Ethanol physiology in the warehouse-staining fungus, *Baudoinia compniacensis*

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**Abstract**

The fungus *Baudoinia compniacensis* colonizes the exterior surfaces of a range of materials, such as buildings, outdoor furnishings, fences, signs, and vegetation, in regions subject to periodic exposure to low levels of ethanol vapour, such as those in the vicinity of distillery aging warehouses and commercial bakeries. Here we investigated the basis of ethanol metabolism in *Baudoinia* and investigate the role of ethanol in cell germination and growth.

Germination of mycelia of *Baudoinia* was enhanced by up to roughly 1 d exposure to low ethanol concentrations, optimally 10 ppm when delivered in vapour form and 5 mM in liquid form. However, growth was strongly inhibited following exposure to higher ethanol concentrations for shorter durations (e.g., 1.7 mM for 6 h). We found that ethanol was catabolized into central metabolism via alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ACDH). Isocitrate dehydrogenases (IDHs) were active in cells grown on glucose, but these enzymes were not expressed when ethanol was provided as a sole or companion carbon source. The glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase (MS) activities observed in cells grown on acetate were comparable to those reported for other microorganisms. By replenishing tricarboxylic acid (TCA) cycle intermediates, it is likely that the functionality of the glyoxylate cycle is important in the establishment of luxuriant growth of *Baudoinia compniacensis* on ethanol-exposed, nutrient-deprived, exposed surfaces. In other fungi, such as *Saccharomyces cerevisiae*, ADH II catalyses the conversion of ethanol to acetaldehyde, which then can be metabolized via the TCA cycle. ADH II is known to be strongly repressed in the presence of glucose.

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**Introduction**

Tourists visiting distillery facilities in France where cognac and armagnac are held for ageing are likely to be told about “la part des anges” (“the angels’ share”), the annual 2% loss of ethanol by distilled spirits stored in traditional wooden casks. At the same time, they are shown a black fungus that forms a conspicuous, sooty covering wherever these escaping ethanol vapours contact nearby walls, fences, tree trunks, and other exterior surfaces. Despite the constant attention, the fungus involved in this growth has received almost no scientific study since it was described as *Torula compniacensis* (literally, “the torula from Cognac”) in 1881 (Richon & Petit 1881). A significant obstacle has been that the microscopically nondescript and very slow growing fungus is difficult to obtain in pure culture, tending to be overgrown by competitors. Recently, the organism was isolated into pure culture from several locations around the world, and was investigated by nuclear ribosomal gene sequencing. It was revealed as belonging to an undescribed genus in the order Capnodiales.
This genus was named Baudoinia, and the fungus was redesignated B. compniacensis. Investigation on the biosystematics of B. compniacensis was coupled with physiological studies, including ones on basic growth and specific responses to ethanol (Ewaze et al. 2007). Baudoinia was found to use ethanol, but not other simple alcohols, as a carbon source for growth; however, it proved to be equally capable of growing on ethanol-free medium using glucose as its carbon source. In addition, ethanol was shown to activate stress response proteins overlapping with those activated by heat shock (Ewaze et al. 2007). It was suggested that this activation of survival responses by ethanol might aid the establishment and growth of Baudoinia in the relatively extreme, bare, sun-exposed habitats where it is often seen around spirit maturation warehouses.

In the present study, further investigation was conducted on the metabolism of ethanol in Baudoinia, as well as on the positive and negative effects of various ethanol levels on growth and colony establishment. The ultimate objective was to study the basis of ethanol metabolism in Baudoinia and investigate the role of ethanol in cell germination and growth, with the goal of disclosing information that could be used to control the growth of this conspicuous organism around aging warehouses.

Materials and methods

Isolates used

Baudoinia compniacensis isolates UAMH 10762 (University of Alberta Microfungus Collection, Edmonton, AB, Canada) and UAMH 10839 were isolated from tree bark and a concrete wall, respectively, near a bond warehouse in Windsor, ON. Isolate UAMH 10764 was from a concrete wall near a distillery warehouse in Loretto, KY. Voucher materials of the fungi on which the paper is based have been permanently preserved in the University of Alberta Microfungus collection and Herbarium (UAMH).

Preparation of inoculum

Stock cultures of the isolates were grown on 90 mm Petri dishes containing modified Leonian's (ML) agar (Malloch 1981). Plates were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI) and incubated inverted for 14 d at 26 °C. These stock plates were used for the preparation for mycelial inocula. Mycelial suspensions for liquid cultures were made by flooding the plate surface with sterile water and gently scraping surface mycelia into suspension. Suspensions were axenically filtered to remove large mycelial aggregates by passage through gauze in a thistle funnel covered with aluminum foil. The short mycelial fragments in the resulting suspension were enumerated and their concentration adjusted in distilled water until haemocytometer counts of $5 \times 10^5$ fragments ml$^{-1}$ were obtained as a standard to be used for all experiments. The fungal suspensions were stored at 4 °C for 4–24 h until use. Fresh material was prepared in the same manner for all experiments.

Media

ML agar was used as a general purpose cultivation medium. This medium was prepared by adding to 1 l distilled water the following ingredients: 6.25 g maltose, 6.25 g malt extract 0.63 g (BioShop, Burlington, ON), MgSO$_4$ 7H$_2$O, 1.25 g KH$_2$PO$_4$, 0.63 g Bacto peptone (Difco, Becton Dickinson, Sparks, MD), 1 g yeast extract (Difco), and 15 g agar (Sigma-Aldrich, St Louis, MO) for solid media.

A completely defined medium, Baudoinia physiological medium (BPM), was devised for use in physiological studies. This medium was prepared by the addition of carbon and nitrogen stock solutions to a basic stock solution of trace elements. The composition of the stock solutions is given below. Stock solution A: trace elements (× 100 concentration). A mixture was made consisting of 50 ml each of the following stock solutions: 50 mg l$^{-1}$ CoCl$_2$, 40 mg l$^{-1}$ CuSO$_4$ 5H$_2$O, 12 mg l$^{-1}$ FeCl$_3$ 6H$_2$O, 17 mg l$^{-1}$ Na$_2$EDTA, 620 mg l$^{-1}$ MnCl$_2$ 4H$_2$O, 30 mg l$^{-1}$ Na$_2$MoO$_4$ 2H$_2$O, and 920 mg l$^{-1}$ ZnSO$_4$ 7H$_2$O. The mixture was made to 1 l total volume with distilled deionized (DDI) water. It was added to all growth media at a rate of 10 ml l$^{-1}$ prior to autoclaving. Stock solution B (nitrogen source): nitrogen source stock solutions of NH$_4$Cl or NH$_4$NO$_3$ were added to media, as required, to a final concentration of 5 mM. Stock solution C (carbon source): a stock solution of 95 % (v/v) ethanol was added to cooled autoclaved culture medium under axenic conditions immediately prior to inoculation to a final concentration governed by the particular experiment. When acetate or glucose was used as a carbon source, it was added at the desired concentration to growth media prior to autoclaving. For carbon sources, either 5 mM acetate or 50 mM glucose were used. Acetate added in the form of ammonium acetate (CH$_3$COONH$_4$), served as sole carbon and nitrogen source. Media were dispensed into sterile 125 ml Erlenmeyer flasks.

Growth measurement

For fungal growth measurement in different nutrient solutions, cultures were incubated at 26 °C unless otherwise stated, on a rotary shaker operating at 120 rev min$^{-1}$, using Gyro and Recipro Shake (Model: SK-300/SK-600, Jeio Tech Co., Ltd., Seoul, South Korea). At the end of the growth period, the contents of each flask were filtered through pre-weighed, 0.8 mm A/F glass fibre filter membranes (Gelman Sciences, Ann Arbor, MI). The filter membranes bearing mycelium were washed thoroughly with distilled water and dried at 60 °C to constant weight. Dry weight was measured using an electro balance (AD-6, Perkin Elmer, Waltham, MA) equipped with a Polonium-210 antistatic ionizing strip. Each nutrient variable was tested in duplicate, at minimum.

Growth in ethanol medium

Mycelial suspension (2 ml) of isolates UAMH 10762, UAMH 10839, and UAMH 10764 were prepared as described under ‘preparation of inoculum’ above, and grown in 50 ml defined medium with NH$_4$Cl or NH$_4$NO$_3$ as the sole nitrogen source and different concentrations of ethanol at 11, 22, 43, 87, and 170 mM (= 0.063, 0.125, 0.25, 0.5 and 1 % (v/v), respectively) as

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(Scott et al. 2007). This genus was named Baudoinia, and the fungus was redesignated B. compniacensis.
sole carbon source. At 7 d, the mycelia were harvested, filtered, washed, and weighed as detailed in the previous section.

Tolerance of elevated ethanol concentrations. Mycelial suspensions were prepared as described under ‘preparation of inoculum’, above, and incubated 6 d in ML broth at 26 °C on a rotary shaker to obtain exponential growth. The medium containing actively growing mycelium was filtered through sterile gauze in a sterile funnel to remove large pieces and the mycelial fragment suspension in the filtrate was collected in a small test tube. Inoculum was adjusted to 5 × 10⁶ fragments ml⁻¹ using a haemocytometer. Inoculum of UAMH 10762 was transferred to 50 ml of ML broth plus different concentrations of ethanol: 0, 500, 900, 1600, 1700, and 2400 mM [−0, 3, 5, 9, 10 and 14 % (v/v), respectively]. Cultures were incubated on an orbital shaker at 26 °C and sampled at 0, 3, 6, and 8 h to assess survival on ML medium. At each sampling time, two flasks for each treatment were removed from the shaker and swirled to resuspend the material; a 200 μl aliquot, calculated to contain 1000 cfu, was removed and plated on an ML agar plate. The plates were incubated 14 d at 26 °C and the number of out-growing colonies was counted. The very restricted, slow-growing, black-pigmented nature of the Baudoinia colonies facilitated accurate counting.

Stimulation of cells by pulse exposure to low ethanol concentrations. Inoculum of UAMH 10762 prepared as for the cell viability experiment was transferred to flasks containing 50 ml of different, relatively low concentrations of ethanol (0, 2.5, 5, 10, and 22.5 mM). The purpose was to determine whether short-term exposure to ethanol had a particularly strong potentiating effect on formation of new colonies in later incubation on ethanol-free medium. Aliquots of 200 μl, calculated to contain 1000 cfu, were removed from each flask after 20 h and inoculated onto ML agar. The plates were incubated at 26 °C and the number of the colonies was counted after 14 d.

Effect of ethanol vapour on dormant cell germination. Five 2 l Erlenmeyer flasks each containing 1 l distilled water, were autoclaved, and different concentrations of ethanol were added to the cooled water under axenic conditions. Using the Henry’s law constant for ethanol in aqueous solution (5 × 10⁻⁵ atm m⁻³ molecule⁻¹ at 25 °C) (Gaffney et al. 1987), ethanol concentrations were calculated to obtain ethanol vapour levels in the head space ranging from 0.1–100 ppm; the actual volumes of 95 % ethanol added to the water were 1.23, 12.3, 123 and 1230 μl. Aliquots of 10 μl calculated to contain 300 cfu of UAMH 10762 were applied to the surface of mixed cellulose ester filter membranes (0.45 μm pore size, 25 mm; SKC, Eighty Four, PA). Each filter was affixed with a pin onto the lower surface of a Neoprene stopper (Fisher Scientific, UTE Tech Park, Singapore), these stoppers were then inserted firmly into the flasks, and covered with Parafilm to avoid ethanol loss. The flasks were incubated stationery for 14 d at 26 °C.

At the end of the incubation period, the filter membranes were removed from the stoppers and dried at room temperature. To facilitate counting the small colonies that had formed on the filters, optical clearing of the filters was performed as per the NIOSH Manual of Analytical Methods (NIOSH 1994), using an acetone vaporizing unit (Wonder Makers Environmental, Kalamazoo, MI). Each filter was placed on a clean slide, which was placed on a cool polypropylene block positioned at the outlet of an aluminum retort (hot block) heated to 70 °C. A 1 ml aliquot of acetone was injected into the hot block with slow, steady pressure on the plunger button while holding the pipette firmly in place. Condensation of the acetone vapour on the membrane rendered the membranes transparent. A drop of clear lactic acid and a glass cover slip were applied to the surface of the cleared membrane. Microcolonies were counted using LM.

**Protein electrophoresis studies**

**Preparation of cell-free extracts for protein studies.** A weighed quantity of Baudoinia inoculum, mixed with a small quantity of perlite baghouse fines (Dicaperl Minerals, Thomason, MA), was ground with a pestle in an ice-cold mortar containing 100 μl Tris–HCl extraction buffer. The crude extract obtained was filtered through filter paper (Whatman No.1) and maintained at 4 °C prior to use.

**Proteins elicited by growth on ethanol medium.** Mycelia of UAMH 10762 and UAMH 10839 grown for 7 d at 26 °C in control ML broth and in 43 mM (0.25 %) ethanol plus 5 μM NH4NO3 and trace elements. Cell-free extracts were prepared as outlined above.

For electrophoresis, 60 μl of cell-free extract was loaded per well. Proteins were separated by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) using a 12 % resolving gel and 4 % stacking gel run in a Mini Protein® 3 Cell (BioRad, Hercules, CA). All steps were carried out at 4 °C at 200 V for 40 min. A detailed analysis of the bands produced was obtained by scanning the gels and analysing the images with the software Scion Image for Windows (Scion, Frederick, MD). Bands were scored as present/absent and strong/weak based on the graphical readouts produced by this reading method (not shown).

**Alcohol dehydrogenase (ADH, NAD form, EC 1.1.1.1).** All three study isolates were grown in a medium consisting of 43 μM ethanol, 5 μM NH4NO3 and trace elements at 26 °C for 7 d on a rotating shaker at 120 rev min⁻¹. As a positive control, Saccharomycetes cerevisiae, in the form of commercial packaged yeast, was grown 7 d on Sabouraud glucose medium (in 1 l distilled water: 50 g glucose, 3 g NaNO3, 1 g K2HPO4, 0.5 g MgSO4 7H2O, 0.5 g KCl, and 0.01 g FeSO4 7H2O). A weighed quantity of yeast, mixed with a small quantity of fine perlite, was ground with a pestle in an ice-cold mortar containing 100 μl Tris–HCl extraction buffer, pH 7.5. The crude extract was filtered through filter paper (Whatman No.1) and maintained at 4 °C prior to use. The Baudoinia and Saccharomycetes cerevisiae extracts were partially purified in an Amicon Ultra 4 centrifugal filter device (Millipore, Billerica, MA).

For electrophoresis, 60 μl of treated cell-free extract was loaded per well. ADH-NAD bands were determined using discontinuous native PAGE as described by Ornstein & Davis (1964). A 10 % resolving gel and 4 % stacking gel were run in a MiniProtein® 3 Cell. All steps were carried out at 4 °C at 200 V. Gels were run until the tracking dye was about 1 cm from the bottom. The gels were stained for ADH-NAD⁺ isozyme activity, according to Wendel & Weeden (1989). They were incubated in 100 ml of reaction mixture consisting of 50 μM Tris–HCl, pH 8, mixed with 1 μM ethanol (substrate),
1 mM NAD\(^+\), 0.7 mM phenazine methosulphate (PMS), and 0.37 mM nitroblue tetrazolium (NBT). Incubation was at 37 °C for 5 min in the dark with gentle rocking and held for an additional 5 min to develop the colour. The reaction was stopped by rinsing the gel with water.

Acetaldehyde dehydrogenase (ACDH, NAD form, EC 1.2.1.4). As above, the three Baudoinia compniacensis isolates were grown in a mixture consisting of 43 mM ethanol, 5 mM NH\(_4\)NO\(_3\), and trace elements at 26 °C for 7 d on a rotating shaker at 120 rev min\(^{-1}\). Mycelia were extracted in 100 mM potassium phosphate buffer at pH 7 containing 2 mM EDTA.

For electrophoresis, 60 μl of treated cell-free extract was loaded per well. ACDH-NAD bands were determined by discontinuous native PAGE as detailed in the previous section. All steps were carried out at 4 °C to 200 V for 50 min. The gels were stained for ACDH-NAD\(^+\) activity by incubating them in 100 ml of reaction mixture. The mixture consisted of 100 mM potassium phosphate buffer (pH 7.6) containing 70 mM acetaldehyde (substrate), 1 mM NAD\(^+\), 0.1 mM PMS, and 0.5 mM NBT. Staining was done overnight to develop the colour, as described by Jendrossek et al. (1987). The reaction was stopped by rinsing the gel with water.

Isocitrate dehydrogenase-NAD\(^+\) (IDH; EC.1.1.1.41) and IDH-NADP\(^+\) (EC.1.1.1.42)

The three Baudoinia compniacensis isolates were grown in two carbon source media, M1 broth, as well as BPM with an ammonium acetate carbon and nitrogen source. Media were incubated at 26 °C for 7 d on a rotating shaker at 120 rev min\(^{-1}\). The mycelia was extracted in a buffer consisting of 100 mM Tris-HCl and 2 mM EDTA buffer at pH 8.

For electrophoresis, 60 μl of treated cell-free extract was loaded per well. IDH-NAD and IDH-NADP bands were determined by discontinuous native PAGE as described above. All steps were carried out at 4 °C to 200 V for 50 min. The gels were stained for IDH-NAD\(^+\) activity using discontinuous native PAGE as described by Soltis et al. (1983), by incubating them in reaction mixture, consisting of: solution A: 50 mM Tris-HCl, pH 8 (15 ml) containing 200 mg agarose (used as an optical enhancer to increase the clarity of stained bands); solution B: 50 mM Tris-HCl, pH 8 (15 ml) containing, 35 mM MgCl\(_2\), 1 mM NAD\(^+\) (alternatively, 0.4 mM NADP\(^+\) was used to test for the IDH-NADP\(^+\) isozyme), 25 mM isocitrate-Na-salt, 0.8 mM NBT, and 0.4 mM PMS. Solution A was first boiled and cooled to 60 °C. Solution B was gently mixed in and the mixture was poured into a gel form so that it covered the existing gels. Once the agarose in the staining mixture had solidified, gels were incubated in dark at 30 °C until blue bands appeared.

Malate synthase (MS, EC 4.1.3.2) and Isocitrate lyase (ICL, EC 4.1.3.1). The three Baudoinia compniacensis isolates were grown on BPM with ammonium acetate and nitrogen source at 26 °C for 7 d on a rotating shaker at 120 rev min\(^{-1}\). For MS enzyme assay, the mycelia was extracted in 50 mM Tris-HCl buffer, pH 7.5. For the ICL assay, the mycelia was extracted in 50 mM potassium phosphate buffer, pH 7. Both enzyme assays were conducted at 26–28 °C in a total volume of 2 ml.

MS activity was assayed by the method of Ono et al. (2003). The MS assay mixture contained 100 mM Tris-HCl (pH 8), 0.1 mM acetyl-CoA, 10 mM MgCl\(_2\), 1 mM glyoxylate, and cell-free extract. The reaction was initiated by the addition of glyoxylate. The disappearance of acetyl-CoA was monitored with a Genova MK3 Life Science Analyser spectrophotometer (Jenway, Dunmow, Essex) at 232 nm.

ICL activity was again assayed by the method of Ono et al. (2003). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 30 mM d,l-isocitrate, 10 mM MgCl\(_2\), 2 mM dithiothreitol, 4 mM phenylhydrazine and cell-free extract. The reaction was initiated by addition of isocitrate. The increase in absorbance resulting from the formation of the phenylhydrazone derivative of glyoxylate was assayed spectrophotometrically at 334 nm.

**Protein determination**

Cell-free extract was analysed for protein quantity, e.g. prior to gel loading, using the method described by Lowry et al. (1951).

**Results**

**Ethanol growth**

Different patterns of ethanol utilization were displayed by the three test Baudoinia compniacensis isolates (Fig 1). On one or more of the two ammonium-based nitrogen sources, isolates UAMH 10762, UAMH 10764, and UAMH 10839 demonstrated a growth peak with ethanol at a concentration of 43 mM. At that concentration, on medium with NH\(_4\)Cl, the total biomass yield per 50 ml medium was relatively high for UAMH 10762 and UAMH 10839 at 4 and 3.5 mg, respectively, and was distinctly lower for UAMH 10764 at 1.5 mg. Growth in all treatments diminished substantially above 170 mM ethanol (Fig 1). No growth was observed for all three isolates on 5 % ethanol with NH\(_4\)Cl.

**Tolerance of elevated ethanol concentrations.** UAMH 10762 demonstrated survival rates of 81, 65, and 36 %, respectively, after 6 h incubation at 520, 870, and 1700 mM ethanol (3, 5, and 10 %, respectively; Fig 2). Twenty percent ethanol was completely lethal at both time intervals. Six percent survival was observed at 2.4 mM (14 %) ethanol for 3 h incubation, and no survival was observed after a 6 h incubation at 3.5 mM (20 %) ethanol (data not shown).

**Stimulation of cells by pulse exposure to low ethanol concentrations.** Volatile organic compounds, such as ethanol, are known to act on fungi as triggers for germination (Fries 1973; Hasen 1980; Zeutzen et al. 1988). Although this experiment was initiated using inoculum in exponential phase, the inoculum was mycelial in nature and in filamentous fungi, only apical cells of mycelium are actively growing, in the sense of extending faces to prepare inoculum is expected to have removed a mixture of apical and non-apical cells. The question addressed in the present experiment was whether relatively brief exposure to low ethanol concentrations might stimulate some otherwise partially or completely dormant cells, e.g. non-apical cells, to grow and produce colonies rapidly on subsequent incubation in ethanol-free medium. Isolate (UAMH 10762) was...
used to determine the optimal ethanol concentration to induce outgrowth from mycelial fragments. Based on these assays we observed a trend towards increased colony formation following ethanol pretreatment at a concentration of 5 mM in aqueous solution for 18–24 h (Fig 3).

Effect of ethanol vapour on germination of dormant mycelial cells. Isolate UAMH 10762 was used to find the optimal ethanol vapour concentration triggering the germination of the typical dark, heavy-walled, chlamydospore-like cells composing mycelial fragments. Optimal germination was observed with 10 ppm of ethanol vapour (Fig 4). When 100 ppm of ethanol was used, germination was not stimulated but rather inhibited to 32% lower than control levels (0-value on the ordinate of Fig 4).

Protein studies

Proteins elicited by growth on ethanol medium. In discontinuous SDS-PAGE gel electrophoresis, more than 28 protein bands were observed for untreated mycelium grown for 7 d on ML broth (Fig 5). Cells grown 7 d on 43 mM ethanol medium proved to have strongly different banding patterns for total protein than those seen for control cells.

ADH. For each Baudoinia compniacensis isolate, one distinct band was identified on gel stained for ADH-NAD activity with ethanol used as substrate (Fig 6). The variation in ADH-NAD band intensity among the B. compniacensis isolates was influenced by the actual presence of ADH activity, as well as the concentration of cell-free extracts applied to the gel.

Various carbon sources were investigated for their influence on ADH activity (Fig 6). A distinct band for ADH-NAD dependent enzyme activity was observed for commercial yeast grown on glucose (in the constituent glucose of Sabouraud broth; data not shown), as well as for Baudoinia isolates grown on 43 mM ethanol with 5 mM NH₄NO₃. In contrast, no such band was detected for UAMH 10839 grown on glucose, in this case supplied as a component of ML broth (data not shown). Similarly, no corresponding band was seen for UAMH 10839 when grown in ML broth with 43 mM ethanol (data not shown).

ACDH. One band was identified for ACDH-NAD activity for the three test isolates grown on 43 mM ethanol with 5 mM NH₄NO₃ (Fig 6). Acetaldehyde-free control gels showed no bands.
IDH, ICL, and MS. Isolates UAMH 10839 and UAMH 10762, showed an intense single band for both IDH-NAD (EC: 1.1.1.41) and IDH-NADP (EC: 1.1.1.42) when grown on ML broth inclusive of its glucose carbon source (Fig 6). Only a faint band was seen for both enzymes with UAMH 10764. The differences in band intensities seen in the figures reflect the amount of protein applied to the gel. Control gels in which isocitrate substrate was absent showed no activity bands. No isozyme activity corresponding to IDH-NAD or IDH-NADP was detected in the three *Baudoinia compniacensis* isolates grown in medium with acetate, a non-fermentable carbon source (Fig 6).

When ICL (EC: 4.1.3.1) and MS (EC: 4.1.3.2) activity levels were examined in the three *B. compniacensis* isolates grown on acetate medium as a carbon source (Table 1), moderately high activities were observed for both enzymes. ICL activity in UAMH 10839 was twice as high as in UAMH 10762 and four times as high as in UAMH 10764. Isolates UAMH 10839 and UAMH 10672 showed higher ICL activity than MS activity, but UAMH 10764 had ICL activity lower than its MS activity.

**Discussion**

Given the known association between *Baudoinia compniacensis* and habitats exposed to ethanol (Scott et al. 2007, Ewaze et al. 2007), it is perhaps surprising that 870 mM (5 %) ethanol completely suppressed the growth of this fungus. This tolerance level is well below that recorded for most alcohol-producing species of *Saccharomyces* (Casey & Ingledew 1986). We also observed that 2.4 M (14 %) ethanol reduced the viability of *Baudoinia* by 95 % in 3 h. This same reduction in viability of a strain *S. cerevisiae* required a significantly higher concentration of ethanol, 3.6 M (21 %) (Casey & Ingledew 1986). Therefore, we consider *Baudoinia* to be relatively intolerant of ethanol, in contrast to *S. cerevisiae*.

Isolate UAMH 10764 exhibited a lower growth rate than the other isolates when grown on ethanol. This observation may be related simply to a correspondingly lower rate of ADH II protein expression in this isolate, as the trend of the curve is the same for the three isolates. It is noteworthy that isolates UAMH 10762 and UAMH 10839 are genetically highly similar.
and were isolated from same area (Scott et al. 2007). Isolate UAMH 10764, although also highly similar, was obtained from a distant geographic locality.

The ethanol concentration most stimulatory to Baudoinia was 43 mM, while 20 h pulse exposure to a much lower concentration, 5 mM, proved optimal in inducing colony formation by mycelial fragments. When ethanol vapour was investigated, optimal germination of dormant Baudoinia mycelia was seen at a low level, 10 ppm. These results suggest that ethanol may play a stimulatory role in the growth of Baudoinia above and beyond its potential function as a source of metabolic carbon. Currently, it is unclear whether growth of Baudoinia in the vicinity of distillery warehouses could be diminished in direct proportion to the reduction of ethanol emission levels from the warehouses, or if even minute levels of ethanol emission would be sufficient to trigger the observed levels of growth of this fungus. If ethanol functioned mainly as a carbon source, any reduction in emission should lead to a corresponding reduction in the growth of Baudoinia. However, if low levels of ethanol triggered colony establishment even where ethanol levels were insufficient to provide all the carbon needed for growth, then only the reduction of ethanol to very low levels would be likely to reduce the environmental proliferation of Baudoinia.

The results obtained on optimal ethanol levels for germination and growth of Baudoinia can be used in practical work. For example, preliminary studies suggest that the amendment of growth medium with 5 ppm ethanol greatly increases the success of isolation from environmental materials (Scott et al. 2007).

Although ethanol can function as a factor modifying the properties of the cell membrane and influence growth that way (Mishra & Prasad 1989; Ceccato-Antonini & Sudbery 2004), positive growth responses usually indicate usage as a respiratory substrate, perhaps via activation of ADH. The normal fungal pathway for ethanol metabolism is via the ADH enzyme ADH II. Ethanol tends to integrate into common metabolic pathways via the intermediates acetate and acetaldehyde (Russell et al. 1983). In S. cerevisiae, ADH II preferentially catalyses the conversion of ethanol to acetaldehyde, which then can be metabolized via the tricarboxylic acid (TCA) cycle and also serve as an intermediate for gluconeogenesis (Russell et al. 1983). In S. cerevisiae, ADH II preferentially catalyses the conversion of ethanol to acetaldehyde, which then can be metabolized via the tricarboxylic acid (TCA) cycle and also serve as an intermediate for gluconeogenesis (Russell et al. 1983). Our finding that ethanol-grown Baudoinia forms an ADH II isozyme suggests that our fungus can also oxidize ethanol to acetate. The formation of ADH II by Baudoinia is not stimulated by growth on medium containing glucose, whether or not ethanol is added. In S. cerevisiae also, ADH II is known to be strongly repressed by the presence of simple, fermentable sugars such as glucose (Denis et al. 1981). Our data also showed the presence of ACDH in Baudoinia, suggesting that NAD-dependent ACDH reduces acetaldehyde to carboxylic acid when the fungus is growing on ethanol.

Kornberg & Krebs (1957) first recognized the ability of some microorganisms to grow efficiently on two-carbon
compounds using the glyoxylate pathway. The key enzymes in this pathway are ICL and MS (Kornberg & Elsden 1961). Our earlier work indicated that the normal TCA cycle functions in Baudoinia when glucose is metabolized (Ewaze et al. 2007). The present study showed that the TCA cycle enzyme IDH was active when Baudoinia was grown on glucose medium; it was detected in two forms, IDH-NAD+ and IDH-NADP+. Neither form of the enzyme, however, was found in extracts of acetate-grown mycelium, suggesting that the glyoxylate pathway was being used in conjunction with this two-carbon substrate. The switching on and off of IDH involves reversible phosphorylation mediated by a bifunctional enzyme, IDH kinase/phosphatase. When IDH is phosphorylated, isocitrate is diverted from the normal TCA cycle to the glyoxylate cycle.

The activities of the glyoxylate cycle enzymes ICL and MS in acetate-grown Baudoinia were comparable with those seen in various other microorganisms (McFadden & Howes 1962; Neumann et al. 1969). These results indicate that the glyoxylate cycle probably plays a crucial role in replenishing TCA intermediates, such as gluconeogenesis precursors, when Baudoinia is grown on acetate or ethanol.

The glyoxylate cycle enzymes may be important in functions other than basic metabolism on two-carbon substrates. They have been reported to contribute to protein synthesis in nitrogen-fixing bacteria (Green 1998), riboflavin biosynthesis in Ashbya gossypii (Schmidt et al. 1996), and oxalate biosynthesis in a plant pathogen, Athelia rolfsii (Maxwell & Bateman 1968). Baudoinia compniacensis needs to be investigated to determine the overall contribution of glyoxylate cycle enzymes to its metabolism.

It is likely that the glyoxylate cycle of Baudoinia, conferring the ability to utilize ethanol, is a critical factor involved in the abundant growth of this organism in the nutrient-deprived conditions found on exposed surfaces around distillery warehouses and bakeries. Whether ethanol serves as a major carbon source under these conditions or only as a signal stimulating growth, it is likely that the glyoxylate cycle is involved: the level of simple sugars in the environment is unlikely to be high enough to switch it off.

### References


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