Mass production of fungi for biopesticides

Bruce A. Auld, Agricultural Research and Veterinary Centre, Forest Road, Orange N.S.W. 2800, Australia.

Summary

The need for scale-up in production of fungi as biopesticides from pilot laboratory processes is discussed. Methods of mass production by submerged culture, solid substrate fermentation and combinations of these are surveyed in relation to biopesticide production. Production capacity is discussed in relation to field use.

Introduction

Production of many fungi for biological pest control may often be relatively easily achieved in quantities suitable for laboratory and glasshouse research and even small scale field trials. However, if large-scale practical use is ultimately intended, mass production techniques for the fungus must be developed.

Demonstration of the feasibility of mass production for a potential biopesticide fungus will increase the likelihood of commercial interest in a project. The purpose of this paper is to introduce the methods of mass production which can be used and indicate where they can be adapted to a pilot scale in a non-specialized laboratory.

Submerged culture fermentation

Many filamentous fungi sporulate in submerged culture (Vezina *et al.* 1965). In the industrialized world at least, liquid fermentation provides the simplest and most economical way to produce large numbers of fungal spores. This is because existing equipment can be used without modification. The commercial biocontrol agents for weed control, Collego® and Devine® are produced by this method (see Churchill 1982, Stowell 1991) as well as *Verticillium lecanii* (as blastospores) for control of aphids (Mycotal®) and whitefly (Vertalec®) (Latge *et al.* 1986) and some strains of *Beaurveria bassiana* for insect control (Thomas *et al.* 1987).

Although a bioreactor (fermentor) would usually be used in industrial submerged culture, it may be possible to produce propagules in flask culture. Oxygen mass transfer is a major problem for aerobic processes as the solubility of oxygen in water is only about 6 ppm. Oxygen transfer can be increased by minimizing boundary layer resistance and maximizing surface area for transfer – this is achieved by agitation.

The type of agitation may be important and the air/liquid ratio as well as the surface contact area may need to be high. Conical flasks of 50–2000 mL may be used. The medium being used is sterilized with the flask, relying on a porous closure, like cotton wool, to act as a filter. Flasks with side indentations to act as baffles will encourage greater turbulence and aeration. Reciprocating shakers are inferior to orbital shakers which can given high oxygen transfer rates (Figure 1). Reciprocating shakers tend to splash the closure increasing the danger of contamination. Sophisticated shakers in which temperature and light are controlled may be necessary.

Another method is to bubble compressed air into flasks to provide oxygen and (limited) agitation (e.g., see Papavizas *et al.* 1984). However a pilot fermentor allows greater oxygen input and control as well as control of temperature, pH, agitation and foaming. The speed and diameter of the impellor has a big influence on oxygen transfer. The diameter of the impellor should be about $^1/_3$ the diameter of the reactor. Baffle plates assist turbulence, breaking up the liquid; they should be about $^1/_{10}$ the diameter of the reactor. However they may be a nuisance with some filamentous fungi. Standard industrial reactors are from 1000 L to 800 000 L. Smaller pilot reactors cannot mimic exactly what larger reactors will do. For instance there is a marked increase in shear at impellor tips but longer mixing times as volume increases. The minimum size of pilot fermentors generally available is 1 litre (Figure 2).

Bioreactor operation may be batch culture, semi-batch or continuous culture (e.g., see Trinci and Wiebe 1990). Batch culture is the simplest procedure with less likelihood of contamination. The output of a product such as fungal spores generally follows a sigmoidal curve with lag, exponential, stationary, and perhaps decline, phases. The growth of product can be affected by substrate limitation and inhibition, product inhibition as well as temperature and pH.

If the production of a given fungus has a long lag phase in batch culture it may be worth investigating semi- (or fed) batch culture to shorten the lag period.

Possibly one of the greatest problems encountered with fermentation will be contamination. Sterilization is a vital part of the process but the degree of "sterility" required is a function of the end use.

Media for growth of the biocontrol organism should be as simple as possible utilizing a standard set of inorganic salts and sources of carbon and nitrogen. Production of spores in the fermentor may be enhanced by changes in media components or simply by diluting the medium (Auld *et al.* 1988). Production may also be increased by beginning the fermentation process with increasing volume or concentration of starter culture. (See Latge and Moletta (1988) for an extended treatment of the production of entomopathogenic fungi in submerged culture and Stowell (1991) for examples of mycoherbicide production).

Within the bioreactor fungal growth may take the following physical forms: small discrete cells; small compact pellets; larger floccose pellets; or a filamentous form (Solomons 1980).

The method of reproduction in submerged culture may differ morphologically and physiologically from in vivo

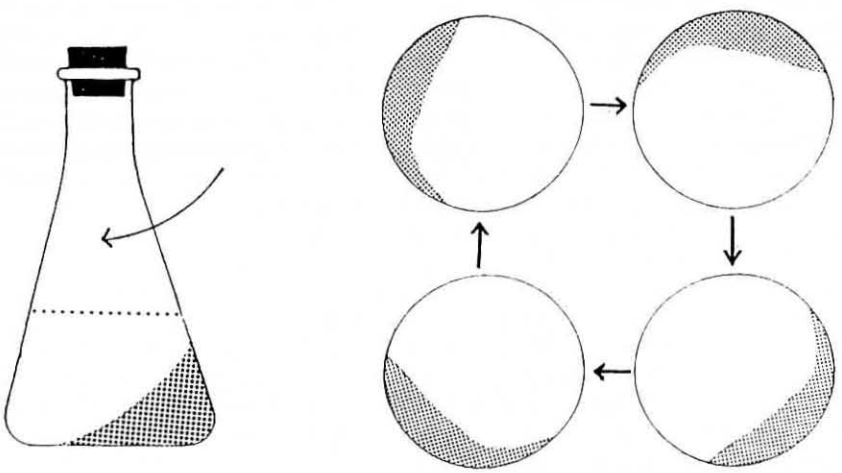

Figure 1. Conical flask – side and aerial views. The movement of liquor in an orbital shaker leaves a trail of material around the flask which maximizes oxygen transfer.

Footnote: This paper is based partly on a talk given to an I.I.T.A. workshop in Cotonou, Benin, West Africa in May 1991.

production. In the production of Collego® only about 8–10% of the spores produced are normal conidia, most of them are fission spores (Churchill, 1982).

Some fungi which do not sporulate in submerged culture may produce mycelium which can be dried (Pereira and Roberts 1990) and applied as fragments or pellets in the field; among them is *M. anisopliae* produced by Bayer as BIO 1020®. Such fungi may sporulate following dew (Roberts and Wraight 1986, Robmach *et al.* 1986, Roberts *et al.* 1987). Walker and Connick (1983) describe the production of sodium alginate pellets of dried mycelium for a mycoherbicide.

It may be necessary to screen isolates for spore productivity under fermentation conditions as well as the virulence of the spores produced. The most virulent isolates may not be the most productive in vitro.

In some cases it may be more appropriate to produce survival propagules rather than infective propagules. Chlamydospores of *Trichoderma* and *Gliocladium* for instance were more effective in field use than conidia (Lewis and Papavizas 1984, Papavizas *et al.* 1984).

Solid substrate fermentation

Solid substrate fermentation has been widely used to produce fermented foods in China, Japan and south-east Asia (Wood and Yong Fook Min, 1975). Media may contain some liquid; the 'solid' substrate itself may be relatively inert (e.g., paper, wood, vermiculite), allowing for use of defined nutrient levels. The fungus *Sporidesmium sclerotivorum* used against the lettuce pathogen *Sclerotinia minor* is grown on vermiculite moistened with liquid medium (Adams and Ayres 1982). On the other hand some nutritive solid substrates may be available locally at low costs (e.g., coffee pulp, sorghum grain, straw, groundnut shells). Particle size, moisture content and temperature may all need to be controlled for successful production. Equipment used may be bags, trays or rotating drums.

Industrial submerged culture fermentation production requires considerable capital investment. Likewise, production on solid substrates may be relatively costly in terms of labour and materials in the western industrial environment but may not be so where labour is less costly and suitable raw material is freely available.

Beauveria bassiana has been produced on solid substrates such as heat sterilized grains in the USSR and the Peoples Republic of China. In the latter system 500 mL flasks of substrate are used to "seed" 5 kg lots of steamed grain which is mixed with ten times the amount of wheat bran for fermentation in flat trays or in outdoor pits (Bartlett and Jaronski 1988). The fungus is produced in liquid surface culture in large inflated plastic bags in Czechoslovakia (Kybal and Vilcek 1976, Samsinakova *et al.* 1981). Solid substrate fermentation is an alternative for fungi which will not sporulate in submerged culture. Goettel (1984) has also described a technique for producing fungi using cellophane sheets on bran in autoclaveable bags. Abbott Laboratories in the USA have made commercial scale-up tests for the fungus, using solid substrate to produce either a wettable powder or granular formulation (Bartlett and Jaronski 1988). R.J. Milner (personal communication, 1991) has developed a solid substrate fermentation system for *M. anisopliae* using rice, inoculating with conidia and harvesting conidia by a washing technique (Australian Patent PK3451/90).

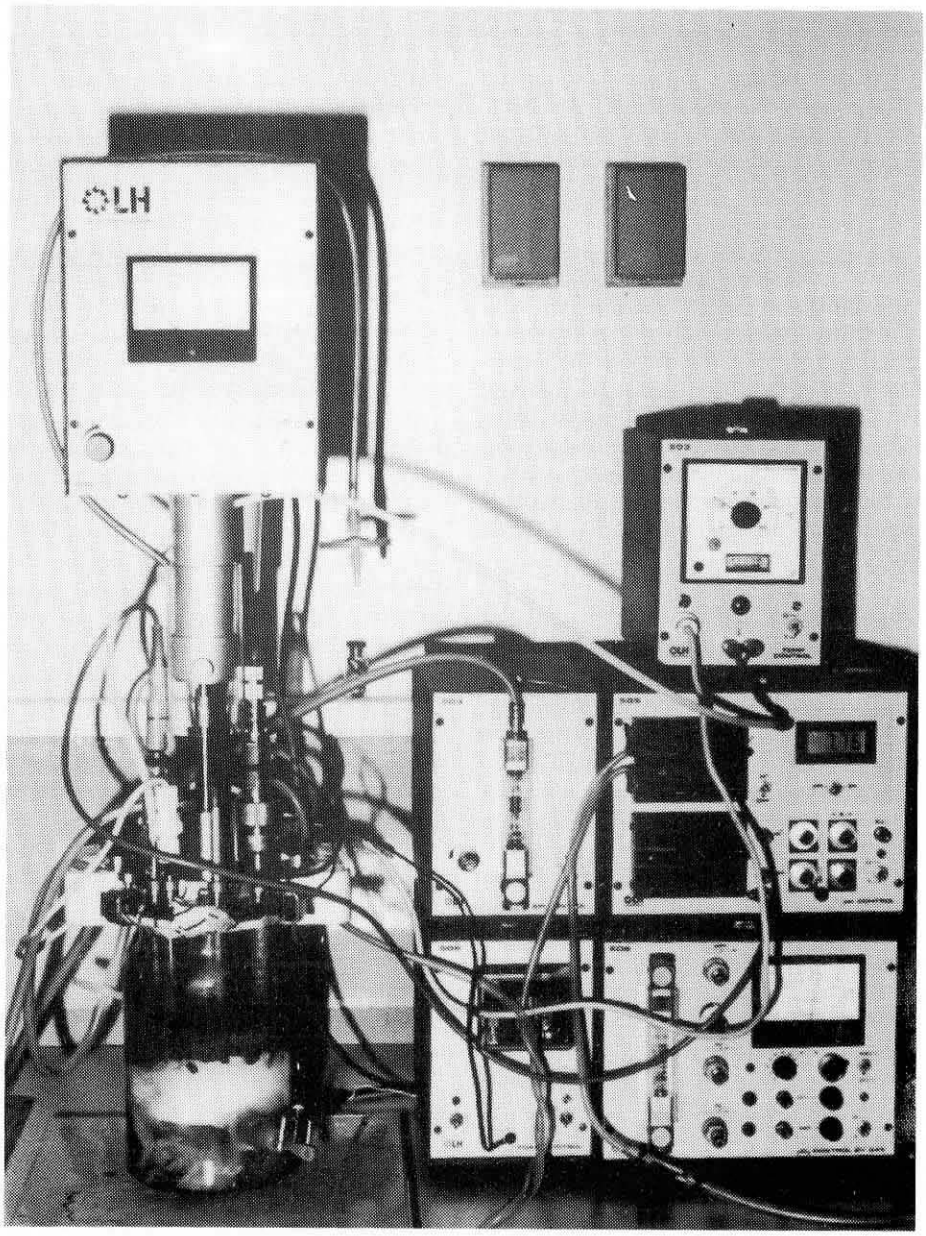

Figure 2. A one litre pilot fermentor with controllers for oxygen, temperature, pH, agitation and foaming.

Two phase systems

A two phase system has been used for *B. bassiana* and *Metarhizium anisopliae* production in the USSR, where mycelium produced in deep tank fermentation is allowed to sporulate in shallow open trays (Roberts and Yendol 1981, Goral and Lappa 1973). Walker and Riley (1982) described a similar preparation method for *Alternaria cassiae* for control of the weed *Cassia obtusifolia*. In our lab we have recently used this system to mass produce *Alternaria zinniae* (Auld and Schrauwen unpublished data). In Brazil *M. anisopliae* is produced on autoclaved rice or wheat bran in autoclavable plastic bags following inoculation with blastospores produced in liquid shake culture (Aquino *et al.* 1975, 1977).

Recovery of product and storage

Recovery of spores from bioreactors may be a problem with filamentous fungi, requiring large centrifuges to spin off spores. Filtration methods often leave a large number of spores behind in the mycelial mass if the latter is formed during fermentation.

Following recovery of spores from a production process it is usually necessary to dry them for long term storage. It is not always possible to do this and retain viability of the organism. It is for this reason that the mycoherbicide Devine® is sold in liquid form like fresh milk. Drying should be done as quickly as possible under "clean" conditions to prevent bacterial contamination. Inert materials such as diatomaceous earth, silica gel or clay minerals may be suitable to hasten drying and to act as carriers.

Production capacity

In a non-specialized fermentation laboratory, the demonstration that sporulation of a given fungus occurs in submerged culture may be an adequate goal. Co-operation of fermentation specialists, usually in industry, could then be sought. However, ultimately, the maximum production per unit volume of fermentation liquor must be established and this related, as bioreactor capacity (time × volume) to concentrations per unit volume required for field use.

Bartlett and Jaronski (1988) cite typical rates of *Beauveria* conidia per hectare are about 1×10^{14}; yields for *Beauveria* conidia obtainable from liquid surface culture are 1×10^{14} m^{-2}, submerged liquid fermentation 3×10^{11} per litre and 7×10^{12} per kg for solid substrate fermentation. Thus if the current submerged liquid production technology were to be used, a fermentation capacity of over 300 litres is required for each hectare treated.

Apart from research on maximizing production per unit volume or area, improved formulation and application techniques may reduce the density of spores required in the field. Given that there will ultimately be physical limitations to the amount of spores that can be produced in a given area or volume, work on improving application may be required in parallel with production research.

Acknowledgment

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