



MASS PRODUCTION OF ENTOMOPATHOGENS *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* USING RICE AS A SUBSTRATE BY DIPHASIC LIQUID-SOLID FERMENTATION TECHNIQUE

^aYadav Seema, ^bTandan Neeraj & ^aKumar Krishan

^aDepartment of Biotechnology, Singhania University, Rajasthan - 333515, India

^bScientific and Applied Research Center, Meerut - 250001, India

ABSTRACT

Beauveria bassiana (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin are entomopathogenic fungi (EPF) that occur naturally in the environment and have a potential as biological control agents against many insect pests. Different solid substrates i.e such as grains, vegetable wastes, maize, bran, cotton seed, rice husk, wheat and liquid media such as coconut water were evaluated at variable moisture content and yeast extract concentration for mass production of two entomopathogenic fungi: *Beauveria bassiana* (Bals.) Vuillemin and *Metarhizium anisopliae*. In the present study rice is used as a best solid substrate for spore production and their viability but fungus also grows equally well on maize or other grains. This gives an overview of the mass production of fungal spores using diphasic liquid-solid fermentation i.e LUBILOSA system which is particularly well adapted to the production of EPF's *B. bassiana* and *M. anisopliae* and provides explanations for the use of various techniques and procedures.

KEY WORDS – *Beauveria bassiana*, *Metarhizium anisopliae*, Entomopathogenic fungi, Mass production, Diphasic Liquid-Solid Fermentation, Solid substrates, Rice.

1. INTRODUCTION

Entomopathogenic fungi (EPF) such as biological control agents can be used as a component of integrated pest management (IPM) of many insect pests. Under natural conditions, these pathogens are a frequent and often cause natural mortalities of insect populations. There is a high potential for the use of Hyphomycetes such as *Metarhizium* or *Beauveria* for biological control because such fungi can be cost-effectively mass-produced locally, and many strains are already commercially available. For example, *Beauveria bassiana* has been mass produced on different solid substrates, including sugarcane wastes (Somasekhar *et al.*, 1998), silkworm pupal powder (Chavan *et al.*, 1998), agar medium (Sergio *et al.*, 2003) and steamed rice (Feng *et al.*, 1994, 2004). Species in the same genus (*B. bassiana* and *B. brongniartii*) are produced by more than 14 companies, and *Metarhizium* (*M. anisopliae* and *M. anisopliae* var. *acridium*) by more than 10 (including some companies in Africa), aimed at controlling various insect pests including termites, cockroaches, black vine weevil, whiteflies, aphids, corn borers, colons and other insects (Strasser *et al.*, 2000; Khetan, 2001; Wraight *et al.*, 2001).

Biopesticides based on bacteria, viruses, entomopathogenic fungi and nematodes are often considerable scope as plant protection agents against several insects (Noris *et al.*, 2002). Use of entomopathogenic fungi as biological control agents for insect species has increased the global attention during the

last few decades. Production of adequate quantities of good quality inoculums is an essential component of the biocontrol programme.

Production of good quality inoculums in an adequate quantity is an essential component of the biocontrol programme. The production of entomopathogenic fungi may be carried out by following techniques based on the quantity and quality of the product desired; (1) relatively small quantities of the inoculum for laboratory experimentation and field-testing during the development of mycopesticide and (2) development of a basic production system for large-scale production by following the labour intensive and economically viable methods for relatively size small markets. China (Feng *et al.*, 1994) and America (Alves and Pereira, 1989) supply fungal pathogens by this method in sufficient quantities for niche markets in their immediate area.

Entomopathogens can be mass produced using the diphasic liquid-solid fermentation technique developed for the LUBILOSA (Lutte Biologique contre les Sauteriaux, www.lubilosa.org) project to produce *Beauveria bassiana* and *Metarhizium anisopliae* (Lomer *et al.*, 1997). The liquid phase provides active growing mycelia and blastospores, while the solid phase provides support for development of the dry aerial conidia. The conidia produced by these fungi can be used directly as natural granules or extracted through sieving and formulated as powder, granules or oil concentrate, or any other suitable formulation depending on the target insect pest for example, *Metarhizium* is applied as conidia or mycelia in

various formulations. Control of insect pests in field after initial application is achieved through the induction of a fungal epizootic, where new spores, and vegetative cells produced in infective insects are spread, naturally, to healthy members of the insect population.

According to Moore *et al.* (2000), fungal spores are living organisms and their viability diminishes with time depending on environmental conditions. It is therefore essential to determine the best substrate for spore production and their viability. This study was conducted to evaluate the mass production of fungal spores using the two stage technique liquid-solid fermentation developed for the LUBILOSA which is particularly well adapted to the production of EPF's *B. bassiana* and *M. anisopliae*. Rice is used as a solid substrate in this experimental system. The fungus also grows equally well on maize or other grains. The author also determined the suitable temperature for spore production as 28°C.

2. General procedures for two stage production of entomopathogens *Beauveria bassiana* & *Metarhizium anisopliae*

2.1 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.

2.2 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 colonization 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 KNO₃, from which proteins can be synthesized) 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.

2.3 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.

2.4 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. 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.

3. MASS PRODUCTION USING THE DIPHASIC LIQUID-SOLID FERMENTATION

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

3.1.1 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:

1. Mix 20 g dried 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. Homogenize 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 aluminum foil.
8. Autoclave at 121°C (15 psi) for 20-30 minutes.

3.1.2 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 6×10^6 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 haemocytometer. 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 uninoculated broth and a sample of inoculated broth for contamination using the contamination check

procedure for liquid samples (described in the quality control section).

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

3.1.3 INOCULATION OF RICE WITH LIQUID MEDIUM

STAGE 2

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.

3.1.4 DRY EXTRACTION OF CONIDIA FROM RICE

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).
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.

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.*

3.1.5 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) desiccators containing 13 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.

4. 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 LUBILOS 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).

5. CONCLUSION

Mass production of entomopathogenic fungi are important steps in successful utilization of EPF's as biocontrol agents. Fungi can be cost-effectively mass-produced on different solid substrates such as maize, bran, wheat, vegetable waste etc. but the findings comes through this study confirms that rice grain was found to be appropriate substrate for higher multiplication of spores of *B. bassiana* and *M. anisopliae* at the suitable temperature 28 °C. The diphasic liquid-solid fermentation i.e LUBILOS technique is an appropriate method for the mass production of mycopesticides of *B. bassiana* and *M. anisopliae* for the commercialization in the small market by many manufacturing companies. The study and

optimization of the mass production process led to the adoption of quality standards for the produced spores, which could be imposed on those who wanted to produce the spores commercially under licence. Various aspects of quality control are important, including: levels of contamination (especially the absence of human pathogens), virulence to target pests, particle size spectrum and, not least, viable spore count. Extensive research was carried out on optimising storage of spores, which should be dry (<5% moisture content) and ideally be maintained under cool conditions.

REFERENCES

Alves, S.B., Pereira, R.M. (1989) Production of *Metarhizium anisopliae* and *Beauveria bassiana*, *Ecosustania*, **14** : 188-192.

Chavan, S., Chinnaswamy, K. P. and Changalarayappa (1998) Silkworm pupal powder as ingredient of culture media of *Beauveria bassiana* (Bals) Vuill. *Insect Environment*, **4** (1), 21.

Feng, M.G., Poprawski, T.J. and Khachatourians, G.G. (1994) Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biological Control Science and Technology*, **4**, 3-34.

Feng, M.G., Poprawski, T.J. and Khachatourians, G.G. (1994) Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biological Control Science and Technology*, **4**, 531-544.

Feng, M.G., Pu, X. Y., Ying, S.H. and Wang, Y.G. (2004) Field trials of an oil based emulsifiable formulation of *Beauveria bassiana* conidia and low application rates of imidacloprid for control of false-eyed leafhopper *Empoasca vitis* in Southern China. *Journal of Crop Protection*, **23** (6), 489-496.

Khetan, S.K. (2001) *Microbial Pest Control*, 1st edition. Marcel Dekker.

Lomer, C. J., Prior, C. and Kooyman C. (1997) Development of *Metarhizium sp.* for the control of grasshoppers and locusts. In: Goettel MS, Johnson DL., editors, *Microbial Control of Grasshoppers and Locust*, *Memoirs of the Entomological Society of Canada*. **171**, 265-286.

Moore, D., Lord, J.C. and Smith, S.M. (2000) *Pathogens*. In: B. Subramanyam and D.W. Hagstrum (Eds.), *Alternatives to pesticides in stored-product IPM*. Kluwer Academic Publishers, Boston, M.A. 193-225.

Noris, R.F., Chen, E.P.S., Kogn, M. (2002) *Concepts of Integrated Pest Management* Premise Hall of India Private Limited, New Delhi.

Somasekhar, N., Mehta, U.K. and Hari, K. (1998) Evaluation of sugarcane by-products for mass multiplication of nematode antagonistic fungi. In: *Nematology: challenges and opportunities in 21st Century*.

Proceedings of the Third International Symposium of Afro Asian Society of Nematologists (TISAASN), Sugarcane Breeding Institute (ICAR), Coimbatore, India, April 16-19, 1998, Afro Asian Society of Nematologists; Luton; UK. 199-202.

Strasser, H., Vey, A. and Butt, T.M. (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Toxopneustes* and *Beauveria* species? *Biocontrol Science and Technology*. **10**, 717-735.

Sergio, O., Pablo, S, and Martin, A. (2003) Native and Introduced Host Plants of *Anastrepha fraterculus* and *Ceratitidis capitata* (Diptera; Tephritidae) in Northwestern Argentina. *Journal of Economic Entomology*.**96** (4), 1108 – 1118.

Wraight, S.P., Jackson, M. A. and De Kock, S.L. (2001) Production, stabilization and formulation of biocontrol agents. In: Butt, T.M., Jackson, C. and Magan, N., (eds). *Fungi as Biocontrol Agents, Progress, Problems and Potential*. CABI Publishing, New York. 253-288.