Upscaling and Formulation of BCA's in Ethiopia

Technical Advice

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Referaat
In dit rapportage bevat technische advies over massaproductie en de formulering van bacteriële isolaten (*Bacillus* en *Pseudomonas*) en entomopathogene schimmels (*Beauveria* en *Metarhizium*). Dit advies zal de eindgebruikers in Ethiopië helpen om weloverwogen beslissingen te nemen wat betreft het meeste passende strategieën voor de productie van biologische bestrijders (BCA) op semi-industriële schaal.

Abstract
In this report the technical advice in given for mass production and formulation of bacterial isolates (*Bacillus* and *Pseudomonas*) and entomopathogenic fungi (*Beauveria* and *Metarhizium*). This advice will allow the end users in Ethiopia to make an informed decision about the strategies for the production of biological control agents (BCA's) on semi-industrial scale. The purpose of the production being application to the crops against plant pathogenic *Fusarium* species and insect pests.

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Summary

This report contains information about mass production of bacterial isolates of *Bacillus* and *Pseudomonas*, as well as entomopathogenic fungi *Beauveria* and *Metarhizium*. These organisms have been isolated from soils in Ethiopia and are used as a biological control agents (BCA's) of *Fusarium* diseases (bacterial isolates) and insect pests (fungal isolates).

This reports describes briefly the methods and techniques currently available for upscaling of the production process of bacterial and fungal isolates. Additionally, possible product formulation options (to increase BCA's survival in the environment) are described in short.

Moreover, specific questions are answered such as: 1) question about the methods of preventing the bacterial isolates from losing their antifungal activity with time or 2) compatibility of isolates in prevention of disease when applied together.

1 Introduction

1.1 The IPM project

The Ethiopian Horticulture Producer Exporters Association (EHPEA) is running a program by the name the Ethio-Dutch Program for Horticulture Development (EDPHD). One of the components of this program is the Integrated Pest Management (IPM) which is executed by Wageningen University and Research, Business Unit Greenhouse Horticulture in collaboration with Ethiopian research organizations and EHPEA. One tasks in this programme is: 1.2.2. Provide technical advice and a document on formulation of the best microbial bca's (Metarhizium anisopliea, Beauveria bassiana, Bacillus subtilis and Pseudomonas flourescence) obtained in the preliminary trials conducted by the Addis Ababa University. This document reports on this task.

1.2 Background information on BCA's obtained in Ethiopia

Bacillus subtilis and Pseudomonas fluorescencens, which had been collected by the team of prof. dr. Fasil of the Addis Ababa University, can be applied together (Prof. Dr. Fasil, personal communication). In general, the bacterial antagonists can be combined without negative effects on each other. Compatibility checks have been performed (isolates with chemical means to ensure good IPM). At present both of bacteria are mixed with local soil or alginates and freshly applied (with best effectivity). Question: Which carrier should be used and what type of formulation for upscaling and commercialization? Preference should be given to local biobased materials, such as rice grains/husks and coffee bran.

Both species (Bacillus and Pseudomonas) are isolated from black soil (clay) from carnation cultivation. They perform well in carnation, chickpea and hydroponics of peas. Owing to problems with Fusarium, freesia cultures have disappeared from that Ethiopian regions with black soil. So, these antagonists contain a promise for horticulture challenged with Fusarium pathogens. Bacillus and Pseudomonas isolates show PGPR activity, i.e., IAA production and siderophore production for enhanced Fe-uptake by plants. However isolates experience loss of virulence with aging cultures. Therefore, researchers from Ethipia think that re-isolation from soil will be needed in the future in order to ensure high quality isolates. Question: What are the options to minimise the loss of antifungal activity in isolated bacterial antagonists? Fast identification tools are necessary for bacteria. For Bacillus, the gyrA gene will be used for alignment and marker construction. For Pseudomonas, 16S rDNA will be used.

Beauvaria bassiana (2) and Metarhizium sp. (1) isolates were also obtained from nature in Ethiopia. They target whitefly, thrips, mealybugs (in roses). Advice on formulation is needed to ensure a long shelf-life and high virulence. Different carriers may be used, which one is the best within context of Ethiopia (costs and availability) and fitness of isolates, and large scale production (upscaling and commercialization).

2 Bacterial antagonists of Fusarium

2.1 Compatibility check between bacterial biocontrol agents

A check was performed by the team of Prof. Dr. Fasil to ascertain that biological isolates are compatible with chemical means of plant protection (pesticides). However, the question is whether checks were performed on compatibility of the two bacteria applied together. There are reports that co-inoculation of Bacillus and Pseudomonas species might not necessarily lead to higher biocontrol activity. This is possibly due to disruption of Pseudomonas biocontrol activity by Bacillus (quorum quenching in Pseudomonas).

Bacterial cells communicate with each other. This process is called quorum sensing. Many metabolic processes in bacteria, such as antibiotics production or virulence, are governed by quorum sensing. This basically means that bacteria will not start producing antibiotics until a certain threshold number of bacterial cells are present. Gram negative bacteria such as Pseudomonas use specific signal molecules for this cell-to-cell communication. Other bacteria, such as Bacillus, may produce enzymes which degrade the Pseudomonas signal molecules, while producing their own signal molecules. Generally quorum quenching (disruption of quorum sensing) has no effect on the numbers/growth of bacteria (in this case Pseudomonas) but its metabolism and biocontrol activity might be severely impaired. More information on quorum sensing can be found in the review article written by Grandclement *et al.,* (2016).

2.2 Loss of biocontrol properties by aging cultures of *Pseudomonas* and *Bacillus*

It is advisable to not only maintain the isolates in a growth medium (on plates or in liquid medium). If they are maintained on nutrient rich medium (for example nutrient broth, Tryptic Soya Broth or Luria Bertani broth) they might lose their ability to produce siderophores and antibiotics over time.
For each isolate, inoculum stocks should be prepared in sterile glycerol by mixing 0.5mL glycerol (80%) and 0.5mL of 24hrs liquid culture of specific isolate into a sterile Eppendorf tube (cryovials; 1.5mL). After thorough mixing stocks should be stored in -80°C freezer. Alternatively cultures of isolates should be freeze-dried. Inoculum samples prepared by freeze-drying can be stored for extended periods of time in the fridge (4°C) or at room temperature (22°C).

2.3 Production of bacterial biocontrol agents on the laboratory and semi-industrial scale

2.3.1 *Bacillus*

Bacillus sp. are aerobic endospore-forming bacteria. Their ability to produce endospores makes them by far the most researched group of bacteria in biocontrol. Endospores are produced when the environmental conditions are not favourable (for example lack of nutrients). Endospores are capable to withstand the stress caused by high temperature, high UV irradiation, desiccation, chemical damage and enzymatic destruction. Therefore using endospores as means of delivery of biocontrol to the soil has gained much attention.

Depletion of carbon, nitrogen, or phosphorous causes the process of sporulation to begin, however, the process needs to start before nutrients are exhausted of. Otherwise, the spore formation cannot be completed due to the fact that the nutrients are too low for the energy-requiring sporulation process.

Production of spores requires high cell density of bacterial cells and good sporulation efficiency. At laboratory scale, sporulation is normally induced by growth and nutrient depletion in media such as Difco Sporulation Medium (DSM) (Monteneiro *et al.,* 2005; Monteneiro *et al.,* 2014). This medium should be prepared as follows (per litre): Bacto nutrient broth (Difco) 8 g, 10% (w/v) KCl 10 ml, 1.2% (w/v) $MgSO_4 \cdot 7H_2O$ 10 ml, 1 M NaOH ~1.5 ml (pH to 7.6). Adjust volume to 1 litre with ddH_20. pH to 7.6. Autoclave and allow to cool to 50°C. Just prior to use, add the following sterile solutions (and antibiotics if required): 1 M $Ca(NO_3)_2$ 1 ml, 0.01 M $MnCl_2$ 1 ml, 1 mM $FeSO_4$ 1 ml.

The process of sporulation usually begins after the exponential phase of bacterial growth, when nutrients become more depleted (however not completely). Under ideal conditions, the culture will initiate sporulation at a cell density of about 10^8 cells mL^{-1}, and typical sporulation efficiencies will be in the range of 30-100%. During the growth phase of bacteria, samples should be taken to enumerate viable bacterial cells and endospores. For viable cell counts a standard method of dilution plating could be used. For counting endospores they should be first stained according to a protocol of differential staining technique (the Schaeffer-Fulton method). A primary stain (malachite green) is used to stain the endospores. Malachite green is forced into the endospores by heating. In this technique heating acts as a mordant.

There is also the possibility to produce bacterial inocula/spores on other substrates available locally. Examples of such substrates are: water extract of rice husks, molasses, organic wastes etc. (Abbasi *et al.,* 2013; Korsten, 1996). In any case it is advisable to compare the production of viable cells/endospores on different substrates to the standard media (such as DSM or Luria Bertani).

2.3.2 Pseudomonas

Bacteria belonging to genus Pseudomonas are gram negative bacteria. In the laboratory they are usually grown on King B medium or standard non selective microbiological media such as nutrient broth, Luria Bertani broth etc. (Bisutti *et al.,* 2015). However they also grow well on media based on agricultural waste products.

These bacteria, in contrast to Bacillus species, are not capable to produce endospores. Vegetative cells of Pseudomonas bacteria are far more susceptible to loss of viability during the process of drying, storage, and rehydration. Nonetheless gram negative bacteria have shown a high potential as a biocontrol agents. Usually they are cultivated in a liquid medium and added to the soil substrate as a liquid of freeze-dried powder. There are some carriers, for example talc, spent mushroom substrate, fly ash and lignite, which support survival of Pseudomonas species for more than 2 months when stored at the room temperature (Gade *et al.,* 2014)

Few modifications to a production process were applied to increase the survivability of Pseudomonas inocula. During the cultivation process osmoprotectants (such as glucose, fructose, trehalose, raffinose and stachyose) are added to growth media (Cabrefiga *et al.,* 2014; Bonaterra *et al.,* 2006). Other osmoprotectants used successfully are for example lactose and skimmed milk (Cabrefiga *et al.,* 2014).

2.4 Up-scaling of the production process (Bacillus and Pseudomonas)

The mass production of both Bacillus and Pseudomonas can be undertaken on a (semi-) industrial scale. Submerged fermentation (SmF) bioreactors are usually used for this purpose. Another option is using solid state fermentation (SSF), but it is less popular for bacterial inoculum production. In case of SmF substrate and organisms are present in bioreactor in a submerged form in large quantity of liquid medium. Advantages of SmF are: possibility to control the process parameters better (such as oxygen supply, management of pH of the medium). SmF can be set up as a batch or as semi-continuous process. For production of endospores of Bacillus batch or fed-batch process should be applied. Production of Pseudomonas could be optimised to be run continuously.

Mediums to use can be prepared from organic waste materials available locally (such as liquid manure, rice bran, cotton meal, molasses, and potato starch/dextrose, coffee husks) (Poopathi and Abidha, 2011; Abbasi *et al.,* 2013). A medium should have a proper C:N ratio (optimal between 5-15). Preferable carbon source should be easily degradable sugars, such as glucose (around 20g/L medium). The choice of the medium for mass production of bacteria should be based on the results of comparison of bacterial performance (e.g. numbers and efficacy of endospore formation) in different media. The bacterial performance in a specific medium will depend on many factors and it is advisable to thoroughly check the bacterial growth parameters and production of active ingredients (such as siderophores or antibiotics) in the medium before starting a large scale production process.

The production process could be undertaken in laboratory based bioreactors with build-in environmental controls (pH, O_2, CO_2, mixing, aeration). Such bioreactors could be for example purchased from New Brunswick company. The disadvantage of this option is their relatively high price. Attempt could be made to design the bioreactor from materials which are cheaper and available locally. Important is to remember about incorporating the environmental controls into the design. Moreover a proper oxygen supply with a pump and means of mixing the growth medium are needed. Bioreactor should be made of a material which could be easily sterilised (for example by autoclaving it).

2.4.1 Formulation possibilities

Formulations are typically a mixture of "active" ingredient (in this case microorganisms, cells, spores in freeze-dried form or viable), carrier material and additives. Suitable carrier materials are fine clay, peat, vermiculite, alginate, and polyacrylamide beads, diatomaceous earth, talc, vermiculite, cellulose (carboxymethyl cellulose), biochar, organic waste materials (such as rice, wheat husks, biochar etc.).
Additives such as gums, silica gel, methyl cellulose, and starch protect the micro-organisms from adverse environment conditions. Furthermore they influence the physico-chemical properties of formulations (Schisler *et al.,* 2004). Extensive list and categories of different formulations available is given in the review paper by Malusa *et al.,* (2012) and Mishra and Arora (2016).

To determine which formulation is the best under the Ethiopian conditions it would be advisable to choose a number the local agricultural waste products (rice husks, fly-ash, biochar etc.) and design the experiments to look at the survival of Bacillus and Pseudomonas in these carrier materials over an extended time period (e.g. 26 to 52 weeks) (Schisler *et al.,* 2004; Hale *et al.,* 2015). Bacteria could be added to the carrier as viable cells/ endospores (after removing the culture medium by for example centrifugation) or in a freeze-dried form.

2.4.2 Technical advice

Produce both bacterial cultures in a bioreactor with liquid medium (5-20L). Optimise Bacillus culture for a maximum production of endospores. Pseudomonas could be produced by continuous culture. Separate the bacteria/spores from the liquid medium by centrifugation at <5000rpm. Bacterial pellet can be re-suspended in buffered saline solution. Alternatively grow bacteria in the medium that would be suitable for freeze-drying the cultures.

3 Entomopathogenic fungi (*Beauveria* and *Metharhizium*)

3.1 Production of entomopathogenic fungi on the laboratory and semi-industrial scale

Beauveria and Metarhizium species are representatives of Ascomycete Hypocreales. In general, these fungi have three major propagule types that can be used. In nature, the aerial conidium is the primary infectious propagule. Conidia are the spores that are produced on the exterior of fungus-killed insects. Blastospores are the proliferative stages within the insect for these fungi and can also be produced in liquid fermentation. Under certain liquid fermentation conditions, mainly substitution of inorganic for organic nitrogen, Beauveria and Metarhizium can produce "microcycle" in which microconidia are produced. These conidia are not true conidia and are produced on the ends of hyphal strands. Conidia can be produced also under submerged culture or by a biphasic system. In the latter, fungus is first grown under submerged conditions (in liquid medium) to produce a large biomass of hyphae and then allowed to produce conidia in solid-state conditions. Conidia produced during solid state fermentation (SSF) are usually performing very well under field conditions, because the production process imitates the environmental conditions during the natural process of fungal multiplication.

3.1.1 Upscaling of the production process (*Beauveria* and *Metarhizium*)

There are two types of SSF used for production of entomopathogenic fungi on (semi-) industrial scale:
Cultivation on solid substrate acting as carrier and carbon source (mostly widely used).
Cultivation on inert carrier with addition of carbon source (rarely used), but could be advantageous. Inert carrier could be re-used after extraction of the spores and sterilisation.

The most common natural substrate for the production of entomopathogenic fungi is rice grains. They are widely available and have good characteristics as a dispersal carrier. The other option is using barley grains. However it would be beneficial to find an alternative substrates, due to the fact that both grains are also a staple food. Therefore, more research is needed into the use of agroindustrial waste such as wheat bran and rice straw, residual potatoes, sugarcane bagasse, coffee husk (Dalla Santa *et al.,* 2005). Some of the substrates used for this purpose so far include: bagasse ± 2% dextrose, barley, beetroot, broken rice, broken rice + $CaCl_2$, carrot tubers, cassava chips, chickpea, coconut cake, cottonseed cake, finger millet, groundnut cake, maize, maize bran ± 2% dextrose; neem cake, potato tubers. Also waste products of animal husbandry could be used. In United States a process was developed for production of spores from whey (a lactose rich waste material from cheese production) (Kassa *et al.,* 2008). There are also a few inorganic substrates in use: calcined diatomaceous earth (diatomite), clay granules (e.g. Seramis®). For the low cost production polyethylene bags filled with agro-industrial wastes could be used. Otherwise it is advisable to determine if building for example a packed bed reactor is a viable option. Conidial yields can vary among strains of each fungus species. For example, Arcas *et al.,* (1999) determined that one strain of B. bassiana produced three times as many spores as a second under identical fermentation conditions. Therefore it is important to determine the conidial yields for each strain used. Jaronski *et al.,* (2012) noted that conidial production of 15 B. bassiana isolates ranged from 1.11×10^{11} to 2.25×10^{13} conidia per gram of initial dry substrate when grown under identical solid substrate fermentation conditions.

3.1.2 Formulation possibilities

If the fungal conidia are not to be used directly the moisture content in the product of SSF should be lowered. Refrigerating of the whole sporulated solid substrate (not dried) will also result in a prolonged shelf life. It will however not be more than a few weeks.

If conidia are to be stored for a longer period of time they must be dried down to a moisture content <9% w/w or aw ≤ 0.3 (Jaronski, 2013). This low moisture is necessary for optimal shelf life regardless of whether conidia are formulated or not.

Drying methods include: 1) opening of plastic fermentation bags; 2) transfer of sporulated substrate to open trays or table tops; 3) transfer to Kraft paper sacks (Jaronski and Jackson, 2012) and 4) use of air-lift devices. In general, Beauveria conidia can be dried relatively quickly (within 2–3 days) without loss in viability, whereas Metarhizium conidia require slower drying (5–9 days) (Jaronski and Jackson, 2012). The most popular method to separate conidia from substrate is sieving (mechanical separation).

Conidia of entomopathogenic fungi could be formulated into the oil-based (eg. emulsifiable adjuvant oils, vegetable oils or mineral oils), water or solid formulations. There are contradictory reports in scientific literature about the possible influence on any type of formulation on survival of conidia. It seems like test are needed to evaluate the survival of conidia each time when starting to use a new formulation.

3.1.3 Technical advice

Consider producing the conidia of both entomopathogenic fungi in biphasic process (start with liquid inoculum; then continue with solid substrate fermentation) on rice as a solid substrate. The detailed protocol for the procedure can be found in an article by Seema *et al.,* (2013). This procedure is relatively easily to adapt for other solid substrates (such as agro-industrial wastes). For the process 500 g polyethylene re-sealable bags could be used.

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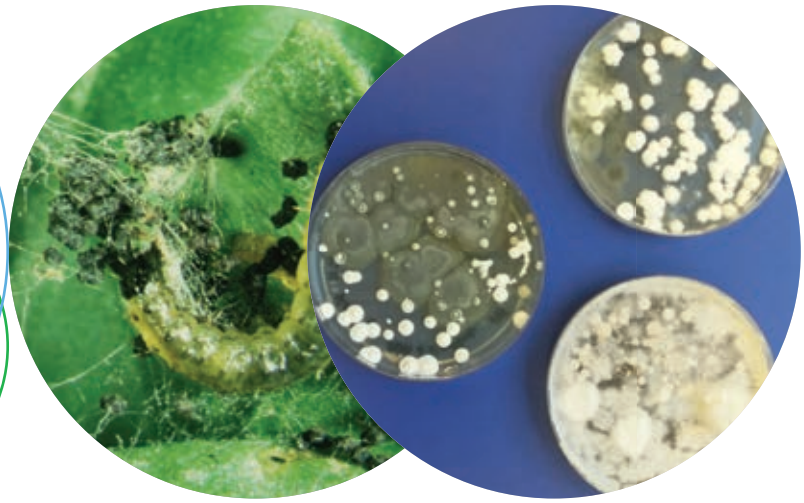

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