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Original Article

Pathogenicity of *Aspergillus clavatus* produced in a fungal biofilm bioreactor toward *Culex quinquefasciatus* (Diptera: Culicidae)

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Many entomopathogenic fungi have been demonstrated to be potential agents for efficiently controlling mosquito populations. In the present study, we investigated a bioreactor system to produce metabolites and conidia by combining technological advantages of submerged and solid-state fermentations. The efficiency of fungal products was tested toward mosquitoes. *Aspergillus clavatus* (Eurotiales: Trichocomaceae) was grown by semi-solid-state fermentation in a bioreactor for up to 7 days. Depending on conidial doses (2.5×10^7, 5×10^7, 7.5×10^7, 10×10^7 and 12.5×10^7 conidia/mL), mortality ranged from 37.2 ± 15.0 to 86.3 ± 5.0% toward larvae and from 35.8 ± 2.0 to 85.2 ± 1.5% toward adults. The metabolites (10, 20, 40, 60, 80 and 100% v/v) yielded mortality from 23.7 ± 15.0 to 100.0 ± 0.1% toward larvae, and two sprayed volumes (5 and 10 mL) reached 45.5 ± 1.4 and 75.6 ± 2.6% mortality, respectively, toward adults.

Keywords: biological control, entomopathogenic fungi, *Aspergillus clavatus*, biofilm bioreactor, solid-state fermentation, submerged fermentation.

Introduction

Mosquitoes (Diptera: Culicidae) are permanent blood sucking vectors of diseases such as dengue, filariasis, and malaria. Many mosquito species and strains are resistant to insecticides commonly used for their control. Biological agents have become increasingly attractive alternative for mosquito control. Among potential microorganisms, entomopathogenic fungi have been used and have provided most interesting results in several pest controls. Conidia are classically applied, but toxic metabolites have also been produced in liquid medium and used against mosquitoes.[1,2] *Metarhizium anisopliae* and *Beauveria bassiana* are promising alternatives to conventional insecticides for eliminating mosquito vectors.[3,4] Recently, many *Aspergillus* species have commonly been used against mosquitoes.[5,6] The larvicidal effect of *Aspergillus flavus* and *Aspergillus parasiticus* metabolites for controlling *Culex quinquefasciatus* has been shown.[7] *Aspergillus clavatus* conidia have also been demonstrated to be efficient against mosquitoes.[8,9] The possibility of using *Aspergillus niger* metabolites against *Cx. quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* larvae has also been shown.[10]

The development of a reliable and efficient fermentation system for large-scale production of insecticidal products is still needed. Classical submerged fermentation of filamentous microorganisms in aqueous suspensions may impair the production of conidia and metabolites of biotechnological interest.[11–13] By contrast, solid-state fermentation is a promising alternative since conidia and metabolites are generally produced in higher quantities when these microorganisms are produced on a solid substrate.[11–16] For example, *Aspergillus oryzae* has been reported to produce a 500-fold higher yield of recombinant chymosin in solid-state fermentation than in submerged fermentation.[17] In this context, efficient utilization of agro-industrial residues as carbon sources has been shown for the mass production of entomopathogenic fungi.[18–21] However, the absence of free water induces parameter variations (such as pH, temperature, moisture, dissolved oxygen or CO_2) that are difficult to control. A complementarity method between solid- and liquid-state fermentations has been attempted in sequential culture.[22]

A fungal biofilm reactor combining the technological advantages of submerged fermentation (*i.e.*, free water facilitating the control of culture parameters) with the biological characteris-

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tics found in solid-state fermentation has been established in the present study. Insecticidal activity of conidia and metabolites secreted by an entomopathogenic *A. clavatus* strain in this culture system was shown against *Cx. quinquefasciatus* larvae and adults.

Materials and Methods

1. Fungal strain

An *A. clavatus* (Eurotiales: Trichocomaceae) strain isolated from the locust cricket *Oedaleus senegalensis* (Orthoptera: Acrididae) at the Laboratory of Reproductive Biology of the University Cheikh Anta Diop (Dakar, Senegal) and shown to be pathogenic against *Anopheles gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* larvae[9] was used (accession number MUCL 55275, Belgian Coordinated Collections of Microorganisms, Mycothèque de l'Université Catholique de Louvain (Belgium)). This strain was cultivated on potato dextrose agar (PDA) in Petri dishes and stored at 4°C.

2. Inoculum

As a preculture, *A. clavatus* mycelia were produced in Erlenmeyer conical flasks (500 mL) containing 300 mL of distilled water, 1% peptone, 1% yeast extract and 2% glucose sterilized at 120°C for 20 min. These cultures were incubated after inoculation for 48 hr on a rotary shaker at 140 rpm and 30°C to produce fungal pellets.

3. Metal structured packing for fungal sporulation

To facilitate pellets fixation and fungal sporulation in a bioreactor, a metal packing was molded. Packing is a cylinder composed of several stainless steel corrugated sheets independent of each other, as previously described.[23] For packing, 28 metal rectangular plates (16×16 to 2×16 cm) with a 2-mm mesh were cut for assembly. The plates were superimposed symmetrically in pairs following the decreasing width of the rectangle in order to form a cylinder approximately 16 cm in diameter. All were then surrounded by a broad plate to form a block of cylindrical packages 16 cm in height and 16 cm in diameter. One of the bases was covered by a circular metal to prevent solid substrates from falling into the liquid medium.

4. Bioreactor system

The fermentation run was carried out in a 20-L bioreactor with 6-L working volume. Wheat bran (200 g) was introduced as a carbon source in the cylinder between the metal plates of the packing, which was suspended in the Biolafitte bioreactor (Fig. 1). Peptone and yeast extract (60 g each) were previously introduced in the total working volume of the reactor containing 6 L of distilled water and 0.05 g/L chloramphenicol as a bacteriostatic agent. The preparation was autoclaved at 120°C. The preculture was aseptically injected into the bioreactor using a syringe. With a peristaltic pump (120 rpm), the culture medium was continuously stirred (connections made with silicone tubing with an internal diameter of 5 mm) and injected on the packing containing wheat bran. The mycelial pellets were then carried out with the liquid medium and fixed to the molded system. The temperature was maintained at 30°C by circulating temperature-controlled water. Air was continuously supplied to the bioreactor at 3 L/min. The pH was allowed to vary freely and was recorded continuously. Cultivation was carried out for 7 days.

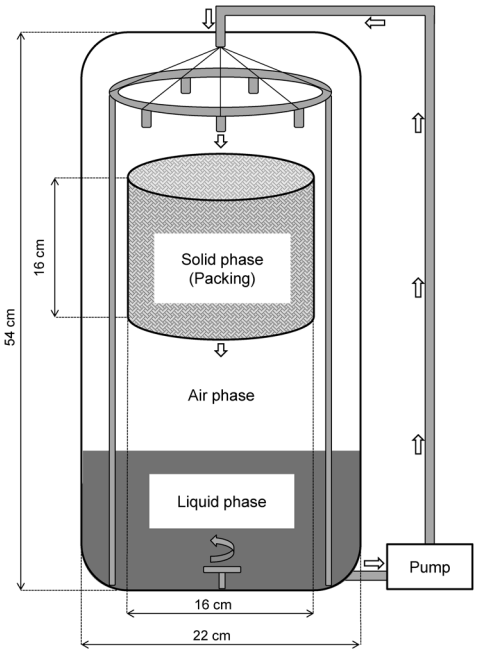

Fig. 1. Schematic diagram of a 20-L bioreactor system with metal packing. The packed structure (16 cm height×16 cm diameter) consisted of a stainless steel cylinder composed of several independent rectangular plates surrounded by a broad plate. The cylinder was closed at the bottom by a circular plate. Uniform liquid media were ensured by mixing. The pump drew the liquid containing the fungal pellets provided from a preculture, and injected them into the packing containing wheat bran as a solid substrate. Biomass immobilization was ensured to avoid liquid viscosity.

5. Conidia recovery

After incubation, the packing that contained fungal conidia and biomass was removed, and the liquid medium was stored at −80°C. The packing was washed with an aqueous solution containing 0.05% Tween 80 (0.05% Tween 80 solution) to detach the conidia. The washing solution was then filtered to discard impurities and centrifuged (4°C, 3,000 *g*, 5 min). The conidia were finally suspended in a 0.05% Tween 80 solution and the dose was adjusted to 10^9 conidia per mL using a hemocytometer (Thoma®).

6. Filtration of metabolites

Liquid medium from the bioreactor was centrifuged (4°C, 7,500 *g*, 30 min) to eliminate impurities. Medium was then filtered with ultrafiltration membranes (Prep/Scale-TFF-1 Cartridge (Millipore) 5.8 cm in diameter and 15.2 cm long with polyethersulfone membrane filtration (column 1 ft2 cartridge))

to remove unconsumed nutrients from the culture medium. Final concentrated metabolites (800 mL) with high molecular weight products (MW>100,000) were obtained and stored at −80°C.

7. Mosquito rearing

Cx. quinquefasciatus (S-Lab) adults were reared in 50-×50-×50-cm cages and fed with a 10% sucrose solution. Blood meal was made using the Hemotek feeding system. Larvae were maintained in distilled water (25-×15-×5-cm containers) in laboratory conditions (25±2°C, 70±5% relative humidity, and 16:8 hr (Light:Dark) photoperiod) and fed with fish food (TetraMin®) and natural brewer's yeast tablets (Biover®).

8. Bioassays

8.1 Larval treatment

First, groups of 20 third-instar larvae were exposed to conidial doses of 2.5×10^7, 5×10^7, 7.5×10^7, 10×10^7 and 12.5×10^7 conidia/mL in separate bottles for 3 days. Control larvae were maintained in 0.05% Tween 80 solution. Second, larvae were exposed to serial dilutions (10, 20, 40, 60, 80, and 100% (v/v)) of concentrated metabolites with distilled water for 3 days. Control larvae were maintained in distilled water. All larvae were fed with the same food as used for rearing.

8.2 Adult treatment

Conidia were formulated with 5% sunflower oil (v/v) in 0.05% Tween 80 solution. The same conidial suspension series used against larvae were directly sprayed on a total of 20 three- and five-day-old sugar-fed adults that were transferred in 25-×25-×25-cm mosquito netting cages and kept for 3 days. For control, a 0.05% Tween 80 solution containing 5% sunflower oil (v/v) was sprayed on the adults. In parallel, 5 and 10 mL of concentrated metabolites were sprayed directly on the groups of 20 adults previously introduced into separate cages and kept for 3 days. For control, distilled water was sprayed on the adults.

8.3 Statistical analysis

Four replicates were performed for each treatment for different durations with new independent fermentation products. Mortality was recorded daily for three days and corrected using Abbott's formula.[24] Dead larvae were removed, rinsed with sterile distilled water to eliminate non-attached conidia, and examined under a microscope for fungal infection. Dead larvae and adults were incubated in Petri dishes containing wet filter paper to monitor the conidial germination. All experiments were done in laboratory conditions with 25±2°C temperature and a 16-hr light photoperiod.

Results

1. Pathogenicity of conidia on Culex quinquefasciatus

Conidia were effective against *Cx. quinquefasciatus* larvae and adults. The percentage of larval mortality increased with increasing conidial concentration and ranged from 37.2±15.0 to 86.3±5.0% for 72 hr of treatment (Table 1). Adult mortality ranged from 35.8±2.0 to 85.3±1.5% after 72 hr post-inoculation (Table 2). Conidial adhesion to the larval cuticle was not observed. Nevertheless, conidia ingested by larvae during bioassays invaded the larval gut, germinated, and emerged thereafter from dead larvae (Fig. 2A–C). By contrast, the conidia penetrated the adult mosquito's cuticle before emerging (Fig. 2D).

2. Toxicity of metabolites on Culex quinquefasciatus

Metabolites produced by *A. clavatus* in a bioreactor showed tox-

Table 1. Mortality of *Culex quinquefasciatus* larvae treated with *Aspergillus clavatus* conidia produced in bioreactor*

Treatment time	Conidial concentration (10^7 conidia/mL)				
	2.5	5	7.5	10	12.5
Cumulative mortality (%)±SD					
24 hr	15.8±14.0	17.1±17.0	30.3±20.0	38.2±15.0	60.5±15.0
48 hr	25.0±6.0	35.5±22.0	42.1±30.0	47.4±9.0	77.6±13.0
72 hr	37.2±15.0	55.3±5.0	65.8±9.0	73.8±6.0	86.3±5.0

* Values are average corrected mortality (%)±standard deviation (SD).

Table 2. Mortality of *Culex quinquefasciatus* adults treated with *Aspergillus clavatus* conidia produced in bioreactor*

Time after inoculation	Conidial concentration (10^7 conidia/mL)				
	2.5	5	7.5	10	12.5
Cumulative mortality (%)±SD					
24 hr	20.0±3.0	20.0±4.0	24.8±2.0	28.3±8.0	37.5±1.0
48 hr	25.8±5.0	55.7±2.0	50.0±0.2	60.0±3.1	75.7±0.8
72 hr	35.8±2.0	56.5±0.9	65.5±0.7	75.0±14.0	85.3±1.5

* Values are average corrected mortality (%)±standard deviation (SD).

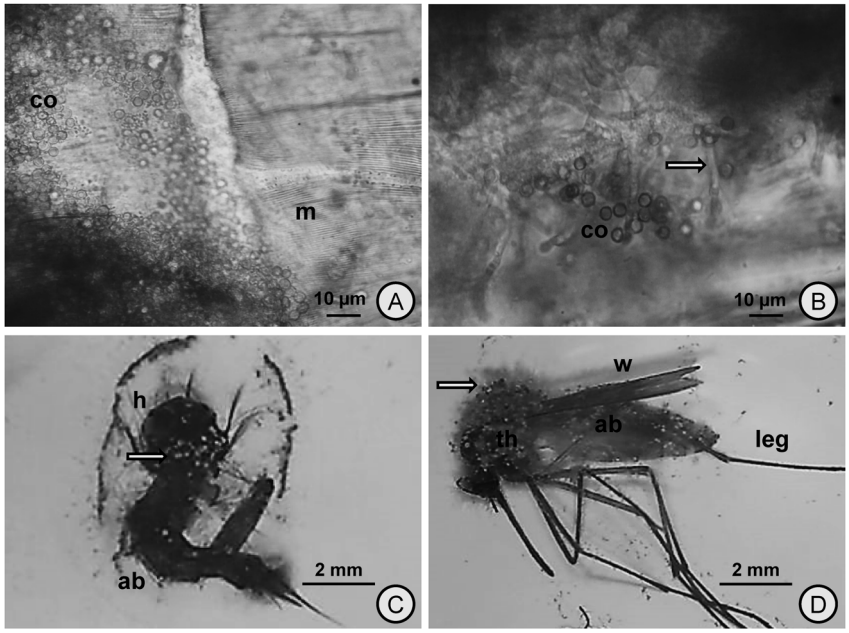

Fig. 2. *Culex quinquefasciatus* larvae and adults infected with *Aspergillus clavatus* cultivated in the bioreactor system. Conidia (A) were observed within the larval gut after 24 hr of treatment. Germ tube (arrow) and filaments within the larval gut (B), and conidial heads (arrow) on the larval cadavers (C) were observed 72 hr after treatment. Conidial formation was observed on the thorax (arrow), legs, and wings of the adults 72 hr post-inoculation (D). co: conidia, m: muscle, h: head, ab: abdomen, th: thorax and: wing.

Table 3. Effect of *Aspergillus clavatus* metabolites on *Culex quinquefasciatus* larvae*

Treatment time	Concentration of metabolites (%)					
	10	20	40	60	80	100
Cumulative mortality (%)±SD						
24 hr	2.6±3.0	22.4±16.0	27.6±10.0	67.1±8.0	76.3±6.0	93.4±5.0
48 hr	11.8±13.0	26.3±15.0	38.2±15.0	73.7±4.0	78.9±4.0	97.4±3.0
72 hr	23.7±15.0	39.5±27.0	55.3±5.0	76.3±6.0	82.9±2.0	100.0±0.1

* Values are average corrected mortality (%)±standard deviation (SD).

Table 4. Effect of *Aspergillus clavatus* metabolites on *Culex quinquefasciatus* adults*

Time after treatment	Sprayed doses of metabolites (mL)	
	5	10
Cumulative mortality (%)±SD		
24 hr	7.7±0.1	18.5±0.2
48 hr	40.1±2.0	65.3±3.6
72 hr	45.5±1.4	75.6±2.6

* Values are average corrected mortality (%)±standard deviation (SD).

icity against *Cx. quinquefasciatus* larvae and adults. The mortality increased with increasing metabolite concentration and ranged from 23.7±15.0 to 100.0±0.1% for larvae after 72 hr post-treatment (Table 3) and 45.5±1.4 to 75.6±2.6% for adults after 72 hr of treatment (Table 4).

Discussion

The system developed in this work shows the possibility to produce simultaneously, with controlled parameters, two kinds of *A. clavatus* products: conidia and metabolites in a bioreactor. The substrate contained in the metal packing allowed for efficient growth of the fungal biomass, which sporulated. The liquid media allowed for easy recovery of metabolites secreted by the fungal biomass. Moreover, the biomass remained confined on the substrate and did not increase the viscosity of the liquid medium. The present process highlights the possibility to control the parameters of metabolites and conidia production for further study. Homogenization of the physical traits in the packed structure (pH, oxygen, temperature), as well as their impact on conidia and metabolites production, must be investigated. Other entomopathogenic fungi, such as *M. anisopliae* and *B. bassiana*, could also be cultivated in this bioreactor system for industrial

production.

Conidia and metabolites were both found to be effective for controlling *Cx. quinquefasciatus* larvae and adults. Unexpectedly, conidia cultivated on wheat bran were not strongly pathogenic toward *Cx. quinquefasciatus* larvae as compared with approximately the same dose of conidia cultivated on wheat powder.[9] Less virulence may be related to the kind of substrate used for fungal production as previously discussed.[25] However, the pathogenicity of the conidia toward adult mosquitoes was not decreased as compared with previous results, where 86.0± 1.7% mortality at day 5 was obtained with 7.9×10^7 conidia/mL.[8] The sunflower oil used for the fungal formulation facilitated the conidial adhesion on the adult cuticle as Neem oil against *Cx. quinquefasciatus*.[8] The observation of dead adults and larvae confirmed that the mosquito mortality was due to the conidia that, respectively, adhered to and penetrated the cuticle or invaded the larval gut. In the latter case, larval death may be due to toxins secreted during germination in the gut of larvae, as the digestive tract was previously reported to be a significant infection site.[26,27]

Our results are similar to those of other studies that showed the toxicity of fungal metabolites against mosquitoes.[2,28,29] *A. clavatus* metabolites were found to be toxic toward adults and larvae with significant mortality rates after 72 hr. Regarding the application approach, metabolites could act by contact with adults and also by ingestion by larvae. Another study revealed that metabolites of fungi, such as *Tolypocladium inflatum* (Hypocreales: Ophiocordycipitaceae), could attack mosquito larvae and be the main cause of histopathological damages.[30] *A. clavatus* germination was associated with the secretion of some toxins.[31–33] These suggest that metabolites produced by the fungus act on mosquito tissues including the midgut of larvae.[9,30,34] These metabolites must be purified and their chemical structure identified. Moreover, the mechanism of the action of these metabolites must be defined on non-target organisms before their use for general biocontrol in the field.

Conclusion

The present study shows the high potential utility of fungal production in a liquid phase continuously recirculated on a metal structured packing in a bioreactor. This new system was developed for fungal growth and metabolite production, combining the technological advantages of submerged and solid-state fermentations. The process allowed facility in recuperation and purification of conidia (confined on the solid substrate) and metabolites (contained in the liquid medium). *A. clavatus* conidia and metabolites were virulent and could be a promising alternative to chemical control of mosquito larvae and adults. Therefore, it is necessary to isolate and show the effect on non-target organisms before use. These results also highlight the possibility to control the culture parameters for further studies. The homogenization of these physical traits in the packed structure, as well as their impact on conidia and metabolites production, must now be investigated.

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