well. The concentration of the resulting spore suspension can be anything from 1 x 10^6 spores/ml upwards.

8 Trickle a small amount (4-5 drops from a sterile Pasteur pipette) of spore suspension over the cold silica gel. The silica beads should be wet but no excess should collect at the bottom of the bottle.

9 Leave the bottles in the laboratory with the lids loose so that the silica can gradually dry out (in humid climates, the bottles should be put in a refrigerator to dry out). This will take about 14 days, the silica beads should become separated from each other and appear dry.

10 Finally, screw the lids of the bottles down tightly and put them in a plastic sandwich box with some freshly dried silica gel. Close the lid of the box tightly and store in a refrigerator.

SPECIALISED METHODS

Freeze drying and liquid nitrogen storage can only be done using specialised equipment which is not generally available in insect pathology labs. However, there are a number of specialist mycology labs which accept fungal isolates for storage and offer freeze drying and liquid nitrogen storage as a service.

Important isolates should always be deposited in a culture collection for safe keeping. See Appendix I for a list of laboratories, and their addresses, which maintain culture collections. Many of these laboratories will also carry out identification services in addition to storing isolates. Some culture collections will accept isolates for deposition free of charge, others will make a nominal fee. All culture collections will charge for identification of fungal isolates, but will then add the culture to their collection free of charge. If it is not possible to deposit important isolates in a recognised culture collection, a good fall back is to send them to another laboratory carrying out work with fungi with whom communications are good. Then, if for whatever reason, problems occur in your own laboratory, there will always be a back up elsewhere.

It is likely that only one or two isolates from the culture collection will be required in such large quantities that mass production is necessary. These isolates will have been selected from a number of different fungi in bioassays. Once selected for mass production, it is essential that the isolate is not sub-cultured more than 5 times from an insect isolation. The following procedures should be carried out for isolates intended for mass production.

Stock cultures for use in mass production

To maintain virulence you must regularly re-isolate the fungus from host insects. This is easily done following the bioassay procedure described in the section on detailed procedures in this bulletin.

1 Once the fungus has sporulated on the cadaver, carry out the re-isolation procedure onto antibiotic agar, the cultures growing on the antibiotic agar are the first subculture (first sub).

2 Once the first sub has formed conidia, the culture should be checked under the microscope to ensure that the culture is pure. These spores may then be used to inoculate a few slopes of PCA, for storage of the isolate. Next, a batch of 'working' cultures (also prepared from the first sub) should be prepared on SDA or malt extract agar (depending on the preference of the isolate). Approximately 20-30 slopes should be prepared, these will be used for mass production. These cultures will be the 'second sub' after the original isolation. It is therefore possible to use the stored PCA cultures to inoculate a further batch of SDA slopes for use in mass production when required - which will be the third sub. If possible, cultures for use in mass production should be restricted to this level of sub-culturing to ensure that virulence is maintained.

3 Once the fungus has conidiated on the surface of the slopes, the lids should be screwed down tightly and the bottles stored in the refrigerator until use (no longer than 3 months).

By preparing these clean stock cultures you will always have viable spores with which to work. Spores from each slope can be expected to produce similar results in mass production. Each time a new set of slopes is used, additional quality control checks should be carried out as part of the mass production procedure (see Quality Control).

NEVER use spores from slopes which have either been stored for over 3 months or which have been sub-cultured more than 5 times.

Use single spore isolations for mass production if available.

If you do not know the history of a slope, DO NOT use it for mass production.

3. MASS PRODUCTION USING THE TWO STAGE

TECHNIQUE PREPARATION AND INOCULATION OF BREWERS' YEAST/SUCROSE (BY/SUC) BROTH

PREPARATION OF BY/SUC BROTH

STAGE 1

This is a simple liquid broth which encourages the production of hyphal bodies and mycelium. These can then be used for inoculation into the second (solid) stage of production. Brewers' yeast is added to this medium in a dry, granulated form using the following procedure:

BY/SUC Broth	g/litre (tap water)
Dried yeast	20
Sucrose	20

1 Mix 20 g yeast and 20 g sucrose in 500 ml of tap water.

2 Heat the broth in a pan of water for 10-15 minutes, this brings the solution to boiling point.

3 Homogenise in a Wareing blender at high speed for 60 seconds or until there are no more lumps of yeast.

4 Leave to settle for 2-3 hours or overnight in the refrigerator, so that the foam can settle out, then add a further 500 ml of tap water to make up to one litre.

5 Mix the broth gently by hand until the yeast is suspended homogeneously in solution.

6 Put 75 ml of the solution into a 250 ml conical flask (This gives optimal aeration during growth of the fungus). If larger conical flasks are used, adjust the amount of liquid medium so that the proportion of liquid to flask capacity is about the same as in the 250 ml flasks above.

7 Plug the flasks with non-absorbent cotton wool bungs or polyurethane bungs and cover with aluminium foil.

8 Autoclave at 121°C (15 psi) for 20-30 minutes.

INOCULATION OF BY/SUC BROTH

It is essential that each production run has a consistent inoculum.

1 Take spores from a stock culture growing on SDA as described above, suspend spores in sterile water containing 0.05% Tween 80. The spore suspension should contain approximately 6 x 10^6 spores per ml. This can be achieved by adding approximately 10 inoculating loops full of spores to 50 ml of 0.05% Tween water. If you are not sure, take a small sample using a sterile pipette and count using a haemocytometer (see Section 3). When you have done this several times, you will soon be able to recognise the concentration of spores in a suspension by the colour of the suspension and you will no longer need to count the spores to get the right concentration.

2 Shake the spore suspension thoroughly and use 1 ml of spore suspension to inoculate each flask (75 ml) of BY/SUC medium.

3 Check the spore suspension, a sample of un-inoculated broth and a sample of inoculated broth for contamination using the contamination check

procedure for liquid samples described in the uuality control section of this section.

4 Put the inoculated flasks on the shaker at approximately 150 rpm for three (3) days at approximately 25-30°C (room temperature).

N.B. Prepare enough sterile water and sterile 1 ml pipette tips in advance. Use aseptic technique throughout this procedure.

PREPARATION AND INOCULATION OF RICE SUBSTRATE

STAGE 2

Preparation of rice

Use broken white rice if possible, as it has the most suitable particle size and is cheaper than whole grain rice.

1 Wash the rice in cold running water to remove any starch dust etc., drain off excess water and place the rice in a large pan.

2 Add approximately 300 ml of water and 20 ml of cooking oil (e.g. peanut oil, maize oil or vegetable oil) for each kilogram of rice. Mix well and heat until all the water has been absorbed. Stir the rice often during the cooking process to prevent sticking and uneven absorption of the water.

3 Distribute the cooked rice into autoclavable bags (500-1000g of dry rice would be enough for one bag when coo ked).

4 Autoclave at 121°C and 15 psi for 40 minutes.

5 Allow to cool to room temperature before inoculation with liquid culture.

It is essential to use aseptic technique during all procedures which follow autoclaving in order to prevent contamination.

Inoculation of rice with liquid medium

1 Take a small sample of the liquid broth to be used for inoculation for contamination control (CC).

2 Take a sample of un-inoculated rice for CC.

3 Use one flask of liquid inoculum (75 ml) for each 500 g bag of rice, or if you used 1000 g of rice, dilute the liquid medium with 75 ml of sterile water. Flame the neck of the flask and pour the whole contents of the flask onto the rice in the bag.

4 Massage the bag from the outside to evenly distribute the inoculum over all the rice grains.

5 Take a small sample of inoculated rice from one bag in every ten that you inoculate for testing the moisture content and for CC.

6 Place the bag in a container such as a plastic washing-up bowl with 6 holes of approximately 20 cm diameter drilled around the circumference. Let the top of the bag fold over loosely to allow air to enter the bag and help aerate the substrate as the fungus grows. Cover the bowl with a lid and plug the 6 holes with polyurethane or non-absorbent cotton wool bungs.

7 Stack the bowls/containers one on top of the other to save space in the incubation area.

8 Incubate the bowls/containers at room temperature (25-30°C) for about 10 days, during which sporulation will occur.

9 Once the fungus has sporulated over the surface of the rice grains open up the bowl and the bag and allow the rice and conidia to dry out. The bowls/containers should be re-stacked to allow as much aeration of the substrate as possible. Drying will take about 7 days depending on the humidity in the atmosphere.

10 Check all the bowls for growth of contaminants. Throw away all contents of any bowls with any visible contamination. Be careful not to spread contamination and ensure that all the contaminated waste is either autoclaved or burnt.

11 Once dried, the conidia can be extracted from the rice.

Dry extraction of conidia from rice

N.B. Always wear full respiratory protection during this procedure. For preference, the extraction should be carried out in a fume hood. Remember that dry spores can easily contaminate locust colonies.

1 Carefully pour the dry rice into the top of a metal sieve with a mesh size of 300 µm or less (if the sieve has a receiving tray at the bottom, you should use this to collect the spore powder. If you do not have a receiving tray, the spore powder can be collected directly inside a plastic bag - see below).

2 Place the top on the sieve and put the entire sieve assembly into a large plastic bag and tie with a knot to seal it.

3 Shake the sieve inside the bag for several minutes, then allow to stand before opening the plastic bag. The spore powder will settle to the bottom of the bag, or if you are using a receiver, the spore powder will have been collected in this.

N.B. If you are using a receiver, the spore powder will have to be removed after each shaking period and collected in a separate container

4 Empty the old rice out and add more sporulated rice to the sieve, seal the bag again and shake well. Repeat the process until you have sieved all of the rice.

5 For larger quantities, use a two-man sieve. This should also be completely contained within plastic during the sieving process.

6 Carefully transfer the conidia from the bag into plastic boxes or other open container for further drying.

N.B. The spore powder should not be too deep to ensure that the spores at the bottom will dry as well as those on the top.

Final drying of the conidial powder

The extracted conidial powder should be dried down to 5% moisture content before being stored in a refrigerator. This can be done on a relatively small scale using a standard glass (or plastic) desiccator containing dried silica gel. The conidial powder can be placed in the desiccator to dry for 5 days.

Fresh dry silica gel beads (non-indicating if possible) should then be added to the conidial powder (these may be contained in small packages made from muslin or nylon netting) at a rate of 20% w/w. The whole product may then be stored in a refrigerator or cold room or a deep freeze until use.

N.B. never use hot silica gel as this will kill the spores.

4. QUALITY CONTROL

There are a number of aspects of quality control in spore production. These include control of contamination (CC) during the production process and quality control (QC) checks on the viability, moisture content, yield per gram of substrate, number of conidia per gram of spore powder, particle size spectrum and virulence of the final product. Each of these aspects is covered in detail below:

CONTAMINATION CONTROL (CC)

One of the most critical aspects of mass production is to ensure that contamination of the product is avoided at all costs. Even low levels of contaminants such as Aspergillus or Penicillium spp. would be completely unacceptable in a registered product. Contamination control is most usefully done by making regular checks for the appearance of contamination during the mass production run and recording the results of these checks on the mass production record sheets. The smallest amount of contamination at the beginning of the procedure will multiply to an unacceptable level in a number of days. If contamination is left unchecked, it can also result in an increase of the contamination load in the incubation room, thus increasing the risk of contamination in future batches. The documentation of all contamination checks allows easy identification of the source of contamination and thus enables appropriate preventative measures to be taken. Contamination control should be carried out at each stage of the procedure, i.e. a check of the spore suspension used as inoculum, the liquid shake flask before and after inoculation, the un-inoculated rice, rice after inoculation and finally a complete quality control procedure should be carried out on the conidia powder (final product) once extracted from the substrate.

CC 1 - EXAMINATION OF SLOPE

Look carefully at the slope and check that the sporulation on the surface looks normal. Do not use slopes which look abnormal or which have visible contamination.

CC 2 - SAMPLES OF SPORE SUSPENSION

1 When you have finished inoculating the liquid shake flasks, take a sample (approximately 0.5 ml) of the remaining spore suspension and aseptically spread over the surface of an agar plate (SDA or Malt)

N.B. Use aseptic technique..

2 Incubate the plates at approximately 25-30°C for 3-5 days.

3 After the incubation period, the results should be logged in the appropriate place on the mass production record sheet.

4 If contamination is found on any of these plates, the whole mass production run should be regarded as suspect and each individual bowl in this mass production run must be checked for contamination as described in CC 4 below.

CC 3 - LIQUID SHAKE FLASK SAMPLES

1 Select 10 flasks at random.

2 Check a small sample from each flask under a microscope for visible signs of contaminating fungi (these will have different sized hyphae to the fungus you are working with) and bacteria (these will appear as tiny even sized dots which often swim about very fast).f any flask shows signs of contamination, you must check ALL flasks in the mass production lot and reject any flasks which show signs of contamination.

3 Now take 0.5 ml from the sample to be tested and aseptically spread over the surface of an agar plate (SDA or Malt)

N.B. Use aseptic technique..

4 Incubate the plates at approximately 25-30°C for 3-5 days.

5 After the incubation period, the results should be logged in the appropriate place on the mass production record sheet.

6 Again, if contamination is found on any of these plates, the whole mass production run should be regarded as suspect and each individual bowl in this mass production run must be checked for contamination as described in CC 4 below.

N.B. The point at which contamination was introduced into the procedure can be easily identified.

7 If the plates inoculated with a sample of the original spore suspension are found to be contaminated, the contamination must have occurred either during the inoculation of the agar slope from which the spores were taken or during the preparation of the spore suspension. If the plates containing a sample of the liquid broth after inoculation are contaminated, but the spore suspension plates and the plates containing a

sample of un-inoculated liquid culture are free of contaminants, the contamination problem must have occurred during the inoculation of the shake flasks.

CC 4 - SOLID SAMPLES (RICE OR OTHER SUBSTRATE)

1 Select approximately 1 (one) bag in 10 for CC.

2 Before inoculating the substrate with liquid medium, carefully remove a few grains of 3 the substrate and place them on the surface of an agar plate (SDA or malt). This procedure may also be carried out on inoculated or colonised grains.

3 Incubate the plates for 3-4 days at 25-30°C and incubate and record as above.

4 After the incubation period, the results should be logged in the appropriate place on the mass production record sheet.

5 If contamination is found on any of these plates, this could indicate that the substrate was not sufficiently sterilised before use. This may result in the whole mass production run being contaminated.

PRODUCT QUALITY CONTROL (QC)

QC 1 - YIELD OF CONIDIA PER GRAM OF SUBSTRATE

As part of the general monitoring of the mass production system, it is important to keep a record of the yield of conidia/g of substrate. A drop in yield can act as an indicator of problems in the system such as unsuitable temperature fluctuations, inefficient extraction or declining productivity of the fungal isolate.

Estimation of yield/g of substrate

During the extraction procedure, a note must be made of the total original weight of substrate to be extracted. In the standard mass production procedure for instance, each bowl would normally contain 1 kg of rice substrate, the number of bowls to be extracted should therefore be noted along with the total weight of substrate (e.g. 3 bowls = 3 kg rice). Conidia should then be extracted by sieving or other selected method and placed into a suitable container.

N.B. If the container is pre-weighed before adding the spore powder, the total weight of conidia inside is easily calculated by subtraction of the weight of the container. The weight of spore powder should be noted after desiccation, i.e. once the spores have equilibrated to approximately 5% moisture content Do this BEFORE ANY samples are taken out for use in other quality control procedures.

Yield of conidia/gram of substrate is calculated by dividing the weight of conidia powder by the total weight of substrate extracted in grams.

e.g. if we obtained 127.6 g of spore powder from 3 bowls of rice (3000 g), the yield of conidia per g substrate would be:

QC 2 - MOISTURE CONTENT OF SPORE POWDER

In order to maintain viability and virulence of conidia during storage, it is essential to reduce the moisture content of the conidia to less than 5%. Conidia stored at higher moisture contents loose viability rapidly and are less resistant to environmental stresses such as elevated temperature and UV radiation. Therefore, conidia are actively dried after extraction using dehumidifiers and silica gel. The moisture content of the conidia powder must be checked after drying to ensure the correct level has been achieved.

Estimation of percent moisture content

1 Label a number of small glass bottles (1, 2, 3 etc.) with permanent marker pen and put them in an oven at approximately 80°C for about 2 hours to ensure that they are completely dry.

2 Remove the bottles from the oven and allow them to cool to room temperature. Weigh each one and make a note of the weight. Use a balance with at least three decimal places.

3 Take a small sample of the dry conidia powder (0.05-0.5 g) and place it in one of the bottles. Weigh the bottle immediately to prevent the spore powder absorbing moisture from the atmosphere and make a note of the new weight. Prepare three bottles for each lot of conidia powder.

4 Put the bottles back in the oven at 80°C for 48 hours. Do not put lids on the bottles, but put some loose lids in the oven to dry alongside the samples.

5 Remove the bottles from the oven and immediately screw a dry lid on each bottle. The lids must be screwed down tightly to prevent the dry spore powder from absorbing any moisture from the atmosphere. Allow the bottles to cool to room temperature but do not leave for more than an hour before re-weighing.

6 Re-weigh each bottle with the lid removed and make a note of this new weight.

7 The percentage moisture content can now be calculated from the three figures obtained above

$$\%\text{ moisture content} = \frac{(W-B)-(D-B)}{W-B} \text{ x } 100$$

Where:

W = Weight of bottle plus wet spores

D = Weight of bottle plus dry spores

B = Weight of bottle

e.g. if:

The empty bottle (B) weighed 15.8682 g

The bottle plus wet spores (W) weighed 16.5302 g

The bottle plus dry spores (D) weighed 16.4970

$$\frac{(16.5302-15.8682)-(16.4970-15.8682)}{16.5302-15.8682} \text{ x } 100 = 5.02\%$$

QC 3 - NUMBER OF CONIDIA PER GRAM OF DRY SPORE POWDER

This figure should be fairly constant for all batches of conidia from the same isolate, providing that the measurement is made with conidia at 5% moisture content (which it should be). This measurement should therefore be made after the moisture content check, in fact it is possible to use the spore powder samples from the moisture content check to carry out these tests.

Determination of number of conidia/g conidia powder

1 If you use the spore samples from the moisture content determination, you must be sure that none of the spore powder has been spilt from the bottle and that you have noted the weight of the spore powder (minus the bottle) before drying. Alternatively, accurately weigh a fresh sample of conidia powder (0.05-0.1 g) into a universal bottle and note the weight (3 reps per sample).

2 Add exactly 10 ml of Tween water (containing between 0.05 & 0.1% Tween 80) to each sample and shake vigorously to suspend all the conidia in the water, sonicating will also help. If all the conidia will not go into suspension, dilute the whole contents of the universal in a larger volume of Tween water,

N.B. note the amount of water used e.g. 10ml plus 50ml

8 Once all the conidia are suspended carry out a dilution series using 1 ml in 9 ml as explained above. In this case it will not be necessary to dilute down to -8, a dilution of -2 or -3 will usually be sufficient to enable the spore suspension to be counted accurately.

9 Select the most appropriate dilution and count the spore concentration using a haemocytometer. Calculate the concentration of conidia/ml using the procedure given in Section 3. Multiply this figure by the dilution to give the spore concentration in the original 'stock' solution.

10 Multiply the concentration of the stock solution by the number of ml in the stock solution i.e. 10 ml or if this was diluted further in order to completely suspend the spores in solution, multiply by the total volume used (e.g. 10+50=60 ml) to give the total number of conidia in that solution. Then divide this number by the weight of conidia powder (wet weight) in grams to get the number of conidia/g powder.

e.g. if the concentration of spores in the -1 dilution was 1.9 x 10^7 spores/ml, the concentration of spores in the stock solution would have been 1.9 x 10^7 x 10(-1 dilution) = 1.9 x 10^8. The stock solution contained a total of 60 ml (10 ml added to the spore powder plus and extra 50 ml to enable all the spores to be suspended) so 1.9 x 10^8 x 60=1.14 x 10^{10} (total number of spores in the stock bottle). Finally, divide this figure by the original weight of the spore powder in the bottle: 1.14 x 10^{10}/0.1808 g=6.29 x 10^{10} spores in 1 g of spore powder.

QC 4 - VIABILITY OF DRY SPORE POWDER

The viability of conidia after extraction and drying should be checked immediately before the conidia are placed in storage, at intervals during the storage period and immediately before use. Aseptic technique is not necessary in this procedure as the spore powder to be tested has not been extracted under sterile conditions and the oil used to suspend the spores will also not be sterile. The agar plates should be prepared under sterile conditions to ensure that contaminants are not growing in the agar before the plates are used. Viability is determined by counting the percentage germination of conidia after 24 hours incubation as follows:

Estimation of percentage germination

1 Take a small sample of spore powder and make a dilute spore suspension (10^5-10^6 conidia/ml) in light mineral oil such as Shellsol T or kerosene.

2 Use a micro-spatula to transfer 2-3 drops of the spore suspension onto the surface of a SDA plate. The oil suspension should be carefully spread over the surface of the agar and the plates incubated at 25-30°C for 24 hours.

3 Count the proportion of germinated and non germinated conidia by counting a total of 300 conidia per plate under the x20 or x40 objective of a light microscope (200 or 400 times magnification). Count all the spores in each field of view, a spore should be considered to be germinated if a germ tube is visible protruding from the side of the spore. If no germ tube is visible the spore should be counted as non-germinated. Use a separate tally counter for germinated and non-germinated spores. A total of 3 plates (300 spores per plate) should be counted to give an accurate estimate of the percentage germination.

4 Record the results in the appropriate place on the mass production record sheet.

QC 5 - BIOLOGICAL PURITY OF DRY SPORE POWDER

1 Take a sample of dry conidia powder (approximately 0.1 g) using a small spatula and suspend the powder in 10 ml sterile water plus 0.05% Tween 80 (accurately measured) in a universal bottle. Replace the lid and shake vigorously.

2 Do a dilution series by taking 1ml of the spore suspension and transferring it to a universal bottle containing 9 ml sterile water plus 0.05% Tween 80. Label this bottle -1 as this is the first one in ten dilution of the dilution series.

3 Shake the -1 bottle vigorously, then transfer 1 ml of this spore suspension to another universal bottle containing 9ml sterile water plus 0.05% Tween 80. Label this bottle -2.

4 Shake the -2 bottle and continue the dilution series down to -8.

5 Now take a 0.2 ml sample from the -8 dilution and spread it over the surface of a malt agar or SDA plate. Prepare two plates for each of the dilutions in the series and label them accordingly.

6 Incubate the plates at room temperature (or approximately 25°C) for 4-5 days. Then remove the plates and count the number of *Metarhizium* colonies present on each of the -8 dilution plates. The average number of colonies per plate is calculated by adding the two counts together and dividing by two.

7 Count the colonies as above for each pair of dilution plates where individual colonies are still distinguishable. At the higher concentrations, the colonies will have merged together and it will not be possible to count the number of *Metarhizium* colonies.

8 Now look through all the plates and search for any contaminating microorganisms. If you find any, count the number on both plates of that dilution and divide by two just as above, note this number and the dilution where the contamination was found. Do this for each pair of plates where any contaminating colonies are found.

9 sing the *Metarhizium* colony counts and the contamination colony counts it is possible to calculate the percentage contamination present in the spore powder product. First, you must calculate the number of *Metarhizium* conidia present in the original concentrated spore suspension. To do this, take the average counts from each of the dilutions where counts were possible. You should see a pattern in these values in that the highest dilution should contain approximately one tenth of the number of conidia in the dilution before and approximately 100th of the number of conidia than the dilution before this.

e.g. the average count of Metarhizium colonies from the -8 dilution might have been 6, the count for the -7 dilution would therefore be expected to be about 60. In reality the count could be anything between 45 and 75. The -6 dilution would therefore contain between 450 and 750 colonies and it is quite likely that it would not have been possible to count these plates. The most accurate way of calculating the concentration of the original spore suspension in this example would be to take the average count from the -8 dilution and multiply it by 10, then add this number to the average count for the -7 dilution and divide by two. This will give an estimated average of the number of conidia in the -7 dilution:

e.g. average count for the -8 dilution = 6

average count for the -7 dilution = 53

6x10=60 (equivalent to the -7 dilution) so 60+53=113/2=56.5

10 The calculation above gives the estimated number of colonies in 0.2ml of the -7 dilution. To calculate the number of conidia in 1ml of the -7 suspension, you must multiply by 5.

e.g. 56.5 x 5=282.5 colonies/ml

11 Now, to calculate the number of colonies in the concentrated solution, you must now multiply the colonies/ml by the dilution.

e.g. 282.5 x 10^7=2.82 x 10^9 colonies(spores)/ml

12 To calculate the number of contaminating colonies in the most concentrated solution, follow steps 9 to 11 for the dilutions where contaminating colonies were found.

e.g. 1 contaminating colony on a -4 dilution plate but none on the other :

1+0/2=0.5 average colonies on a plate: 0.5 x 5=2.5 colonies/ml:

2.5 x 10^4 (i.e. the dilution) = number of contaminants in concentrated solution

13 Finally, divide the number of contaminating colonies/ml by the number of *Metarhizium* colonies/ml and multiply by 100, to get the % contamination of the dry spore powder.

e.g.. 2.5 x 10^4/2.82 x 10^9 x 100=0.00089%

14 Contamination should be less than 0.001%. (i.e. 1 in 10 000)

QC 6 - PARTICLE SIZE SPECTRUM OF DRY SPORE POWDER

The size spectrum of particles in the conidia powder has a profound effect on the spray properties of ULV formulations. Large particles can cause blockages in restrictors and the presence of a high proportion of non-conidial 'matter' can cause clumping and rapid settling of the formulation during spraying. Ultimately, conidial powder should contain mostly single spores with only a small proportion of double and short chains of conidia. The composition and particle size spectrum can be accurately measured using the 'Malvern' laser analyser. However, if there is no access to a Malvern laser analyser, the size spectrum of a conidial formulation can be controlled during extraction using either a cyclone extractor or by using a series of fine mesh sieves to ensure that all large particles are removed, e.g. initial extraction of spores from the rice using a 300 µm mesh sieve followed by a second sieving using a 106 µm sieve.

QC 7 - VIRULENCE

Regular checks for virulence of the conidia product to the target organism should be made at specific points during the mass production calendar. For instance on the first use of a new 'batch' of slopes, approximately half way through the use of that batch and finally at the end. All other mass production runs during the period of use of this bank can be assumed to be the same, providing no decline in virulence is detected in the above tests.

Test for virulence

The test takes the form of a standard bioassay where conidia from the mass production run are compared directly with fresh conidia from an SDA stock culture at the standard bioassay concentration (N.B. a blank oil control should always be included). 25 insects should be used per treatment and inoculated with 0.2 µl of 3.75 x 10^7 conidia/ml. Mortality should be recorded daily over a 14 day period and the data analysed using a statistical package such as SPSS. The best method of comparing the virulence of the two spore types is to perform Kaplan-Meier survival analysis on the daily mortality readings of the treatments. This analysis will give an accurate estimate of average (mean) survival time (AST) and enable a comparison of the two treatments using a log-rank statistic for significant difference in the results.

If no statistical software is available the daily mortality results of the two spore treatments should be compared side by side for obvious differences in mortality patterns. Be very suspicious of any batches of spores which result in big differences in mortality in comparison with the original stock culture.

If reduced virulence is detected, there are a number of factors which could be responsible, these are listed below and should be checked and rectified if possible.

Factors which can be responsible for loss of virulence

1 **Fungal culture has been sub-cultured on agar too many times.**

Sub-culturing should be kept to a minimum and the isolate passaged through the host or related insect at regular intervals - in LUBILOSA mass production, the maximum number of subcultures is 5.

2 **Problems with a new batch of slopes.**

The factors which affect virulence through sub-culturing are not well understood. If virulence is low after using a slope from a new batch of stock cultures, the virulence of future batches of spores from this inoculum is also likely to be low. If possible, set up a new batch of cultures from a different spore inoculum. This may be a good time to re-isolate your fungus through the host or related insect.

3 **Physical conditions such as temperature and humidity during production.**

Temperature should be maintained close to the optimum for growth and spore production, high temperatures during the production process can damage spores.

4 Low spore viability. If the bioassay was set up with spores of low viability, this will reduce the number of conidia in the application which are capable of initiating disease. Thus the insects in this treatment will have received a lower dose than the standard. This should be cross checked with the viability test.

An example of a quality control record sheet is given on the next page. Always keep records of each mass production run and the quality control checks carried out.

5. DETAILED PROCEDURES

This section gives full details of some of the techniques used in the mass production procedure.

PREPARATION OF YEAST GRANULES FROM YEAST SLURRY

Waste brewers' yeast can generally be obtained free from local breweries providing the manager is an agreeable soul!

The yeast waste usually looks like a thick slurry and must be processed soon after collection.

1 Put the yeast slurry into large glass beakers.DO NOT fill above the half-way mark, as the yeast expands during autoclaving.

2 Cover the beakers with foil and autoclave for 30 minutes to destroy the yeast cells. Leave to cool and settle. You can leave the beakers for a day or so in this form before the next stage of processing.

3 Remove the water from the surface of the yeast.

4 Filter the yeast through a double layer of laboratory paper towel (the passage of water is faster than with conventional filter paper). The filtration process takes a number of hours (e.g. overnight) and is finished once the yeast looks like putty.

5 Open out the paper towel and let the lump of yeast dry, preferably in a flow cabinet. As the yeast dries out, break up the lump into small pieces by hand.

6 Crumble the small pieces by hand regularly throughout the drying process. The finished product should look like coffee granules in both size and hydration.

7 It is possible to store the dried product in a cool dry place in a sealed container for up to 6 months.

PREPARING STERILE WATER WITH 0.05% TWEEN 80

Sterile water with Tween is used when making up spore suspensions with hydrophobic spores. Tween is a concentrated detergent which helps to wet the surface of the spores so that they are more easily suspended in solution. ween is usually made up in distilled water, although for most applications you can also use tap water.

It is very difficult to accurately measure the proportions of water to Tween when you prepare a solution of Tween.

1 One (1) droplet from a Pasteur pipette in 100ml water is approximately 0.05%. For one (1) litre of 0.05% Tween solution add 10 drops to one (1) litre of water.

2 Shake to dissolve.

3 Put into bottles of an appropriate size.

4 Prepare enough Tween water to inoculate all the flasks of liquid medium you have prepared. You will need 1 ml of Tween solution for each 75 ml flask, plus 5 ml extra for a margin of error

5 e.g. for 50 flasks you will need 55 ml of Tween water in a 100 ml conical flask or similar)

6 Autoclave the Tween solution for 20 minutes at 121°C and 15 psi. You can store the sterile solution for a few months in a screw topped bottle.

DETERMINATION AND ADJUSTMENT OF THE MOISTURE CONTENT OF CEREAL PRODUCTS.

The recommended moisture content of rice for production of *Metarhizium* spp. is 50%. However, some fungi differ in the amount of water that they require for growth . Also, if you decide to try using an alternative cereal substrate, you will need to determine the optimum moisture content of this substrate for growth and sporulation of your fungal isolate. In order to do this, you will need to be able to calculate the resulting moisture content of the substrate after the addition of various amounts of water and then use these figures to calculate the amount of water required to achieve a specific moisture content.

As a rough guide, whole grains should contain between 40% and 50% water after inoculation and bran products between 50% and 60%. Using rice as an example, dry rice has a moisture content of between 8 and 15%, it is therefore necessary to add water to the rice before autoclaving. This is best done by cooking or par boiling the rice as described in the section on preparation of rice above.

CALCULATING THE MOISTURE CONTENT OF CEREAL PRODUCTS

To calculate the resulting moisture content of a given sample of cereal (normally done after autoclaving and inoculation):

1 Take at least 3 clean, dry Universal bottles and give each an individual label,

e.g. 1, 2, 3 etc.

2 Note the exact weight of each bottle (without lid) to at least 2 decimal places (preferably 3 or 4).

3 Half fill each bottle with a sample of cereal substrate and re-weigh the bottles (without lids). Note the new weight for each bottle.

4 Place all the bottles in the oven at 80°C for at least 24 hours but no more than 72 hours. Put the lids of the bottles in the oven to dry, but don't put them on top of the bottles.

5 Once dry, put the lids on the bottles and screw down tightly to prevent moisture absorption from the atmosphere. Leave the bottles to cool down slowly at room temperature.

6 Once cool, take the lid off of each bottle just before weighing and note the weight quickly as the dry cereal will start to absorb moisture from the atmosphere as soon as the lid is removed.

7 Using these three measurements you can calculate the wet weight and the dry weight of the cereal substrate and from these you can calculate the moisture content of the wet substrate as a percentage.

8 To obtain the wet weight of the substrate, subtract the weight of the empty bottle from that of the total weight of the bottle plus wet substrate.

9 Do the same for the bottle containing dry substrate to find the dry weight.

10 Now, to calculate the percentage moisture content, subtract the dry weight from the wet weight, make a note of this figure, then divide this by the wet weight of the substrate and finally, multiply by 100.

e.g.

$$\frac{WET - DRY}{WET} \text{ x } 100$$

Once you have found out the moisture content of your inoculated substrate, you should make a note in the mass production records sheet. If the moisture content was too low e.g. 30%, you should adjust the amount of water that you add to the rice or other substrate next time you do a mass production run. This can be done accurately by using the calculation given below.

ADJUSTING THE MOISTURE CONTENT OF CEREAL PRODUCTS

To calculate the amount of extra water needed to achieve a specific moisture content, use the following equation:

$$A = \frac{B \text{ x } (C - D)}{100 - C}$$

Where:

A = Amount of extra water needed (ml)

B = Total weight of substrate (g) before the addition of any water

C = % moisture content required

D = % moisture content in current sample (from the calculation above).

e.g. for a bag containing 500g of rice, with a known moisture content (after cooking, autoclaving and inoculation) of 30% and a required moisture content of 50%, the calculation would be:

$$\frac{500x(50-30)}{100-50} = 200ml$$

This is the amount of extra water you will need to add to each 500 g of rice to get a moisture content of 50% next time you start preparing rice for mass production.

You can use the same calculations for any cereal substrate.

PREPARATION OF ANTIBIOTIC AGAR

Only use this agar for isolating fungal isolates from dead insects or if you have found bacterial contamination in your stock cultures.

1 Prepare agar medium using the standard lab procedure (see Section 3 for media recipes). For isolation from insects, PCA is normally used.

2 Autoclave the prepared media in large bottles for 15 minutes at 121°C and 15 psi.

3 Meanwhile prepare the antibiotic solution as follows: Weigh out 0.05 g Chloramphenicol dry powder into a small bottle such as a Universal bottle and dissolve the powder in 10 ml of 90-95% alcohol (ethanol). DO NOT sterilise this solution.

4 Add antibiotic solution to the sterilised agar at a rate of 1 ml in 100 ml of agar medium (i.e. 10 ml in 1 litre).

5 Gently invert the bottle several times to distribute the solution evenly throughout the medium, but don't shake the bottle as this will create air bubbles.

6 Now autoclave the medium again for 10 minutes but at 118°C, (this can be done in a pressure cooker using the small part of the weight only) to ensure total sterility.

N.B. For agar slopes, distribute the medium in sterile universals before the second autoclave run. This reduces the possibility of contamination.

N.B. Wear gloves at all times when handling concentrated antibiotics. Chloramphenicol is poisonous

6. EQUIPMENT LIST FOR THE ROUTINE MAINTENANCE AND MASS PRODUCTION OF ENTOMOPATHOGENIC FUNGI

ROUTINE MAINTENANCE OF FUNGAL PATHOGENS

ESSENTIAL EQUIPMENT:

9 cm **Petri dishes** (glass or plastic[i])

Titanium inoculating loops

Pressure cooker or **autoclave**

Refrigerator or **10°C incubator**

Access to a balance (2-3 decimal places)

Bunsen or **spirit burner**: 500 ml beakers, 250 ml conical flasks, medical flats, universal bottles, funnels etc.

Access to microscope

Microscopy equipment (slides, cover slips, stains etc.)

Haemocytometer (preferably, an Improved Neubauer)

Pasteur pipettes

Saucepan

Cooker or **gas ring**

CONSUMABLES:

Agar

Peptone, yeast extract or **supply of brewers' yeast** *(is there a brewery in town?)*

Sucrose/Glucose

Tween 80 or **similar surfactant**

Alcohol

Chemicals, general e.g.. K_2HPO_4, $MgSO_4$, NaCl etc.

Mineral oil (High grade paraffinic oil)

Non-indicating silica gel

ESSENTIAL EQUIPMENT:

Rotary shaker or **fermenter**

Conical flasks (preferably 250 ml capacity - twice as many as there are places on the shaker)

Pipette fillers

Sterile 1ml pipettes

(The above two items are best replaced with an automatic pipette and autoclavable tips which will save time and facilitate handling)

Laminar air flow cabinet

Shelving

300 and 106 µm sieves

Large airtight containers for storage of conidia powder (end product)

Access to Wareing blender or similar (e.g. pestle and mortar)

Access to sonicator (bath or probe)

Respiratory protection for fine dusts (these should be full face or half face masks with specialised filters for fine particles - paper masks are not adequate)

Access to a Whirlimixer/Vortex

CONSUMABLES:

White rice or other suitable source of starchy substrate

Non-absorbent cotton wool/polyurethane bungs for flasks

Aluminium foil

Silica gel (preferably non-indicating)

Odourless (deodorised) kerosene (Only for oil formulation)

Viscous oil e.g. groundnut oil (Only for oil formulation)

Autoclavable bags (Some types of cheap carrier bags are fully autoclavable, try the local market)