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Semiselective isolation of the ethanol-imbibing sooty mould *Baudoinia* of distillery aging warehouses

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Abstract: *Baudoinia compniacensis* is a darkly pigmented microfungus that grows conspicuously on environmental surfaces around warehouses where alcoholic spirits are stored in wooden casks. This fungus has long been ignored because its primary isolation is very difficult. The present study describes a new semiselective isolation medium for this fungus based on the use of ethanol as a sole carbon source and low levels of nitrogen and trace elements.

Key words: Baudoinia compniacensis, alcohol dehydrogenase, ethanol, spirit maturation, sooty mould, warehouse staining.

Résumé : *Baudoinia compniacensis* est un champignon microscopique à pigmentation foncée qui croît ostensiblement sur des surfaces environnementales autour des entrepôts où des spiritueux sont gardés dans des fûts de bois. Ce champignon a longtemps été ignoré car il est très difficile à isoler. La présente étude décrit un nouveau milieu d'isolement semi-sélectif pour ce champignon, basé sur l'utilisation d'éthanol comme seule source de carbone et sur de faibles niveaux d'azote et d'éléments traces.

Mots-clés : Baudoinia compniacensis, alcool déshydrogénase, éthanol, maturation des spiritueux, fumagine, maculage d'entrepôts.

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Baudoinia compniacensis (Richon) J.A. Scott & Unter., the black fungus responsible for "warehouse staining" in the spirits industry, appears to grow only where the environment is exposed to ethanolic vapors transpired from stored wooden barrels of distilled spirits. It was described as *Torula compniacensis* Richon in 1881 (Richon and Petit 1881), but since then, it has remained almost completely unstudied, probably because of its nondescript morphology and its failure to grow on most artificial media used in primary isolation. Attempts to isolate a specialized organism into pure culture often require the testing of various types of media. We investigated which media best supported the primary isolation and further cultivation of *B. compniacensis*. Our results led to the development of a novel medium that greatly facilitates work with this organism.

Media

Modified Leonian's agar (MLA) (Malloch 1981) was used as a general purpose cultivation medium. This medium was prepared by adding to 1.0 L of distilled water the following ingredients: 6.25 g maltose, 6.25 g malt extract (Bioshop, Burlington, Ontario, Canada), 0.63 g $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 0.63 g Bacto peptone (Difco, Becton Dickinson & Co., Sparks, Maryland), 1.0 g yeast extract (Difco), 0.05 g chloramphenicol, and 15.0 g agar (Sigma-Aldrich, St. Louis, Missouri) for solid media.

After extensive physiological investigations (Ewaze et al. 2007), a semiselective medium was devised for isolating *Baudoinia* J.A. Scott & Unter. from environmental materials. This medium was prepared by adding carbon and nitrogen stock solutions to a basic stock solution of trace elements plus 0.05 g of chloramphenicol and 15.0 g of agar. The composition of the stock solutions is given below.

Trace element stock solution (100×)

A mixture was made consisting of 50 mL each of the following stock solutions: 50 $mg \cdot L^{-1}$ $CoCl_2$, 40 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 12 $mg \cdot L^{-1}$ $FeCl_3 \cdot 6H_2O$ chelated with 17 $mg \cdot L^{-1}$ Na_2EDTA, 620 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 30 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, and 920 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$. The mixture

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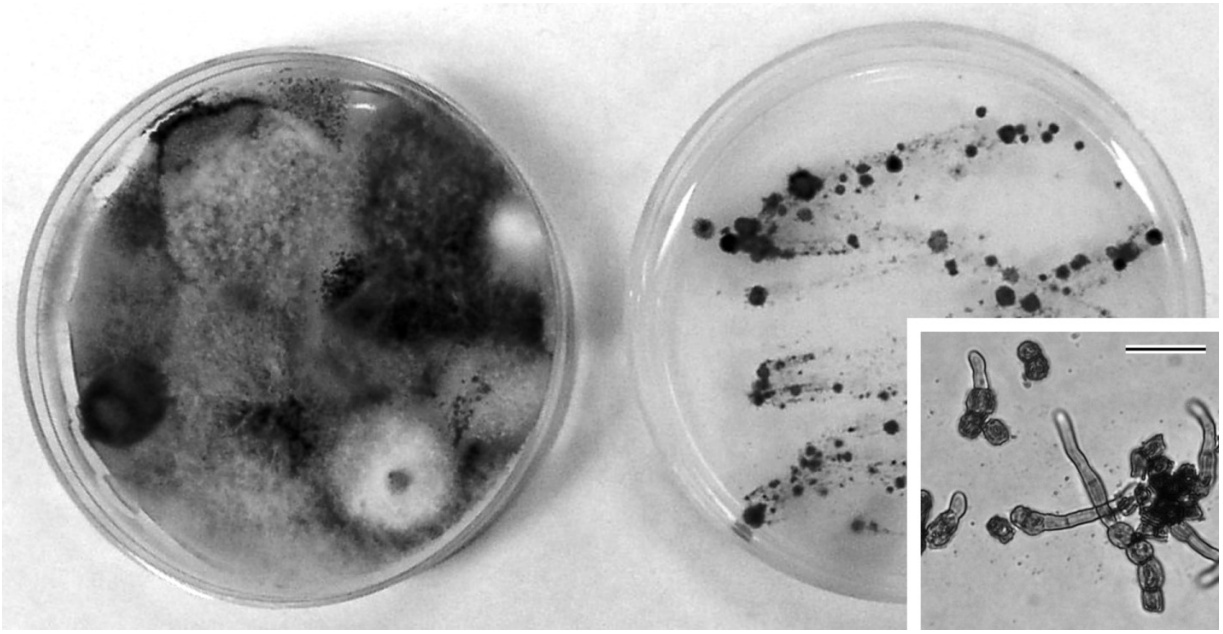
Fig. 1. Environmental swab streak samples grown for 18 days at 26 °C on 2 media: modified Leonian's agar with chloramphenicol (left) and semiselective medium (right). Inset shows cell germination and developing microcolony on the surface of semiselective medium at 2 days. Bar = 10 μm.



was made to a total volume of 1 L with distilled water. It was added to all growth media at a rate of 10 mL·L^{-1} prior to autoclaving.

Nitrogen stock solution

The nitrogen source in the medium consisted of 5 mmol·L^{-1} NH_4NO_3. It was added to the growth medium prior to autoclaving. A variation with a 5 mmol·L^{-1} NH_4Cl nitrogen source was also tested but proved equivalent (data not shown).

Carbon stock solution

Ethanol (95%), calculated to yield a final concentration of 3% (v·v^{-1}) in the medium, was added to cooled autoclaved medium under axenic conditions immediately before plates were poured. Variations with 1% and 2% ethanol were also tested (data not shown).

Isolation trials

Environmental materials consisting of scrapings from barrel staves, galvanized tin roofing, a fire hydrant, and polyvinyl chloride guttering were collected from a Caribbean distillery using a sterile cotton swab and streaked onto test media. Five plates of each type of medium was streaked for each sample. The media used were the nonselective MLA and the new semiselective medium. Plates were incubated at 26 °C for 18 days. The surfaces of the plates were examined over time using compound microscopy at low power (e.g., 100×) to locate germinated conidia morphologically conforming to *Baudoinia*. Pure cultures of *Baudoinia* were obtained by transferring germinated conidia to fresh medium using a sterile, finely pointed tungsten needle. The identities of pure cultures were verified by sequencing of nucSSU and nucITS regions (data not shown).

Swab streaks onto MLA invariably yielded a heavy growth of common environmental fungi, such as species of *Alternaria* Nees:Fr., *Aureobasidium* Viala & G. Boyer, *Aspergillus* Fr.:Fr., *Cladosporium* Link, *Epicoccum* Link, *Paecilomyces* Bainier, *Penicillium* Link, and *Pithomyces* Berk. & Broome (Fig. 1, left). These fungi appeared within 4–5 days and covered plate surfaces completely. The overgrowth effectively prevented isolation of the slow growing *B. compniacensis*, though it was seen growing on the medium. By contrast, our semiselective medium in all cases showed dominant growth of *Baudoinia* and weak growth of *Cladosporium* (Fig. 1, right). Conidia of *B. compniacensis* from environmental materials could be seen to germinate after 2 days of incubation at 26 °C on our semiselective medium (Fig. 1, inset).

Similar isolation attempts using this semiselective medium were carried out on environmental materials from other sites worldwide and gave results essentially indistinguishable from those seen here, whenever *Baudoinia* was present in the materials sampled (data not shown).

Once isolated, *B. compniacensis* could be subcultured to MLA and other conventional laboratory media and generally showed good growth, though at its normal, very slow rate (Ewaze et al. 2007).

Many fungi thrive on MLA, but it is a relatively rich medium that may promote excessive mycelial growth. *Baudoinia compniacensis* is difficult to isolate from environmental substrata on MLA because it grows much more slowly than the cosmopolitan fungi co-occurring with it, as either niche inhabitants or as sedimented dormant propagules. It is likely that this overgrowth problem, combined with the possibility that *B. compniacensis* can be confused with *Aureobasidium pullulans* chlamydospores or black *Aspergillus* conidia in direct examination, has strongly contributed to the lack of study on this dramatic and locally infamous fungus, which conspicuously coats inert and arboreal surfaces with a sooty

layer in the vicinity of distillery warehouses (Scott et al. 2007). Under current sociopolitical conditions, the heavy growth of black fungal material in response to industrial emissions can become controversial, and this fungus, though apparently long accepted as a fact of life in ancient distillery regions like Cognac, France, is now regarded as a potential public relations problem, particularly when neighbouring houses, businesses, gardens, and parks exhibit heavy sooty mould growth. Currently, the implications of this growth for persons with asthma and other allergic problems have not been investigated. Further study of this organism and its control is clearly needed, and development of a semiselective isolation procedure is a necessary step.

We have shown that *B. compniacensis* can be efficiently isolated on a nutritionally depauperate medium, with relatively low amounts of carbon, nitrogen, and salts. Ethanol is readily utilized by *B. compniacensis*, which has the necessary enzymes to degrade this material (Ewaze et al. 2008[2]). *Baudoinia compniacensis* also utilizes glucose (Ewaze et al. 2008[2]), but ethanol concentrations of 3% tend to select against co-occurring environmental fungi in an isolation medium, as seen in the present study, making use of ethanol preferable.

Apart from use in distillery warehouse isolations, our semiselective medium may also enable the discovery of the currently unknown primary environmental habitat of *B. compniacensis*.

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