Production of Conidia of Alternaria cassiae with Alginate Pellets

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Received May 7, 1992; accepted December 2, 1992

Formulations of alginate-encapsulated mycelia are used to generate spores for mycoherbicidal application to weed-infested fields and for bulk production of spore-based products. Spore yield of such formulations is a primary determinant of product efficacy. A number of parameters of the alginate process were studied to develop an optimal alginate formulation for field application of Alternaria cassiae, a mycoherbicide for sicklepod (Cassia obtusifolia). The composition of the fermentation medium and of the filler used in formulation and the fermentation time were important variables. The addition of nutrients to the mycelial homogenate after fermentation increased sporulation but the amount and ratio of nutrients in the fermentation medium had a greater influence on spore yield from pellets. Optimal sporulation resulted from mycelia produced during a 60- to 70-h fermentation in 2.4% dehydrated potato dextrose broth and 14% V-8 vegetable juice and entrapped in pellets containing corn cob grits as the filler.

Key Words: sporulation; V-8; potato dextrose broth; corn grits; Alternaria cassiae; fermentation; sodium alginate; encapsulation.

INTRODUCTION

Entrapment of cells within spheres of calcium alginate has become one of the most widely used techniques for applications ranging from immobilization of living or dead cells in bioreactors (Smidsrød and Skjåk-Bræk, 1990), immobilization of plant protoplasts for micropropagation (Draget et al., 1988), and immobilization of hybridoma cells for production of monoclonal antibodies (Duff, 1985). In 1983, the use of alginate pellets for production and formulation of mycoherbicides was first reported (Walker and Connick, 1983; Connick et al., 1983). Biocontrol agents encapsulated in an alginate matrix have included Alternaria macrospora, A. cassiae, Fusarium lateritium, Colletotrichum malvarum (Walker and Connick, 1983; Connick et al., 1983), Fusarium solani f. sp. cucurbitae (Boyette and Walker, 1986; Weidemann, 1988; Weidemann and Templeton, 1988a, 1988b), Talaromyces flavus, Gliocladium virens, Penicillium oxalicum, Trichoderma viride, and Pseudomonas cepacia (Fravel et al., 1985; Lewis and Papavizas, 1987; Papavizas et al., 1987). Formulations have contained nutritional amendments (ground oatmeal, soy flour, and cornmeal) (Weidemann, 1988; Weidemann and Templeton, 1988a, 1988b), fillers (kaolin [Boyette and Walker, 1986] and hydrous aluminum silicate [Fravel et al., 1985]), and different gellants (calcium chloride [Walker and Connick, 1983] and calcium gluconate [Fravel et al., 1985]).

The alginate process can be altered in many ways to increase the efficacy of biocontrol agents. These include modification of the fermentation product to be formulated, addition of filler constituents to modify pellet bulk and weight, adjuvants to influence the entrapped agent’s growth and release from the pellet, and the gelant. This study examines modifications of the basic formulation and process for making alginate pellets with the goal of optimizing the procedure for spore production by the mycoherbicide A. cassiae.

MATERIALS AND METHODS

A. cassiae cultures originating from a monoconidial transfer of Mycogen's strain MXY-104 (Mycogen Corp., San Diego, CA) were used in all tests. The fungus was maintained on a modified V-8 agar (Cotty and Misaghi, 1984) consisting of 5% (w/w) V-8 vegetable juice and 2% (w/w) agar adjusted to pH 5.2 with 20% (w/w) KOH prior to autoclaving. Cultures were grown at 25 to 27°C under fluorescent light on a 12-h diurnal light cycle. For long-term storage, 3-mm-diameter plugs were maintained at 4°C in 25-ml vials containing 5 ml sterile distilled water (Cotty and Misaghi, 1984). Conidial suspensions used to seed fermentation flasks were made by flooding plates with sterile water and gently rubbing spores from the culture surface with a rubber policeman.

Erlenmeyer flasks (250 ml) containing 70 g of a test fermentation medium were adjusted to pH 6.5, autoclaved for 20 min, cooled to room temperature, and seeded to a final concentration of 500 to 1000 conidia of A. cassiae per gram. The fungus was maintained on a modified V-8 agar (Cotty and Misaghi, 1984) consisting of 5% (w/w) V-8 vegetable juice and 2% (w/w) agar adjusted to pH 5.2 with 20% (w/w) KOH prior to autoclaving. Cultures were grown at 25 to 27°C under fluorescent light on a 12-h diurnal light cycle. For long-term storage, 3-mm-diameter plugs were maintained at 4°C in 25-ml vials containing 5 ml sterile distilled water (Cotty and Misaghi, 1984). Conidial suspensions used to seed fermentation flasks were made by flooding plates with sterile water and gently rubbing spores from the culture surface with a rubber policeman.
Four alginate pellets were weighed and placed on top of 2.5 g sand and 0.5 ml water in each well. Plates were transferred. To test the influence of these nutrients and performed. In the first experiment media. Cultures were grown in either the standard medium or in media containing i, t, and the nutrients of the standard medium. Homogenates of cultures from the latter three media were added to the alginate-kaolin mixture described above. Nutrient treatments were also added to the mixture in multiples of the equivalent quantity of nutrients (PDB and V-8) found in 10 ml of unfermented standard medium (SM). The treatments were: no nutrients, 1X SM, 2X SM, and 4X SM. The second experiment was performed to determine if nutrients added after fermentation could compensate for deficient fermentation media. Cultures were grown in either the standard medium or in media containing 1/4, 1/2, and 1/4 the nutrients of the standard medium. Homogenates of cultures from the latter three media were added to the alginate-kaolin mixture described above containing 1X SM; homogenate of the culture from the standard medium was added to unsupplemented alginate-kaolin.

Spore yield was assessed in 24-well multwell plates. Four alginate pellets were weighed and placed on top of 2.5 g sand and 0.5 ml water in each well. Plates were covered but not sealed. After 4 days incubation under 12 h diurnal fluorescent light, pellets from each replicate were combined in a glass bottle (4 ml, 4.5 mm[h] × 0.5 mm[d]) containing lactophenol aniline blue solution (50 µl) as previously described (Daigle and Cotty, 1991). Water (2 ml) was subsequently added and the mixture was agitated for 10 s on a vortex mixer. For each replicate, three separate drops (10 µl each) of the solution were placed on a slide and the number of conidia in each was counted at 100× magnification. The treatments were replicated three times and the experiments were performed twice.

Analyses were performed manually or with the statistical analysis system (SAS Institute, 1985). All multiple comparisons were first subjected to analysis of variance. Linear regressions were calculated by the least squares method.

RESULTS AND DISCUSSION

In preliminary experiments, substitution of dehydrated PDB for sucrose in the original mycoherbicidal fermentation medium (Connick et al., 1983; Walker and Connick, 1983) composed of soybean flour (1.5%), corn meal (1.5%), sucrose (2.9%), and calcium carbonate (0.3%) resulted in pellets that yielded 30% more spores. A medium containing only dehydrated PDB (2.4%), however, gave a lower spore yield. A medium of V-8 (14%, v/w) and dehydrated PDB (2.4%, w/w) gave the best spore yield, a 300% increase over the original medium (data not shown).

The spore yield increased with V-8 concentration of the fermentation medium from 0 to 20% (v/w). This trend held both for media containing V-8 alone and for media supplemented with 2.4% (w/w) dehydrated PDB.

FIG. 1. The effect of PDB (1.68 g/70 g medium) with increasing quantities of V-8 juice in the fermentation medium on sporulation of A. cassiae in alginate pellets made from the fermentation product. Fermentation time = 66 h. Filler, kaolin clay at 5% (w/w) concentration of alginate solution (1.33%, w/w). Linear regressions were significant for spore yield versus V-8 concentration in media both with and without PDB (P < 0.0001, r² > 0.87).
FIG. 2. The effect of increasing quantities of PDB in a fermentation medium containing 14.3% (v/w) V-8 juice on sporulation of A. cassiae in alginate pellets made from the fermentation product. Fermentation time, 66 h. Filler, kaolin clay at 5% concentration of alginate solution (1.33%, w/w). Linear regression for spore yield with PDB concentration was significant ($P < 0.0001, r^2 = 0.86$).

of dehydrated PDB increased from 0 to 7.2% (w/w) in media containing PDB as the sole nutrient (Fig. 2). In general, increased nutrient content of the medium resulted in an increase in spore yield of pellets made from cultures grown in that medium. This differs from spore production on solid agar media; on agar, increased nutrients cause an increased release of respiratory carbon dioxide and this represses sporulation of wild type Alternaria strains but not mutants insensitive to carbon dioxide (Cotty, 1987). The results suggest that either respiration is reduced in pellets made with high-nutrient fermentation media compared to that on agar media or that alginate-entrapped mycelia are relatively insensitive to carbon dioxide repression. Preliminary results suggest that the former is probably the case because sealing assay plates resulted in markedly reduced spore yield, and the effects of sealing on sporulation of Alternaria species have been previously attributed to carbon dioxide (Cotty, 1987). The results suggest that either respiration is reduced in pellets made with high-nutrient fermentation media compared to that on agar media or that alginate-entrapped mycelia are relatively insensitive to carbon dioxide repression. Preliminary results suggest that the former is probably the case because sealing assay plates resulted in markedly reduced spore yield, and the effects of sealing on sporulation of Alternaria species have been previously attributed to carbon dioxide (Cotty, 1987).

Nutrients added during pellet formation can substitute, to some extent, for suboptimal nutrient levels in the fermentation medium. Reducing the nutrient content of the fermentation medium to $\frac{1}{3}, \frac{1}{2},$ or $\frac{1}{3}$ the standard medium resulted in no significant reduction in final spore yield from the pellet if the equivalent nutrients found in the unfermented standard medium were added to the homogenate just prior to pellet formation. Pellets produced from such supplemented homogenates, however, contained a total amount of nutrients greater than that in the control. Thus, suboptimal media can be at least partially compensated by the addition of nutrients during pellet formation. Economic considerations may dictate at which step the nutrients should be added. Some beneficial nutrients may be inappropriate for the fermentation media due to insolubility or incompatibility with sterilization. However, these may still be useful if added after fermentation.

To formulate fungi into effective mycoherbicides, the formulation components must be physically and biologically compatible with the fungi. One of the principal components of the alginate pellet is the filler that provides bulk for handling. Kaolin clay has been previously favored because of its compatibility and economy (Walker and Connick, 1983; Connick et al., 1983). It does not, however, add any nutritional value to the pellet. A direct comparison of kaolin and other fillers for formulating mycoherbicides containing alginate pellets has not been reported. Therefore, we investigated a number of fillers for compatibility within the alginate system.

FIG. 3. The effect of nutrients added after fermentation on sporulation of A. cassiae in alginate pellets made from the fermentation product. Nutrients were equivalent to 0, 1X, 3X, and 4X the quantity of nutrients in unfermented standard medium. Fermentation time, 66 h. Filler, kaolin clay at 5% (w/w) concentration of alginate solution (1.33%, w/w). Bars with different letters differ significantly by Fischer's protected LSD test ($P = 0.05$).
the fermentation medium consisting of 14% V-8 juice, and filler type (corn cob grits or kaolin) on sporulation of A. cassiae in alginate pellets many spores after 2 or 3 days as unsupplemented kaolin pellets were too viscous or lumpy after autoclaving and unsuitable for pellet making. Furthermore, products made from these materials at 5% concentration did not give high spore yields. Wheat bran at 2% concentration has been used recently with Beauveria bassiana in the alginate process (Knudsen et al., 1990). Nonnutritive fillers such as Solka Floc (hydroxyethylcellulose), vermiculite, and celite were compatible with the alginate process and pellets made with these materials gave approximately the same spore production as those made with kaolin (data not shown). The final filler tested, corn cob grits, a waste product of the corn industry, performed better than the other fillers.

The results of experiments comparing kaolin and corn cob grits are illustrated in Fig. 4. Unsupplemented (no PDB) corn cob grits yielded about four times as many spores after 2 or 3 days as unsupplemented kaolin. Addition of PDB permits kaolin pellets to produce spore levels equivalent to unsupplemented corn cob grits. Thus, the benefits of corn cob grits may be at least partially attributed to nutrition.

The length of fermentation influences both the quality of mycelia produced and the nutrients remaining after fermentation. In all of the previously discussed experiments, a 65- to 70-h fermentation at 27°C was used. The optimum incubation time for A. cassiae in the standard medium at 27°C was between 48 and 72 h (Fig. 4). Short incubations of 24 h or less resulted in pellets that gave low spore yields. Spore yield also decreased after 3 days of fermentation (Fig. 4).

The alginate process for solid formulation of mycoherbicides is complex and several factors interact to influence product performance. Quantity and quality of nutrients and fermentation conditions influence the spore production potential of the final pellet. Furthermore, nutrients accessible to the entrapped mycelia may be added during pellet formation either in the form of a degradable filler (i.e., corn cob grits) or as freely diffusible nutrients (i.e., PDB), and these nutrients can improve spore yield from the pellet.

REFERENCES


