Research Paper

Nutritional requirements and strain heterogeneity in *Ashbya gossypii*

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Colony radial growth rates and specific growth rates of three related *Ashbya gossypii* strains ATCC10895, IMI31268, MUCL29450 and an unrelated strain, CBS109.26, were measured on various carbon and nitrogen sources at pH 4.5 and pH 6.5 to elucidate physiological growth requirements and strain differences. All strains grew on yeast extract or ammonium as nitrogen sources, but not on nitrate. Substantial growth at pH 4.5 was observed only on complex medium. D-Glucose, glycerol and starch were utilised as carbon sources. Ethanol was produced during growth on glycerol. Conversion of xylose into xylitol demonstrates that the xylose reductase is active. Phenotypic differences between related strains were greater than expected. We demonstrate that *A. gossypii* utilizes ammonium as sole nitrogen source at pH 6.5, facilitating further physiological studies using chemically defined media in the future.

Keywords: *Ashbya gossypii* / Nitrogen utilization / Carbon utilization / Colony radial growth rate / Ethanol / pH

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Introduction

The filamentous hemiascomycete *Ashbya gossypii* [1, 2] was one of four species infecting cotton in the British West Indies, first characterised by Nowell (1915) (as cited in Pridham and Raper 1950). The fungus infects the developing lint fibers which become dirty-yellowish and mat onto the seeds. The yellow pigment was later identified as riboflavin.

A. gossypii is a natural producer of riboflavin [3, 4] and is used for industrial riboflavin production [5]. Its genome has been sequenced and annotated [6, 7], revealing an evolutionary relationship with *Saccharomyces cerevisiae*, with remarkable similarities at the synteny level but lacking sequence duplications that are present in *S. cerevisiae*. *A. gossypii* has primarily been studied in the context of riboflavin production [8–13] and as a model to investigate polarized hyphal growth on the molecular level [14–19].

A. gossypii as a riboflavin producer represents an example of environmentally-friendly white biotechnology. Other, more novel applications of *A. gossypii* could be envisioned, for which it is useful to clarify its nutritional requirements, reports of which have been incomplete or contradictory and may reflect strain differences. The ability to utilize polymeric carbohydrates, pentoses and glycerol, which are currently readily available from plant biomass and the biodiesel industry [20], is of particular interest. Although *A. gossypii* is known to grow on glycerol, its ability to produce ethanol from glycerol, converting it into a useful fuel, has not previously been reported.

In this paper, we demonstrate the similarities and differences of three related *A. gossypii* strains, ATCC10895, MUCL29450, IMI31268 and the more distantly related CBS109.26 and assess their ability to grow in chemically defined medium on various carbon and nitrogen sources, in order to gain insights on *A. gossypii* physiology. ATCC10895 and MUCL29450 were deposited at ATCC and MUCL as the same strain, and both should thus be derived from the parent strain ATCC8717, also deposited as IMI31268. The sequenced strain ATCC10895 and derivatives of it are now the most commonly used *A. gossypii* strains.

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Materials and methods

Strains

The four *Ashbya gossypii* (sometimes also referred as *Eremothecium gossypii*) strains used in this study were ATCC 10895 (kindly provided by Prof. P. Philippsen, University of Basel, the sequenced strain), MUCL29450 (equivalent of ATCC10895, CBS109.51, or NRRL Y-1056), IMI31268 (equivalent of ATCC8717, the parent strain of ATCC10895, isolated from cotton) and CBS109.26, an independent strain of *A. gossypii* isolated from Asclepias fruit in Trinidad and Tobago.

Media

YP medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone), defined medium [21] with minor modifications as indicated, synthetic complete (SC) defined medium, containing complete amino acid supplement [22] and yeast nitrogen base (Difco) and *Ashbya* Full Medium (AFM; 10 g l^{-1} peptone, 10 g l^{-1} yeast extract, 1 g l^{-1} myo-inositol plus a carbon source) were used for growth of *A. gossypii*. D-Glucose, glycerol, starch, carboxymethyl cellulose (CMC) (20 g l^{-1}), D-xylose or L-arabinose (10 g l^{-1}) were provided as carbon sources. Ammonium sulphate in Verduyn defined medium was in some cases replaced with potassium nitrate (5 g l^{-1}) or yeast extract (5 g l^{-1}).

When used for submerged growth, SC medium was supplemented with 1 g l^{-1} $CaCO_3$ for buffering and 1 g l^{-1} agar to facilitate filamentous growth. For measurement of colony radial growth rate, media were solidified with 20 g l^{-1} agar. The initial pH of agar-solidified defined media was adjusted to 4.5 or 6.5.

Inoculum preparation

Strains MUCL 29450, IMI31268 and CBS 109.26 did not sporulate on any of the media used in this study, nor on a variety of other fungal sporulation media. Therefore, all strains were preserved as mycelium in glycerol (20% v/v) at –80 °C to provide reproducible inoculum for liquid cultures. ATCC10895, which does sporulate, was also stored as spores in glycerol (20% v/v) at –80 °C. Mycelia were prepared by collecting 8–10 d old mycelia from agar-solidified medium, digesting with zymolyase (150 mg ml^{-1}) for 2 h, and washing with a solution of 0.8% (w/v) NaCl, 20% (v/v) glycerol and 0.025% (v/v) Tween 20. Fragmented mycelia were inoculated into YPD in flasks and allowed to grow (200 rpm. 30 °C) for approximately 20 h. The mycelial suspension was diluted with an equal volume of 40% (v/v) glycerol and frozen in aliquots at –80 °C. Long-term viability of frozen mycelia was not assessed, but mycelia remained viable for at least 2 years.

Culture conditions

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml medium and incubated at 30 °C, 200 r.p.m. Flasks were inoculated with 0.6 ml mycelia which had been stored at –80 °C. Agar-solidified medium was inoculated with 10 µl mycelia at up to three locations per 9 cm diam. Petri dish.

Colony radial growth rate, culture optical density and dry weight

Colony radial growth rates (K_r; [23, 24]) were determined by measuring diameters of colonies growing on agar-solidified medium in 9 cm diameter Petri dishes.

Culture optical density at 600 nm (OD_{600}) was used as a measure of biomass for submerged cultures with dispersed, filamentous growth [25]. The relationship between OD_{600} and mycelial biomass was determined by collecting and washing mycelia on glass fibre GF/C filters and drying at 100 °C. One OD_{600} unit corresponded to 1.3 g l^{-1} DW of *A. gossypii*.

Extracellular substrates and metabolite concentrations

Extracellular substrates and metabolites (ethanol, glycerol, D-glucose, D-xylose, L-arabinose and xylitol) were analyzed by HPLC as previously described [26].

The production of starch degrading enzymes on agar-solidified medium was detected by staining the surface with iodine solution [27] to visualize zones in which starch had been broken down.

Results

Use of inorganic nitrogen by *A. gossypii*

The K_r of *A. gossypii* ATCC10895, MUCL29450, IMI31268 and CBS109.26 was determined on Verduyn defined medium containing $(NH_4)_2SO_4$, KNO_3, or YE as the nitrogen source or lacking a nitrogen source, with the initial pH adjusted to 4.5 or 6.5. Although all strains showed measurable colony expansion on KNO_3 at pH 6.5, the density of mycelium was similar to that on medium lacking nitrogen and clearly represented only background growth on the nitrogen available in the inocula (Fig. 1).

All four *Ashbya* strains in this study grew well on Verduyn defined medium with only ammonium as nitrogen source at pH 6.5 (Fig. 1), but not at pH 4.5, although they grew at both pH values when YE was provided as the nitrogen source (Table 1). Subsequently, experiments with $(NH_4)_2SO_4$ as sole nitrogen source were supplemented with $CaCO_3$ to maintain the pH above 6.0.

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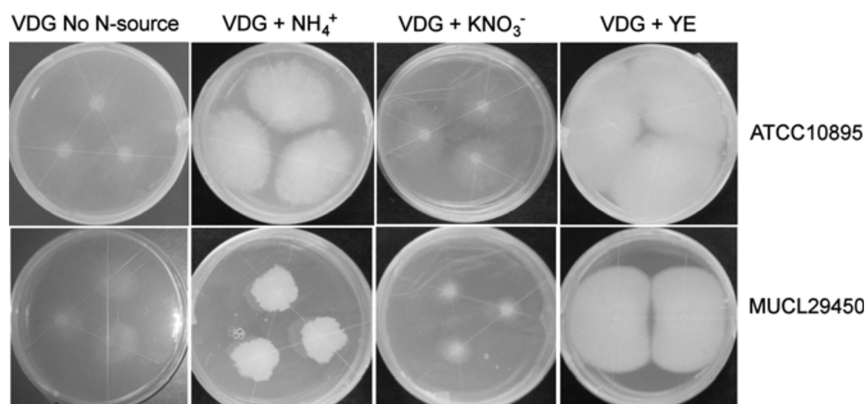

Figure 1. Colonies of *A. gossypii* ATCC10895 and MUCL29450 on chemically defined medium at pH 6.5 containing no nitrogen source, or ammonium, nitrate or YE as nitrogen source. Colonies were incubated for 7 d.

Table 1. Colony radial growth rate (K_r, μm h^{-1}) of *A. gossypii* ATCC10895, MUCL29450, IMI31268 and CBS109.26 on Verduyn defined medium containing $(NH_4)_2SO_4$ or YE as nitrogen sources, and with the initial pH adjusted to 4.5 or 6.5. Values in the same row with the same superscript (a to d) did not differ significantly ($p < 0.05$).

pH	Nitrogen source	ATCC10895	MUCL29450	IMI31268	CBS109.26
6.5	YE	183 ± 4^b	149 ± 2^a	195 ± 4^c	154 ± 3^a
	$(NH_4)_2SO_4$	165 ± 2^c	76 ± 2^a	220 ± 5^d	134 ± 1^b
4.5	YE	152 ± 1^c	117 ± 1^a	160 ± 1^d	137 ± 2^c
	$(NH_4)_2SO_4$*	4 ± 1^a	3 ± 1^a	11 ± 1^b	4 ± 1^a

*sparse growth

The K_r of ATCC10895 was generally lower than that of the parental strain IMI31268, although it was more similar with YE as the nitrogen source (183 ± 4 μm h^{-1} and 195 ± 4 μm h^{-1}, respectively, $p > 0.01$) than with ammonium (165 ± 2 μm h^{-1} and 220 ± 5 μm h^{-1}, respectively, $p < 0.01$, Table 1). The K_r of MUCL29450 was significantly lower ($p < 0.05$) than that of ATCC10895 on all media, indicating that the two strains are phenotypically different. The K_r of CBS109.26 was also lower ($p < 0.05$) than that of ATCC10895 with either YE or ammonium as nitrogen source.

Comparison of strains on different carbon sources

All four strains grew in chemically defined medium with D-glucose, glycerol or starch as carbon sources and $(NH_4)_2SO_4$ as nitrogen source at pH 6.5 (Table 2). As on D-glucose, MUCL29450 had a significantly lower K_r ($p < 0.05$) than ATCC10895 or IMI31268 on both glycerol and starch (Table 2). The K_r of ATCC10895 and IMI31268 also differed. None of the strains grew on CMC, although background growth, comparable to that on medium lacking a carbon source, was observed.

A. gossypii did not grow as well on starch as on D-glucose or glycerol, but produced more biomass than on CMC or medium lacking the carbon source. When grown on starch-containing AFM medium, zones from which starch had been degraded were visible around ATCC10895 (K_r = 69 ± 3 μm h^{-1}), MUCL29450 (K_r = 68 ± 1 μm h^{-1}) and IMI31268 (K_r = 105 ± 5 μm h^{-1}), indicating the secretion of amylase. Amylase activity has also been observed in culture supernatant of ATCC10895 grown

Table 2. Colony radial growth rate (K_r, μm h^{-1}) of *A. gossypii* ATCC10895, MUCL29450, IMI31268 and CBS109.26 on Verduyn defined medium containing D-glucose, glycerol or starch as carbon sources, and with the initial pH adjusted to 6.5. Values in the same row with the same superscript (a to d) did not differ significantly ($p < 0.05$). Cultures were incubated at 30 °C. Data represents the average ± SEM for 12 replicates.

Carbon source	ATCC10895	MUCL29450	IMI31268	CBS109.26
D-glucose	165 ± 2^c	76 ± 2^a	220 ± 5^d	134 ± 1^b
Glycerol	99 ± 2^c	56 ± 1^a	119 ± 3^c	116 ± 4^c
Starch	115 ± 3^d	44 ± 5^a	92 ± 2^b	52 ± 3^a
no C*	41 ± 2^a	–	–	–

*sparse growth
– represents no growth

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on starch in submerged culture (O. Ribeiro, unpublished result). No clearing zone was observed for CBS109.26 on AFM with starch as the carbon source, although it grew (K_r = 71 ± 2 μm h^{-1}), suggesting it differed in amylase production from the other, more closely related strains.

Use of pentose sugars as sole carbon source by *A. gossypii* ATCC10895

A. gossypii ATCC10895 did not grow with either D-xylose or L-arabinose as sole carbon source. When subcultured sequentially from YP or SC medium containing D-xylose or L-arabinose as carbon source to the same medium, growth visibly decreased. Thus, while some yeast and filamentous fungi have improved growth on pentose sugars when they have been previously exposed to the sugars, *A. gossypii* did not. When grown in submerged culture at pH ~6, L-arabinose was not taken up by the mycelium and arabitol was not produced. D-Xylose was converted to xylitol (Fig. 2) with a yield of 0.99 g xylitol [g D-xylose consumed]$^{-1}$, indicating that the xylose reductase (XR) was active. Although some growth was observed (μ = 0.09 ± 0.004 h^{-1}, ~3 g DW l^{-1}; Fig. 2), this resulted from the glycerol present in the inoculum and storage compounds present in the mycelia. In subsequent experiments, mycelial inocula were washed by centrifugation to remove glycerol and reduce the extent of background growth.

Growth and metabolite production in submerged cultures

ATCC10895 and MUCL29450 grew at the same specific rate (0.33 ± 0.02 h^{-1}) in YPD in submerged culture. The specific growth rate of both strains was slightly (p < 0.10) lower than that of their parent strain, IMI31268 (0.39 ± 0.01 h^{-1}). CBS109.26 (0.22 ± 0.01) grew at a significantly (p < 0.05) slower specific growth rate than the other strains.

ATCC10895, MUCL29450 and CBS109.26 produced small amounts (<1 g l^{-1}) of ethanol aerobically from D-glucose in YPD during batch cultures in flasks. Ethanol production was also detected when ATCC10895 was grown in bioreactor batch cultures in AFM. Ethanol was produced (2.5 ± 0.05 g l^{-1}) at a yield of 0.13 g [g glycerol consumed]$^{-1}$ when ATCC10895 was grown in YP with glycerol as carbon source, but not in SC with glycerol (Fig. 3). Specific growth rates of 0.12 ± 0.01 and 0.11 ± 0.003 h^{-1} were observed in YP-glycerol and SC-glycerol media, respectively. The parent strain IMI31268 consumed glycerol at a similar rate to ATCC10895 in SC-glycerol medium (Fig. 3), but had a specific growth rate of 0.20 ± 0.01 h^{-1}.

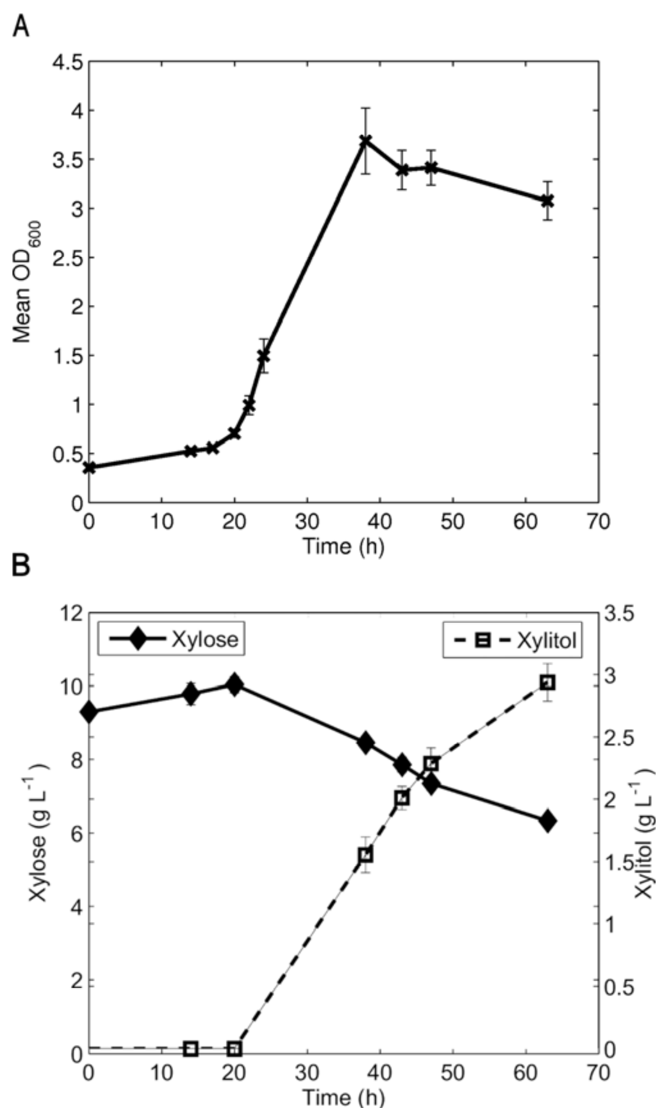

Figure 2. Biomass (A) and xylitol (B) production by *A. gossypii* ATCC10895, and D-xylose consumption (B), in SC medium containing D-xylose (10 g l^{-1}) as carbon source and $(NH_4)_2SO_4$ as nitrogen source buffered with 1 g $CaCO_3$ l^{-1}. Flasks were inoculated with mycelia and filamentous growth was sustained by the addition of 1 g agar l^{-1}. Flasks were incubated at 30 °C, 200 r.p.m. Results are mean ± SEM for 3 cultures.

Discussion

Cultivation media for *A. gossypii* are typically complex (e.g. AFM; [28]) or supplemented with yeast extract or an amino acid [29]. However, here we demonstrate that *A. gossypii* does grow on inorganic nitrogen without supplements. Although Farries and Bell [30], Buston *et al.* [31] and Wright and Philippsen [32] have indicated that *A. gossypii* (strains Ashby and Nowell and ATCC10895) did not grow or grew poorly on ammonium

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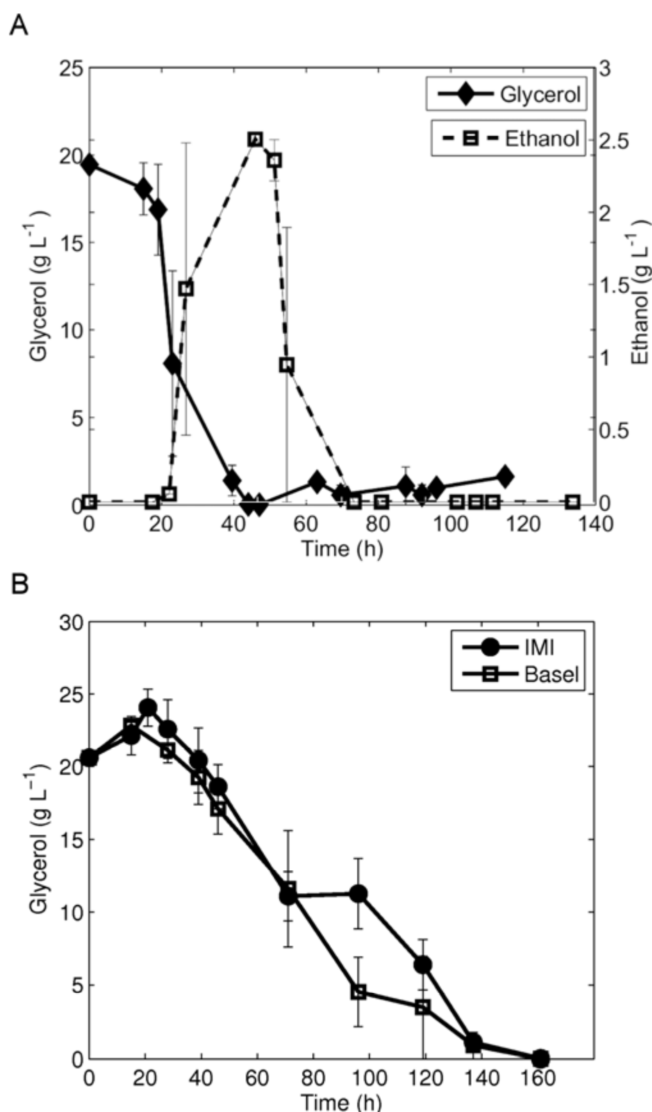

Figure 3. Glycerol consumption and ethanol production of *A. gossypii* ATCC10895 in YP medium (A) and SC medium (B) with glycerol as carbon source in flask cultures incubated at 30 °C, 200 rpm. Error bars represent ± SEM for 3 cultures. Glycerol consumption of IMI31268 in SC medium with glycerol is also shown.

as nitrogen source, we found that all four strains of *A. gossypii* used here grew well on ammonium as sole nitrogen source when the initial pH was 6.5 (Fig. 1). Growth was poor on chemically defined medium at pH 4.5 (Table 1), and associated with very long lag phases in submerged cultures (Yvonne Nygård, VTT Finland, personal communication), indicating that the lack of growth or limited growth previously reported [30–32] probably reflects low medium pH. Growth on ammonium is improved by buffering to reduce the pH decrease as ammonium is taken into the cells.

Ammonium could be used by *A. gossypii* as sole nitrogen source, but nitrate could not (Fig. 1). The *A. gossypii* genome contains genes orthologous to those of *S. cerevisiae* for energy dependent ammonium assimilation (*GLN1* and *GLT1* [33]), including gene sequences encoding both glutamine synthetase (GS) (ACR182C; EC,6.3.1.2), and glutamate synthase (GOGAT) (ADR290W; EC, 1.4.1.13), confirming that *A. gossypii* has the genetic capacity for ammonium assimilation. Although NAD^+ dependent glutamate dehydrogenase (*S. cerevisiae* homolog GDH2) (AGL040C; EC,1.4.1.2) is present, the $NADP^+$ dependent form of the enzyme (*S. cerevisiae* homologs GDH1 or GDH3) is not, suggesting that like the basidiomycete *Agaricus bisporus* [34], *A. gossypii* lacks the glutamate dehydrogenase ammonium assimilation route which is dominant in *S. cerevisiae* [35]. The relative importance of the GS/GOGAT and glutamate dehydrogenase pathways for ammonium assimilation in fungi is species dependent, with the glutamate dehydrogenase pathway dominant in *S. cerevisiae*, *Neurospora crassa*, *Kluyveromyces aerogenes* [36] and *Aspergillus nidulans* [37], but the GS/GOGAT pathway dominant in *A. bisporus* [34], *Candida albicans*, and *Schizoccharomyces pombe* [38–40]. Even in organisms in which the glutamate dehydrogenase pathway is dominant, such as *A. nidulans*, the GS/GOGAT pathway is sufficient for ammonium assimilation in mutants lacking NADP-GDH activity [37]. In contrast, no sequence orthologous to a nitrate reductase is found in the *A. gossypii* genome, explaining the lack of growth on nitrate observed here and by Farries and Bell [30].

As a fungus initially isolated as a pathogen of cotton plants, *A. gossypii* might be expected to produce cellulases, but there are no genes encoding putative cellulolytic enzymes in the genome [6]. Early studies of *A. gossypii* (Ashby and Nowell) similarly found no growth on cellulose [41, 42]. However, Marsh [42] and Tanner *et al.* [1] also reported that *A. gossypii* (Ashby and Nowell) was unable to degrade starch, while Farries and Bell [30] observed a small amount of degradation. Here we observed growth on starch and clear amylolytic activity, although only one gene (AEL276C) containing a putative amylolytic catalytic domain has been identified in the genome.

Although Farries and Bell [30] did not observe growth of *A. gossypii* (Ashby and Nowell) on either D-xylose or L-arabinose, growth on these substrates was re-evaluated here since a putative aldose or D-xylose reductase (XR – ACL107c, EC 1.1.1.21), xylitol dehydrogenase (XDH – ABR229c, EC 1.1.1.9) and xylulokinase (XK – AGR324c, EC 2.7.1.17) are present in the *A. gossypii* ATCC10895 genome (manual annotation). Most fungi which utilise D-xylose as a carbon source do so by reducing D-xylose to xylitol with XR, oxidizing xylitol to D-xylulose with XDH and phosphorylating D-xylulose to xylulose-5-

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phosphate with XK. Although the necessary putative genes are present in the genome, they are not necessarily active, as in *S. cerevisiae*, which does not grow on D-xylose or grows extremely slowly [43, 44]. *A. gossypii* ATCC10895 resembled *S. cerevisiae* in D-xylose metabolism, being able to produce xylitol from D-xylose, but not to grow.

The catabolic pathways of L-arabinose and D-xylose metabolism in fungi share many enzymes [45–47]. L-Arabinose is reduced by a non-specific aldose reductase (EC 1.1.1.21) to L-arabitol, which is converted to xylitol in two consecutive redox steps catalyzed by L-arabitol 4-dehydrogenase (LAD; EC 1.1.1.12) and L-xylulose reductase (LXR; EC 1.1.1.10). The genes encoding LAD and LXR are not present in the *A. gossypii* genome (manual annotation) so that utilization of L-arabinose by *A. gossypii* was not expected. The lack of arabitol production probably reflects poor arabinose uptake under the growth conditions used here.

Although *A. gossypii* ATCC10895 is frequently described as a wild type, it was isolated as a highly pigmented variant of ATCC8717 (equivalent of IMI31268) and clearly differed from this strain in specific growth rate and in colony radial growth rate on various carbon or nitrogen sources. K_r is specific for specific strains in specific environments [23, 48] and reflects a combination of specific growth rate and branch density. In addition, although MUCL29450 was originally deposited as ATCC10895, it clearly differed phenotypically from the strain sequenced as ATCC10895 and showed greater differences from the parental strain IMI31268 than ATCC10895. MUCL29450 and ATCC10895 were more similar in submerged culture, where the same specific growth rate was observed for both (0.34 ± 0.02). Each of these strains has been maintained in independent culture collections for an extensive time, and it should be noted that the parent IMI31268 may also show phenotypic divergence from its equivalent strain ATCC8717.

The specific growth rate of 0.33 ± 0.02 h^{-1} observed here for ATCC10895 in YPD was comparable to that expected of *S. cerevisiae* in complex medium. It was higher than previously reported specific growth rates of *A. gossypii*, which have been between 0.02 h^{-1} (NRRL1363/ATCC12995; [49, 50]) and 0.16 h^{-1} (ATCC10895 and derivatives; [51], including that of an ATCC10895 transformant (0.09 ± 0.00 h^{-1}) in AFM in flasks [52]. However, we have observed similar rates in other cultivations (e.g. 0.32 h^{-1} in YPD and 0.40 h^{-1} in AFM, unpublished data). Specific growth rates were slower with glycerol than with D-glucose as carbon source.

Since glycerol is becoming a considerable waste product of biodiesel production, the ability of *A. gossypii* to convert glycerol to ethanol is interesting. Although the yield of ethanol on glycerol was only 0.13 g [g glycerol consumed]$^{-1}$, it was comparable to that achieved with genetically engineered *S. cerevisae* (2.4 g l^{-1} at a yield of 0.14 g [g glycerol consumed]$^{-1}$ with a strain overexpressing glycerol dehydrogenase and dihydroxyacetone kinase [53]. Thus non-recombinant *A. gossypii* is as efficient at converting glycerol to ethanol as engineered *S. cerevisiae*, but would need further improvement to achieve the yields observed in bacterial systems.

A. gossypii strains grew well on chemically defined medium at high pH (e.g. 6.5), but had greater sensitivity to low pH than many filamentous fungi. D-glucose, glycerol and starch were all utilised as carbon sources and high specific growth rates (comparable to those of *S. cerevisiae*) were achieved in complex medium. Although D-xylose was not used as a carbon source, the presence of active D-xylose reductase was demonstrated.

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