

Full Length Research Paper

Production of mycelium and blastospores of *Hirsutella* sp. in submerged culture

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Hirsutella sp. was grown in four liquid media containing either casamino acids, corn steep liquor, collagen peptone or casein peptone. These media were inoculated with a 7 day-old culture of mycelia and blastospores of *Hirsutella* sp. and the cultures incubated with shaking at 250 rpm at 26°C. The media containing corn steep liquor, casamino acids or collagen peptone produced abundant mycelium, varying from 64 to 76.3 mg/ml at different fermentation days, whereas the medium containing casein peptone produced less biomass. Additionally, the casamino acids and collagen peptone media showed similar biomass values, as well as blastospore counts after 14 fermentation days, without significant differences between the two media. The highest number of blastospores produced was 3.8×10^7 blastospores/ml using medium with casamino acids. This medium, as well as that with collagen peptone gave suitable results for the preparation of an inoculum for the production of conidia in solid medium.

Key words: *Hirsutella*, liquid media, fermentation, blastospores, microbial control, mass propagation.

INTRODUCTION

Hirsutella is a fungus belonging to the order Hypocreales, recently reclassified within the family Ophiocordycipitaceae, which has been tested as a microbial control agent against several eriophid and tetranychid mites. The genus *Hirsutella* includes about 94 species, some of which are pathogenic to insects, mites and other invertebrates. Thus, they have been considered in the biological control of plant-parasitic nematodes, such as *Hirsutella rhossiliensis* and *H. minnesotensis*, which have been used in the control of the soybean cyst nematode (Chen and Liu, 2005; Zhang et al., 2008), and *H. thompsonii* has also been tested as a potential microbial control (Kanga and James, 2002; Kumar and Singh, 2008). *H. thompsonii* has been

isolated from the citrus rust mite *Phyllocoptruta oleivora*, and its successful culture on various synthetic media (McCoy and Kanavel, 1969) introduced the possibility of large-scale laboratory production for later dissemination in the field; however, growth and sporulation of this fungus on solid media is slow compared to many other entomogenous fungi.

Hirsutella has been cultured in some liquid and solid media, although in liquid media, the production of mycelium and spores has been low, as reported by McCoy et al. (1972), for example, who used dextrose and sucrose at 5 and 10 mg/ml, respectively, as carbon sources, and yeast extract and peptone as nitrogen sources at 5 and 0.5 mg/ml, respectively. However, the combination of these sources produced only 22.43 mg/ml mycelium biomass, and without the formation of blastospores. Winkelhoff and McCoy (1984) demonstrated conidiation of *H. thompsonii* var. *synnematos* in submerged culture, after 6 to 11 days of

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Table 1. Composition of culture media for the production of *Hirsutella* sp.

Medium used	Nitrogen source (g/L)	Carbon source (g/L)	Mineral salt
^a CAA Jackson et al. (1997)	25 Casamino acids	80 Glucose	2.0 g KH ₂ PO ₄ , 0.4 g CaCl ₂ ·H ₂ O, 0.3 g MgSO ₄ , 37 mg Co Cl ₂ ·6H ₂ O, 50 mg FeSO ₄ , 16 mg MnSO ₄ , 14 mg ZnSO ₄
CP Rivas-Morales (1998)	15 Collagen peptone 5 Yeast extract	80 Glucose	4 g KH ₂ PO ₄ , 0.8 g CaCl ₂ ·2H ₂ O, 50 mg MgSO ₄ ·7H ₂ O, 50 mg FeSO ₄ ·7H ₂ O
PCS (casein peptone)	15 Casein peptone	15 Corn syrup	0.2 g CoCl ₂ ·6H ₂ O, 16 mg MnSO ₄ ·2H ₂ O 14 mg ZnSO ₄ ·7H ₂ O
CSL (Corn steep liquor)	20 Corn steep liquor	50 Sucrose	5 g KH ₂ PO ₄ , 2 g MgSO ₄ , 0.8 g CaCl ₂ ·2H ₂ O, 50 mg FeSO ₄ ·7H ₂ O, 37 mg CoCl ₂ ·6H ₂ O, 16 mg MnSO ₄ ·2H ₂ O, 14 mg ZnSO ₄ ·7H ₂ O

^aThe medium was modified in concentration of amino acids not supplemented with vitamins.

incubation with shaking at 26°C. The medium contained 10 g/l corn steep liquor and 0.2% Tween 80, which was considered essential for maximum conidiation. Recent studies used diverse sources of carbon and nitrogen such as the one by Li et al. (2010) with medium containing 2.5% sucrose and 0.5% peptone, which produced an average *Hirsutella* sp. biomass of 10.06 g/l and also exopolysaccharides with antibacterial activity. An alternative method for the commercial production of entomopathogenic fungus involves products based on fragmented mycelium or blastospores, which can be produced in liquid media. However, the production of blastospores in these media has not been reported. To produce a commercially successful microbial insecticide, the pathogen must have low-cost mass propagation, for it to be applied in field. Therefore, the development of new solid or liquid culture media in the production of bioinsecticides is a biotechnological tool to control and to decrease the incidence of pests on agricultural crops.

The mass propagation of fungus for use as a biopesticide is a goal for all researchers in pest control because it has advantages mainly in the scale up process, such as control parameters, including temperature, pH, aeration and agitation, and reduction of costs as well. This work reports the results of the production of mycelium biomass and blastospores of a strain of *Hirsutella*, testing four liquid media.

MATERIALS AND METHODS

Strain

The INIFAP-Hir-1 strain was isolated from *Diaphorina citri* (Asian citrus psyllid), which is from the state of Tabasco, Mexico, provided by Forest Research Institute of Agriculture and Livestock (INIFAP). It was then activated as single-spore isolates on potato dextrose agar (PDA), incubated at 25 ± 2°C for 30 to 40 days.

Preparation of inoculum

The monoconidial strain was reseeded on PDA plates and then incubated at 25 ± 2°C for 4 weeks. Afterwards, four 1 cm² agar squares were cut out and each placed in a 250 ml Erlenmeyer baffled flask with 50 ml of medium containing casamino acids (Jackson et al., 1997). The flasks were incubated at 25 ± 2°C in a rotary shaker at 250 rpm for 7 days, after which microscopic observations were made to confirm the purity of the culture. An aliquot of 10 ml was withdrawn to inoculate 250 ml Erlenmeyer flasks each containing 50 ml of culture medium.

Composition of liquid media

Four different liquid media were prepared for the propagation of *Hirsutella* sp, which are described in Table 1. All components of the media were obtained from Difco Laboratories, Detroit, MI, USA, except the corn steep liquor, which was obtained from CP Ingredients S.A. de C. V. (Jalisco, Mexico), corn syrup (Industrializadora de maíz, S. A., Jalisco, Mexico) and collagen peptone (Sensient Lab. Jalisco, Mexico). The flasks containing each liquid medium were autoclaved at 121°C for 15 min. Glucose and sucrose were autoclaved separately. Three replicate flasks were used for each medium and were incubated as previously mentioned for 14 days in a New Brunswick scientific shaker. All experiments were repeated at least thrice.

Dry weight

For the determination of mycelium biomass and blastospores, samples of the different media including their replicates were taken every two days for microscopic observations and dry weight determination. The mycelium was separated from the medium by centrifugation at 4000 rpm for 30 min, using tared glass tubes. Afterwards, the mycelium was dried for 48 h at 80°C.

Statistical analysis

Data obtained from dry biomass were analyzed using one-way ANOVA, and later differences between means were compared by the least significant difference method (LSD) at the 0.05 level of

significance.

Fermentation in casamino acids and collagen peptone media

The fermentation experiments in casamino acids and collagen peptone media were repeated to determine differences in the formation of blastospores between the two media.

Data analysis of mycelium and number of blastospores

The average number of blastospores and mycelium yield were analyzed using Student's t-test at the 0.05 level of significance to determine differences between the two media on each sampling day.

Germination of blastospores produced

The germination rate of blastospores obtained in liquid culture was determined by the inoculation of aliquots from culture dilutions from 14-day samples, using agar plates (3 g of bacteriological agar in 200 ml water). Petri dishes were prepared with 1 cm² marked areas, in which 20 drops of appropriate dilution were placed. The plates were incubated at 25°C for 24 to 48 h. The 1 cm squares were placed on slides, along with a drop of lactophenol blue solution, to visualize the blastospores under a light microscope. The germination of blastospores was assessed by counting a group of 100 blastospores per aliquot, and the viable blastospores were recognized by the presence of a germ tube of half-blastospore size.

RESULTS AND DISCUSSION

The inocula prepared for the four liquid media showed biomass values of 78.3 ± 6.3 mg/ml. The inoculum also contained blastospores formed in the medium with casamino acids. The biomass yields obtained are shown in Figure 1, where we found abundant mycelium production in three of the media tested, namely those containing either corn steep liquor, casamino acids or collagen peptone, whereas the medium composed of casein peptone had low production. Also, the maximum value shown by each medium was obtained at different fermentation times. The medium containing corn steep liquor had a maximum amount of 66.6 ± 1.7 mg/ml at day 8 of fermentation, whereas media with casamino acids or collagen peptone showed maximum values of 76.3 ± 5.9 and 64 ± 17.3 mg/ml at 12 and 14 fermentation days, respectively. On the other hand, the medium containing casein peptone showed a maximum of 15.6 ± 4.5 mg/ml on day 6 of fermentation. Analysis of variance indicated that at 2, 4, 6 and 8 fermentation days, the medium containing corn steep liquor displayed mycelium biomass significantly higher values compared to the other media ($F = 34.67$, $df = 3, 6$, $p < 0.001$ for day 2; $F = 46.77$, $df = 3, 6$, $p < 0.0001$ for day 4; $F = 34.42$, $df = 3, 6$, $p < 0.001$ for day 6; and $F = 98.75$, $df = 3, 6$, $p < 0.0001$ for day 8). On the other hand, on days 10 and 12 of fermentation, the medium containing casamino acids showed significantly higher biomass values compared to the other

media ($F = 56.71$, $df = 3, 6$, $p < 0.0001$ for day 10; $F = 44.08$, $df = 3, 6$, $p < 0.001$ for day 12), while for day 14 of fermentation, casamino acids and collagen peptone media produced similar amounts of mycelium biomass, higher than with the other media ($F = 22.97$, $df = 3, 6$, $p = 0.002$). In addition, blastospores were found to be present in the media containing casamino acids or collagen peptone.

Blastospore production under these conditions had not been previously reported. Therefore, we examined whether the production of blastospores was similar in the two media with casamino acids and collagen peptone. The production of mycelium and blastospores of *Hirsutella* in casamino acids and collagen peptone media was analyzed by Student's t-test. Figure 2 shows the mycelium biomass in the two media which were statistically similar up to day 12 of fermentation (t values found were 0.0413, 2.3410, 0.0351, 0.2552, 1.6020 and 1.8053, for days 2, 4, 6, 8, 10 and 12, respectively, whereas $t_{0.05} = 2.571$, $t_{0.01} = 4.032$, $df = 5$). The highest biomass values found for the two media were on day 14 of fermentation, where the casamino acids medium showed significantly higher values compared to collagen peptone medium ($t = 2.6565$, $t_{0.05} = 2.571$, $t_{0.01} = 4.032$, $df = 5$).

Figure 3 shows the number of blastospores obtained with casamino acids and collagen peptone media during fermentation, where blastospore counts were statistically similar for the two media at all sampling times (values $t = 1.4639$, 0.9703, 0.0485, 1.0579, 1.6940, 0.8043, 1.3399 and 0.6406 for days 0, 2, 4, 6, 8, 10, 12 and 14, respectively, and $t_{0.05} = 2.571$, $t_{0.01} = 4.032$, $df = 5$).

The biomass production obtained in media with corn steep liquor, casamino acids and collagen peptone (66.6, 76.3 and 64 mg/ml, respectively) was higher compared to reported values in earlier studies, using *Hirsutella* strains. For example, McCoy et al. (1972) obtained 22.43 mg/ml biomass, although without the formation of blastospores, using media containing dextrose or sucrose, in addition to yeast extract or peptone, whereas Winkelhoff and McCoy (1984) reported a maximum value of 15.8 ± 3.4 mg/ml in medium with corn steep liquor and 0.2% Tween 80; meanwhile, Li et al. (2010) obtained a biomass average of 10.06 g/L in medium with 2.5% sucrose and 0.5% peptone. The production of mycelium biomass and blastospores obtained here was improved in comparison to the values reported earlier. This is an important finding, since this contributes to increased yields in the production of conidia, mostly in two-phase culture, liquid and solid. An initial liquid culture could be used as an inoculum for solid medium to produce infective conidia, thus achieving greater and more rapid production of fungal conidia to be used in biological pest control programs. The production of blastospores in liquid media, to our knowledge, had not been previously reported in the literature. Thus, it may be important to verify whether these blastospores are equally infective as conidia, and if

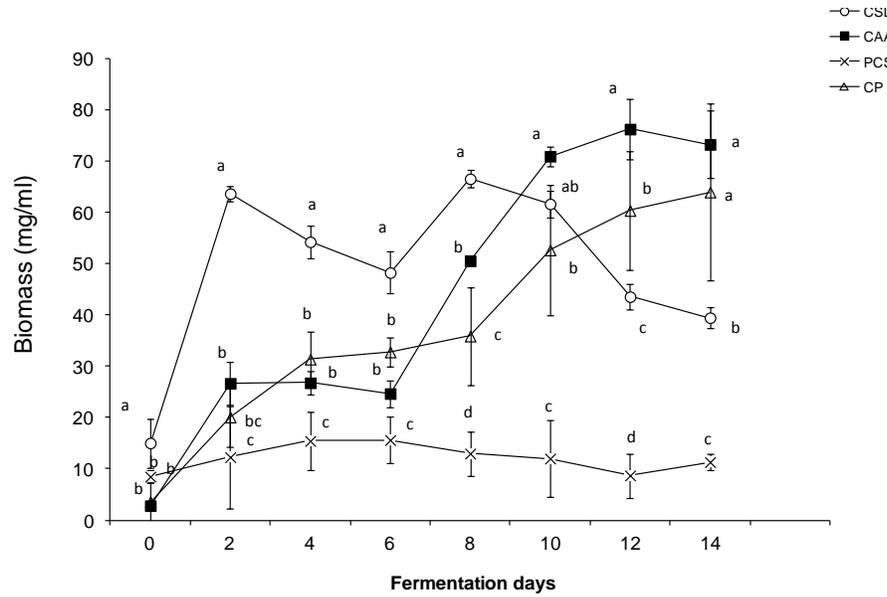


Figure 1. Mycelium biomass produced by *Hirsutella* sp. in four liquid media containing corn steep liquor (CSL), casaminoacids (CAA), collagen peptone (CP) or casein peptone (PCS), incubated at 25 ± 2°C with shaking at 250 rpm for 14 days. Different letters on each sampling day indicate significant differences in the dry biomass produced.

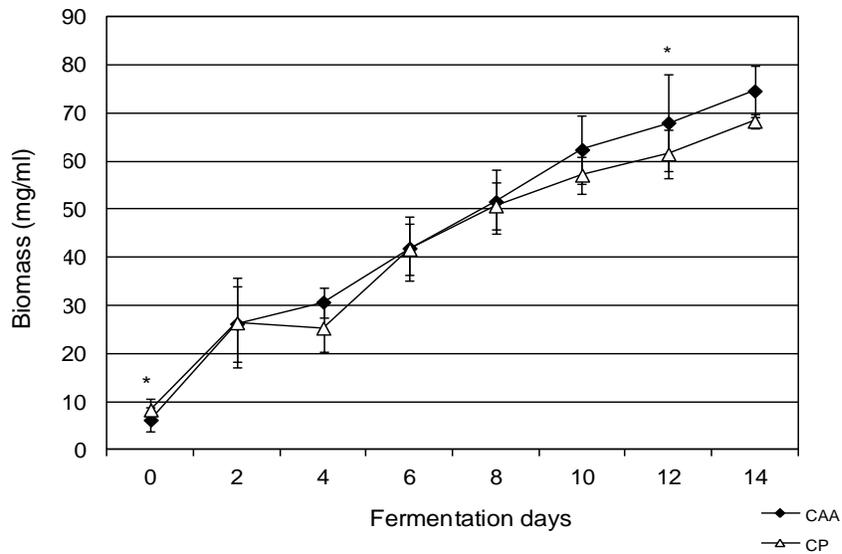


Figure 2. Mycelium biomass produced by *Hirsutella* sp. in casamino acids (CAA) and collagen peptone (CP) media incubated at 25 ± 2°C with shaking at 250 rpm for 14 days. * indicates significant difference in the production of biomass determined by Student's t-test at the 0.05 level of significance.

the infection of target insects or mites could be faster than with conidia, as *Hirsutella* is a fungus that grows and produces conidia very slowly.

Regarding production costs, we mentioned that corn

steep liquor is the least expensive medium of the four tested, and it is the medium that showed increased production of mycelium in less time (8 days) but without the formation of blastospores. The second least

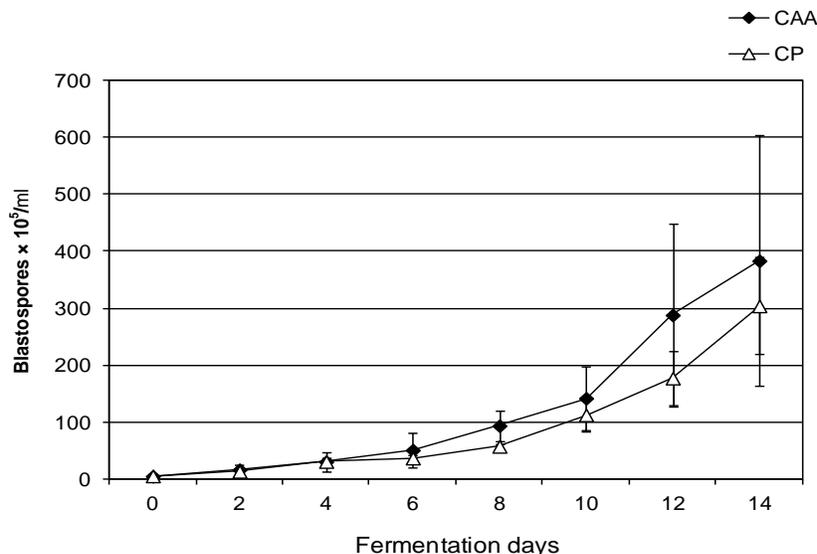


Figure 3. Blastospores/ml produced by *Hirsutella* sp. in casamino acids (CAA) and collagen peptone (CP) media, incubated at $25 \pm 2^\circ\text{C}$ with shaking at 250 rpm for 14 days.

expensive is collagen peptone, followed by casein peptone, and the most expensive is casamino acids. Therefore, we could opt for corn steep liquor or collagen peptone media for mass production, since they are cheaper.

On the other hand, in the blastospore germination test, 14-day fermentation samples for casamino acids and collagen peptone media showed germination rates of 18.6 and 18%, respectively, after 48 h of incubation at $25 \pm 2^\circ\text{C}$, where no significant differences between the two media (t calculated = 0.1538, $t_{0.05} = 4.303$, $t_{0.01} = 9.925$, $df = 2$) were found. This germination percentage was considered low, but Winkelhoff and McCoy (1984) reported lower germination levels between 5.2 and 12.9% for submerged conidia from *Hirsutella thompsonii* var. *sinnematosa*.

Conclusion

We found abundant mycelium with three of the media tested, those containing corn steep liquor, casamino acids or collagen peptone, whereas low production was seen in the medium composed of casein peptone; also, the maximum values shown by each medium were obtained at different fermentation times, with values varying from 64 to 76.3 mg/ml. The casamino acids and collagen peptone media promoted the production of blastospores at a similar level, after 14 fermentation days, without significant differences between the two media, where the highest level was 3.8×10^7 blastospores/ml using casamino acids medium.

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