Optimization of liquid culture medium for commercial production of *Colletotrichum truncatum*

Robert W. Silman and Terry C. Nelsen

*Fermentation Biochemistry Research Unit, National Center for Agricultural Utilization Research, and Midwest Area Office, USDA, Agricultural Research Service, Peoria, Illinois, USA*

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Abstract: A commercial type liquid culture medium for the production of *Colletotrichum truncatum* NRRL 13737, a pathogen of the weed hemp sesbania, was developed. The concentrations of culture ingredients: 20 g glucose l⁻¹, 2.5 g Tastone yeast extract l⁻¹, 7.5 g Pharmamedia l⁻¹ are optimum for batch cultures. Initial glucose concentration determined total dry weight which was largely composed of mycelia: Pharmamedia concentration influenced the yield and rate of spore production; Tastone 154 yeast extract apparently supplied a nutritional factor which also affected the yield and rate of spore production but to a lesser extent. Batch culture with the described media yielded 6.2 × 10⁷ spores per ml in three days. Studies of spore recovery, drying and storage can now be conducted with a commercial type culture medium.

Key words: Biocontrol; Mycoherbicide; Weed control; Hemp sesbania; *Colletotrichum truncatum*

Introduction

The public desire to eliminate chemical pesticides has precipitated an interest in biological pesticides against insects, nematodes and weeds. Our laboratory began studies aimed at filling these needs. Studies in the Fermentation Biochemistry Research Unit (FBRU) have centered on a potential mycoherbicide. This chosen mycoherbicide is *Colletotrichum truncatum*, a pathogen of hemp sesbania (*Sesbania exaltata*) which is a weed of rice and cotton fields. It was discovered by C.D. Boyette, who also evaluated its herbicidal potential [1,2]. To produce a marketable mycoherbicide for inundative control [3] wherein the weed is sprayed annually, many requirements must be met. An already commercialized mycoherbicide, Coluego, is the dried spores of *Colletotrichum gloeosporioides* fsp. *aeschnomene*, but its production development has been only partially described [4,5].

Two avenues leading to the use of *C. truncatum* as a mycoherbicide have been taken in the studies of the FBRU. Jackson and Bothast [6], Schisler et al. [7] and Jackson and Schisler [8]...
have shown the influence of total carbon to total nitrogen ratio upon conidiation, the efficiency in inciting disease and compositional differences in biomass and conidia. At low C:N ratio (10:1) more pathogenic spores were produced than at 30:1, whereas at higher ratios (30:1) more conidia were produced than at 10:1. The other approach has been more empirical. Silman et al. [1] compared liquid shake flask culture, solid-state cultures and cultures on cellophane membranes over media-soaked pads. The important medium ingredients for all three methods were found to be carbohydrates and two nitrogen sources, KH₂PO₄ and CaCO₃ [1]. Because all three culture methods gave nearly the same number of spores per ml of medium, and liquid culture is the easiest of the three to sample and assay, liquid culture in shake flasks was selected for optimization of media. The goal to exceed was 2.75 x 10⁷ spores per ml of liquid culture obtained after 4 days incubation. In the experiments reported in this paper we varied the concentration of media ingredients in order both to find optima for, and to provide a liquid culture for, spore isolation, drying, storage and formulation studies.

Materials and Methods

Preparation of inoculum

Colletotrichum truncatum NRRL 13737 isolated by Boyette [1] was described previously [9]. Inoculum for flask cultures was prepared as follows: 5 ml of 0.01% Triton was added to each 6-day-old slant and mixed with a Vortex mixer to suspend the spores. Then 3 ml of the pooled suspension from 2–4 slants was added to 3–5 2800-ml Fernbach flasks, each containing 250 ml agar media and spread over the entire surface. Incubation was for 6 days at 25°C after which 70 ml 0.01% Triton was added to each flask. A sterilized stiff artist’s brush was used to dislodge the spores from the agar surface. These conidial suspensions averaged 3.34 ± 0.29 x 10⁷ spores per ml based on microscopic counts (Mc) and 2.50 ± 0.40 x 10⁷ spores per ml based on plate counts (Pc).

Media and culture conditions

Media consisted of various concentrations of glucose; Tastone 154 yeast extract (Universal Foods Corp., Milwaukee, WI); Pharmamedia, a cottonseed meal, (Traders Protein, Memphis, TN); KH₂PO₄; and CaCO₃. Table 1 gives the concentrations of ingredients used in the four experiments. Five-hundred ml media was sterilized 30 min at 121°C in 2800-ml Fernbach flask and inoculated (2.5% v/v) to give 8.2 x 10⁵ spores per ml by microscope count (Mc) and 6.1 x 10⁵ spores per ml by plate count (Pc). Incubation was at 25°C and 200 rpm on a rotary shaker of 2 inches eccentricity, under fluorescent lights until microscopic spore count stopped increasing, or decreased.

Analytical

Samples were removed daily from the Fernbach flasks using an aseptic technique. The num-

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² 2 level-5 factor partial factorial [14].
³ 3 level-3 factor Box-Behnken [15].
³ 3 level-3 factor partial factorial [14].
ber of conidia was determined microscopically using a Petroff-Hauser counter. The pH of the whole broth was measured with an electronic pH meter. Ten ml whole broth was centrifuged. The supernatant fluid was used to determine residual glucose concentrations using a Waters ALC-201 HPLC with a Bio Rad Aminex HPX-87 H column and degassed water as a mobile phase. Peaks were detected with a Waters R401 differential refractometer.

Centrifuged solids (mycelia, spores and unused solid media) were dried at 80°C under vacuum to give total dry weight (TDW). Density (g spores/l) of the spore inoculum was obtained in the same way.

Spore weight was calculated from the microscope count by the following equation:

\[
\text{Spore weight (g/l)} = \frac{\text{no. of spores per ml of sample}}{\text{no. of spores per l inoculum}} \times \frac{\text{g spores per l inoculum}}{	ext{g spores per l inoculum}}
\]

The spores in the inoculum averaged 0.98 g/10⁷ spores. Cascino et al. [10] reported 0.63 g/10⁷ for *C. gloeosporioides* but those spores, while having similar density, are smaller in size than *C. trun-catum*.

**Experimental design and statistical analysis**

Five experiments were run using fractional factorial experimental designs as described by Box et al. [11]. The basic experimental design was a balanced fractional factorial set up to measure simple effects of the main factors. The ability to examine interactions between and among some factors was sacrificed in order to gain efficiency in time and materials. The specific designs for the individual experiments are described in Table 1. Results were analyzed by least squares analysis of variance using PROC GLM in version 6.05 of SAS [12].

**Results and Discussion**

**Establishment of important medium ingredients**

In a preliminary experiment two levels each of five medium ingredients (glucose, Tastone 154 yeast extract, Pharmamedia, KH₂PO₄ and CaCO₃) were compared. Glucose and Pharmamedia levels influenced \((P < 0.10)\) Mc on 2, 3 and 4 days and Tastone YE level affected \((P < 0.01)\) Mc on days 3 and 4. The KH₂PO₄ and CaCO₃ levels did not \((P > 0.10)\) affect the Mc. The overall mean Mc was \(8.80 \times 10^6\) on day 2, \(18.86 \times 10^6\) on day 3 and \(22.32 \times 10^6\) on day 4.

**Optimization of important media ingredients**

Having established that glucose, Tastone YE and Pharmamedia were the three most important ingredients at the levels tested, four more experiments were run with different levels of these three ingredients. Experiments I, II, III, and IV were run to bracket the optimum levels of each of the three ingredients and to look for finer and finer distinctions within the overall optimum levels of interest.

Overall least squares means for each of the levels of glucose, Tastone YE and Pharmamedia were calculated and are shown in Fig. 1a, b and c for Mc assays run on 2, 3, and 4 days. It can be seen that 2 days incubation was insufficient for maximum spore production. The optimum glucose level was in the range 20 to 30 g/l (Fig. 1a). None of the individual experiments found significant \((P < 0.10)\) differences between any two levels between 18 and 30 g/l. The optimum Tastone level was in the range 2.0 to 2.5 g/l (Fig. 1b). The optimum level of Pharmamedia was in the 7.0 to 8.0 g/l range (Fig. 1c). No differences were found among Pharmamedia levels of 7.0, 7.5 or 8.0 g/l \((P > 0.10)\).

The derived optimum levels of 20 g/l glucose, 2.5 g/l Tastone YE and 7.5 g/l Pharmamedia gave \(61.6 \times 10^6\) spores/ml after only 3 days of culture. The course of this culture is shown in Fig. 2. Glucose was consumed within 2 days. After the maximum spore concentration was attained in 3 days there was a decline in the spore count from \(61.6 \times 10^6\) spores/ml. The decline can be explained by germination of some of the spores but no subsequent mycelial growth since there is no more glucose and it is known that spores germinate on plain water agar plates but with little subsequent growth. The total dry weight (TDW) increased from 8 g/l (the initial solids are
from CaCO₃ (2.8 g/l) and Pharmamedia (4.6 g/l) to about 20 g/l by the first day sample and remained nearly constant thereafter. The TDW minus the spore weight is also shown. The spore weight for \(6.16 \times 10^6\) spores/ml (3 day culture) was calculated to be 6.03 g/l. If it is assumed that CaCO₃ solids did not change during culture and since microscopic examination showed that Pharmamedia particles were present in samples of all ages, it is then quite probable that the plot of TDW minus spore weight indicates behavior of \(C.\) truncatum that is similar to \(C.\) gloeosporoides i.e. "sporulation was achieved at least in part by use of endogenous material" (Cascino et al. [10]).

**Effects of glucose, Tastone YE and Pharmamedia**

Cultures were run in order to determine the function of glucose, Tastone YE and Pharmamedia. The time courses of changes in total dry weights (TDW), microscopic spore counts (Mc) and glucose concentration (G) during culture are depicted in Fig. 3 for experiments in which glucose, Pharmamedia and Tastone YE respectively were varied. When glucose was 10, 20 or 30 g/l with constant Tastone YE (2 g/l) and Pharmamedia (6 g/l), TDW was proportional to initial glucose concentration (Fig. 3a). Also, glucose may have determined when spore production would
cease, which was about one day later than when TDW stopped increasing (Fig. 3b,c). However, when Pharmamedia was 0, 3, and 6 g/l with constant glucose (20 g/l) and Tastone YE (2 g/l) the results suggested that Pharmamedia apparently is required for the production of new spores since none were made when it was excluded (Fig. 3e). Also, at 3 and 6 g/l, yield and rate of spores production increased despite similar TDW levels (Fig. 3d,e) indicating that initial Pharmamedia directly affects both yield and rate. In contrast, the effects of increased Tastone YE (0, 1, and 2 g/l) at constant glucose (20 g/l) and Pharmamedia (6 g/l) levels are less clear. While it is evident that the amount of spores at 2 and 1 g/l Tastone YE are equivalent, at 0 g/l Tastone YE the total number of spores finally reaches about 2/3 of that at 1 and 2 g/l (Fig. 3h) which implies that Tastone YE also affects the yield and rate of spore production but the nature of the effect is different from that for Pharmamedia.

**Total C : total N ratio**

The total C : total N (C : N) ratio of the optimized media (20 g/l glucose, 2.5 g/l Tastone YE, 7.5 g/l Pharmamedia) was calculated based upon estimates of carbohydrate, fat and protein in Tastone YE and Pharmamedia as supplied by the manufacturers and found to be about 12 : 1 which is near the value of 10 : 1 reported by Schisler et al. [7] for most efficacious conidia. This was both unexpected and fortuitous. However, the observation that Tastone YE and Pharmamedia directly affect the spore yield and production rate, but to

![Fig. 3. Effects of varying glucose, Tastone 154 or Pharmamedia on total dry weight, spore count, and glucose concentrations.](image-url)
different degrees and seemingly in different ways, raises the possibility that C:N ratio alone may not control spore yield and efficacy.

Conclusions and Future Work

The first commercial type culture medium for production of *C. truncatum* spores has been optimized at 20 g/l glucose, 2.5 g/l Tastone 154 YE, 7.5 g/l Pharmamedia, 5 g/l CaCO₃, and 1.0 g/l KH₂PO₄. Also, double the spore concentration was produced in 3 days instead of 4. Using this medium, studies on spore recovery, drying and storage can now be realistically pursued. Specific attention will be given to desiccation tolerance [13] since media modifications can alter this important factor.

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References