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A predictor variable for efficacy of *Lagenidium giganteum* produced in solid-state cultivation

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Lagenidium giganteum **was cultivated on solid media in the absence of free water and evaluated for efficacy against second-instar *Aedes aegypti* mosquito larvae in 100-ml bioassays. Bioassay variables included level of media addition, CO_2 evolution rate (CER) and cell density. Logistic regression was performed on bioassay infection observations to determine if the tested variables were correlated to the probability of attaining at least 80% infection. Both CER (p=0.003) and number of cells (p=0.017) were significantly correlated, while level of media addition was not (p=0.42). Although media addition did not correlate with efficacy, media levels greater than 2 g/l reduced water clarity and infection. Media from cultures younger than 3 days performed poorly under all conditions tested.** *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 203–207.

Keywords: *Lagenidium giganteum*; solid-state cultivation; biocontrol; mosquito larvicide

Introduction

Lagenidium giganteum (Oömycetes: Lagenidailes) is an aquatic fungus that parasitizes mosquito larvae. Due to its effectiveness and host specificity, it has been registered as a biological control agent by both the United States and California EPA (USEPA registration No. 56984-2). When its mycelium is diluted in water, asexual motile zoospores are produced that swim in search of a mosquito larvae. Once encountered, the zoospore encysts and begins to grow vegetatively within the host mosquito. The fungus consumes the larvae as a substrate, killing it within a period of approximately 2 days. Up to a million more fungal zoospores are produced from the cadaver to continue the infection cycle [10]. Infection of other larvae can be observed throughout a whole season and even into the next [3]. Although zoospores are effective at finding and encysting on mosquito larvae, they are too fragile to be applied directly in the field.

In addition to zoospores, *L. giganteum* produces oospores within the mosquito host. Oospores are sexually produced resting spores, capable of withstanding desiccation and abrasion, and germinating when conditions are suitable. Current large-scale production efforts have been unable to provide optimal conditions for economical oospore production. Therefore, vegetative mycelia produced in liquid or solid cultivation systems are applied as the formulation for biological control.

Challenges to applying *L. giganteum* for biological control include evaluating product efficacy and determining the amount of product to apply. Since the product is a living organism, efficacy will decrease during storage due to cell death. Additionally, excessive product application can reduce efficacy due to inhibition of zoospore formation from media components [6]. Thus, efficient application of *L. giganteum* mycelia requires knowledge of product efficacy at the time of application and the influence of product concentration on efficacy.

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In previous studies with *L. giganteum*, application rates were based upon attributes such as number of cells [5,6], volume of liquid growth media [5], number of petri dishes with *L. giganteum* growth [2–4], and number of infected, fragmented mosquito cadavers [10]. While infection was monitored over a period of time in these studies, no attempts were made to estimate efficacy from the product attributes. Since viable cells are responsible for the asexual production of zoospores from mycelia-based products, measurement of viable biomass may provide a means of estimating product efficacy. Accurate quantification of viable biomass, however, is difficult to attain [1,8,13]. Carbon dioxide evolution rate (CER) and oxygen consumption rate have been used as indirect measures of active biomass in solid-state cultivation systems [11,12]. Thus, measurement of CER may offer a rapid method for estimating the efficacy of *L. giganteum* produced in solid-state or other cultivation processes. The goal of the experiments presented here was to determine if the efficacy of *L. giganteum* products in laboratory bioassays could be estimated from measurements of levels of media, cells, and CER added to the bioassays.

Materials and methods

Source and maintenance of L. giganteum

L. giganteum (ATCC 52675) was obtained from AgraQuest (Davis, CA). The fungus was maintained in both liquid and agar media containing 0.025 g cholesterol, 1.25 g peptone, 1.25 g Ardamine pH (autolyzed yeast extract), 3 g glucose, 0.075 g $CaCl_2$, 0.075 g $MgCl_2$, 3 g corn oil, and 0.1 g soybean lecithin in 1 L of distilled deionized water. Corn oil was added aseptically after autoclaving the medium to prevent thermal degradation. Liquid cultures consisted of 75 ml of medium in covered 250-ml Erlenmeyer flasks maintained in an incubated shaker at 30°C and 150 rpm. Agar cultures (for long-term storage) were maintained at 26°C.

Solid-state cultivation and harvesting

Medium used in the solid-state cultivation experiments consisted of 5 g (dry weight) wheat bran (initially at 10% moisture content)

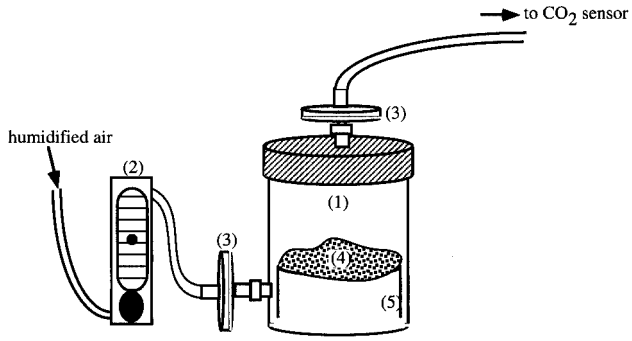

Figure 1 Reactor used for solid-state cultivation studies and CER measurement where (1) is the 250-ml autoclavable reactor, (2) is an air flowmeter, (3) is a 0.22-μm pore-size air filter, (4) is the solid medium at 73% moisture content colonized by *L. giganteum*, and (5) is the perforated medium support.

wetted to 73% with the liquid medium described above. Cultivation studies were completed in reactors constructed from 250-ml Nalgene cups containing perforated media support and airflow inlet and outlet fittings (Figure 1). To prevent contamination during cultivation, 0.22-μm pore-size air filters were attached to the inlet and outlet fittings. Solid medium was inoculated with 2.3×10^5 to 2.3×10^6 cells from 3-day-old liquid maintenance cultures. Cell concentrations were determined from four independent counts of four fields using a hemacytometer.

After inoculation, solid culture reactors were transferred to a 30–32°C incubator and supplied humidified air at 20 ml/min. Carbon dioxide concentrations at both the inlet and outlet of each reactor were measured daily using an infrared CO_2 sensor (Telaire, Goleta, CA). The CO_2 sensor had an uncertainty of ±2% of the observed measurement.

At various times throughout a 1- to 21-day growth period, medium was removed from the reactors and analyzed for efficacy. Just prior to medium removal, CO_2 measurements were made on the influent and effluent gas. The specific CER was estimated from a CO_2 mass balance on the solid cultivation reactor using the following equation:

$$\text{Specific } CO_2 \text{ evolution rate} = F(CO_{2_{outlet}} - CO_{2_{inlet}})$$

where F=specific flow rate of air supplied to the reactor (μmol air/min per gram), $CO_{2_{outlet}}$=molar fraction CO_2 in outlet air (mol CO_2/mol air), and $CO_{2_{inlet}}$=molar fraction CO_2 in inlet air (mol CO_2/mol air).

After CO_2 measurements were done the reactor contents were mixed and a 0.5-g subsample taken to assess cell density. The sample was blended in 20 ml water for 1 s and cells were counted using a hemacytometer. Four independent counts were taken on each sample and an average and standard error were calculated from these data.

Mosquito rearing

Aedes aegypti (Gainesville strain) mosquitoes were reared from eggs purchased from Benzon Research (Carlisle, PA) and all rearing was completed in a 29°C incubator. Mosquito eggs were hatched in 500 ml of 100 mg/l bovine liver powder solution (Sigma Chemical, St. Louis, MO) in 15×23×5 cm plastic pans. Each pan contained less than 200 larvae. For each pan, 80–90% of the liquid was decanted and replenished with water and 1 ml of a 100 g/l fish-food slurry (Aquadine, Healdsburg, CA) was added daily. Larvae were ready for bioassays 72 h after initiating egg hatching.

Bioassays

All bioassays were performed in triplicate and consisted of exposing 10 second-instar *A. aegypti* mosquito larvae to diluted media taken from the 250-ml reactors containing *L. giganteum* mycelia (Figure 1). Bioassays were completed in plastic drink cups containing the diluted media and mosquitoes in a total volume of 100 ml. Three to five dilution levels of the solid medium were tested per bioassay. Controls were prepared without medium or food addition. Bioassays were terminated after 7 days.

The amount of medium added to the bioassays was based upon cell counts, CER and wet weight of material. Cell, CER and wet medium concentration in the bioassays were varied between 1×10^5 and 3.9×10^6 cells/100 ml, 0.0004 and 0.16 μmol CO_2/min per 100 ml, and 4.9 and 833 mg/100 ml, respectively. For every bioassay, calculations were made to determine the amount of medium needed to deliver the desired number of cells or CER. The small amounts of medium that needed to be added necessitated making a large dilution of the solid media and subsampling from that for the 100-ml bioassays.

Dead mosquitoes were removed from the bioassay daily and examined microscopically for the presence of hyphal proliferation. Pupae were also removed. Only those mosquitoes displaying *L. giganteum* hyphae were counted as infected.

Variability analysis

Since solid medium for the bioassays was taken from a concentrated solution, variability in aliquots of diluted medium was assessed on selected samples. Target amounts (dry weight) of the solution were pipetted in triplicate, dried for 24 h at 101°C and then weighed.

Statistical analysis

Logistic regressions were conducted on the potential indicators of efficacy (amount of medium, CER and number of cells) and bioassay infection observations to determine if they could predict the probability of achieving at least 80% infection. The data were separated into two groups: those bioassays that achieved at least 80% infection (success) and those that did not (failure). The binomial data sets were analyzed by three independent logistic regressions (one for each potential indicator of efficacy). Regressions were completed using the statistical software package JMP IN v3.2.6 (Cary, NC) using the following equation:

$$p(x) = \frac{1}{1 + 10^{-[(b_0 + b_1 x)]}}$$

where $p(x)$=probability of achieving at least 80% infection; b_0=intercept determined by logistic regression; b_1=slope determined by logistic regression, 100 ml/g, or 100 ml/number of cells, or 100 ml/min per μmol CO_2; and x=potential indicator variable (independent variable), g/100 ml, or number of cells/100 ml or μmol CO_2/min per 100 ml.

Suspended and dissolved solids

Since high solids levels can inhibit infection [4,7], it was necessary to determine the solids concentration of the medium. The cultivated solid medium was analyzed for total suspended solids, total dissolved solids, suspended volatile solids and dissolved volatile solids. A 0.5-g subsample of wet material was removed from the reactor and shaken with 20 ml water for 10 min. The resulting solution was filtered through a 47-mm-diameter

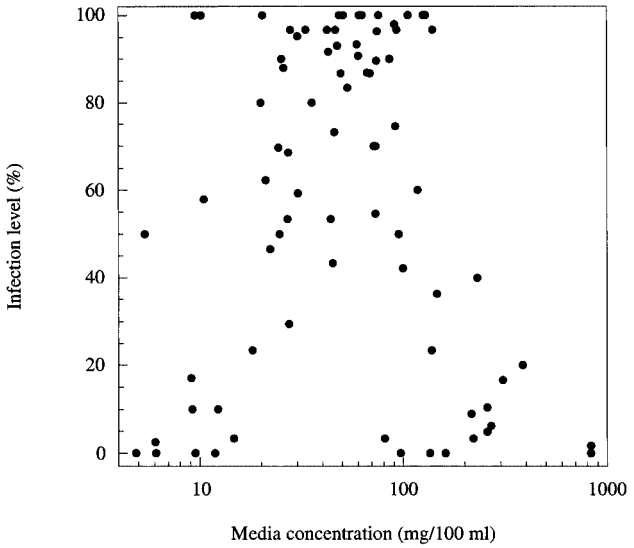

Figure 2 Influence of *L. giganteum* wet solid culture concentration on infection of *A. aegypti* larvae in 100-ml bioassays.

glass fiber filter (Environmental Express, Mt. Pleasant, SC), defined for use in standard methods of solids determination, and washed with an additional 20 ml water. This material was dried at 101°C to yield total suspended solids and then combusted at 550°C to obtain volatile suspended solids. Total dissolved solids were determined by pipetting 20 ml of the filtrate and drying it at 101°C. Volatile dissolved solids were determined by combusting the resulting material at 550°C.

Results

As illustrated in Figure 2, infection levels generally increased as the amount of medium added to the bioassays increased. Above 150 mg, the relationship was not sustained and, when the medium added exceeded 200 mg, water clarity was reduced and infection level dropped below 80%. Therefore, only those bioassays containing less than 200 mg of wet medium were considered for statistical analysis.

Each gram of wet solid medium provided approximately 270 mg of total dry solids to the bioassay. Of the total solids, 74–82% were suspended, and approximately 97% of these suspended solids were volatile. The remaining 18–16% of the total solids were dissolved and were approximately 85–90% volatile. These levels did not change appreciably throughout the cultivation period.

The variability in the amount of medium delivered to bioassays for selected aliquots is shown in Figure 3. In general, the medium delivered varied from the target amount by a small percentage. However, the variability in medium delivered was less than that of the cell counts (data not shown).

Cultures younger than 3 days did not perform well in the bioassays, regardless of concentration (data not shown). These younger cultures may not have developed the presporangial structures necessary for zoosporogenesis to occur upon dilution [9]. Although the absence of presporangial structures was not confirmed microscopically in the studies presented here, bioassay data from cultures younger than 3 days were removed from the data set used for logistic regression.

The p values for the logistic regression models with CER and cell concentration as the independent variables were 0.003 and 0.017, respectively, indicating CER and cell density were significantly correlated to the probability of achieving at least 80% infection. Logistic regression analyses with cell concentration as the independent variable yielded intercept and slope values of -0.92 and 1.57×10^{-6} 100 ml/cells, respectively,

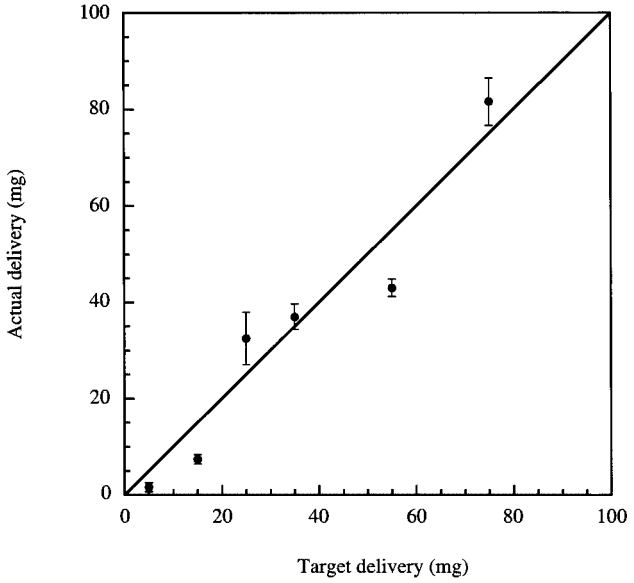

Figure 3 Comparison of target medium delivery (mg) and actual delivery (mg) to bioassays from solid medium dilutions. The average and standard error of three replicate analyses are shown. The solid line represents the expected 1:1 relationship.

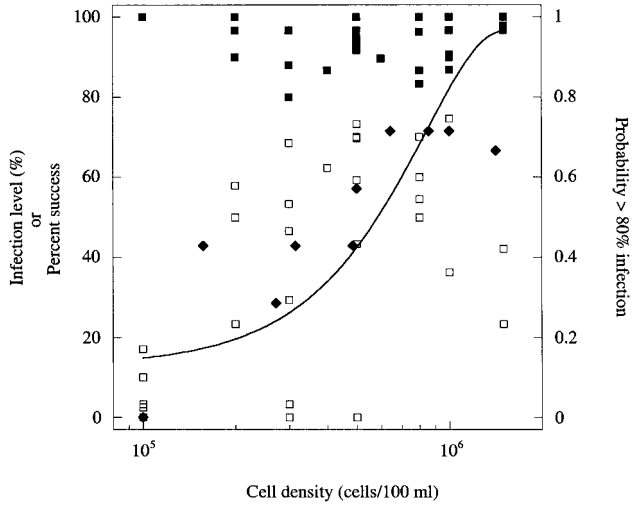

Figure 4 Effect of *L.giganteum* cell density on infection of *A. aegypti* mosquito larvae in 100-ml bioassays. Only results from bioassays of cultures at least 3 days old and bioassays containing less than 200 mg wet medium are shown. Infection levels of bioassays that attained at least 80% infection (success) are represented by ■ points, while infection levels of bioassays that attained less than 80% infection (failure) are represented by □ points. The solid line represents the probability curve for attaining at least 80% infection, as determined by the logistic regression analysis. Percent success, represented by ◆ points, was found by grouping six to seven successive values of infection level, finding the average cell density for the group and the percentage of those bioassays that had at least 80% infection.

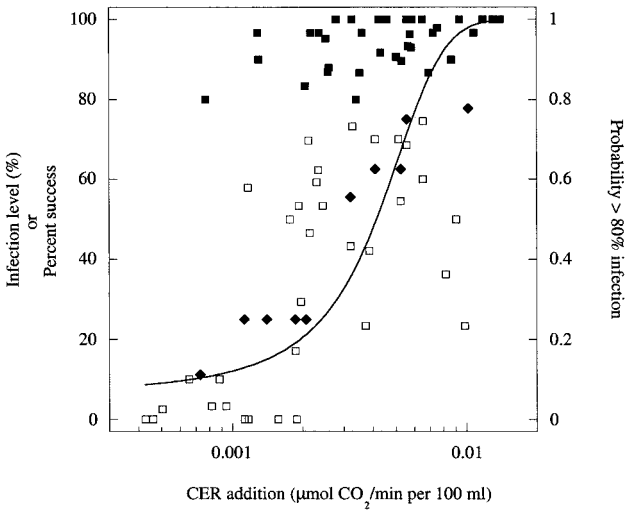

Figure 5 Influence of CER addition on infection of *A. aegypti* mosquito larvae in 100-ml bioassays. Only results from bioassays of cultures at least 3 days old and bioassays containing less than 200 mg wet medium are shown. Infection levels of bioassays that attained at least 80% infection (success) are represented by ■ points, while infection levels of bioassays that attained less than 80% infection (failure) are represented by □ points. The solid line represents the probability curve for attaining at least 80% infection, as determined by the logistic regression analysis. Percent success, represented by ◆ points, was found by grouping eight to nine successive values of infection level, finding the average CER for the group and the percentage of those bioassays that had at least 80% infection.

while analyses with CER concentration as the independent variable yielded values of -1.14 and 274 100 ml/min per μmol CO_2, respectively. The p value for the logistic regression model with media concentration as the independent variable was 0.422 indicating that medium concentration was not significantly correlated to the probability of achieving at least 80% infection.

The probability curves generated from the logistic regressions of cell concentration and CER concentration as independent variables are presented in Figures 4 and 5, respectively. As long as wet medium addition remained below 200 mg and only cultures older than 3 days were considered, the probability of achieving greater than 80% infection in mosquito bioassays increased as CER and cell addition increased. Adding more than 10^6 cells or 0.01 μmol/min CER to the 100-ml bioassays offered little increase in the probability of achieving greater than 80% infection.

Discussion

The amount of solid medium added to the bioassay was a poor predictor of the probability of achieving greater than 80% infection. This was expected, as the amount of medium offers no insight into the viability of *L. giganteum* growing on the medium. Solids addition did, however, affect the water quality characteristics and the mosquitoes in the bioassay. The percentage of pupation increased with the amount of medium added to the system (data not shown). While these results suggest that the increase in solid material provided a food source for the larvae as observed by the accelerated growth rate, mosquito death (e.g., due to starvation) was not observed in the controls.

Wet medium concentrations greater than 200 mg/100 ml bioassay (42 mg total suspended solids/100 ml and 12 mg total dissolved solids/100 ml) adversely affected both infection level and water clarity. The decrease in infection at these higher concentrations may have been due to inhibition of zoospore production as a result of solids concentration. Decreased infection has been observed at high levels of chemical oxygen demand [4] and zoospore production can be inhibited by solutes associated with growth media [7]. Based on these results, an upper limit of 200 mg wet medium (41 mg total suspended solids/100 ml and 12 mg total dissolved solids/100 ml) per bioassay was set. All bioassays that contained more than 200 mg wet medium were removed from the data set used for logistic regression.

Although both cell number and CER were correlated with the probability of achieving greater than 80% infection in mosquito bioassays, cell number had a much weaker correlation than CER. This may have been due to errors in cell density measurements and in measurements being obtained from a small subsample of the total cultivation product. Furthermore, there may have been errors in differentiating viable and nonviable cells during counting. Thus, CER is recommended over cell counts for estimating the efficacy of *L. giganteum* produced in solid-state cultivation. Measurement of CER may offer additional practical advantages over cell counting, such as on-line, noninvasive monitoring of efficacy. For instance, CER could be monitored on a product during storage to determine efficacy on-line without compromising the product. This is particularly important considering the main limitation of *L. giganteum* application for mosquito control is its short shelf life making frequent efficacy monitoring of products imperative to formulation development. On-line monitoring of CER could provide a more rapid and efficient assessment of product efficacy than 7-day-long mosquito bioassays.

While measurement of CER offers producers and end-users a tool for monitoring efficacy of *L. giganteum* formulations, certainly water quality characteristics and prohibitively high or low environmental temperatures would also impact the efficacy of applied products. These would need to be considered in parallel with CER measurements prior to application of a formulation in the field.

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