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SHORT CONTRIBUTION

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Isolation and characterization of a *Trichoderma* strain capable of fermenting cellulose to ethanol

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Abstract The direct fermentation of cellulosic biomass to ethanol has long been a desired goal. To this end, we screened the environment for fungal strains capable of this conversion when grown on minimal medium. One strain, identified as a member of the genus *Trichoderma* and designated strain A10, was isolated from cow dung and initially produced about 0.4 g ethanol l^{-1}. This strain cannot grow on any substrate under anaerobic conditions, but can ferment microcrystalline cellulose or several sugars to ethanol. Ethanol accumulation was eventually increased, by selection and the use of a vented fermentation flask, to 2 g l^{-1} when the fermentation was carried out in submerged culture in minimal medium. The highest levels of ethanol, >5.0 g l^{-1}, were obtained by the fermentation of glucose. Little ethanol was produced by the fermentation of xylose, although other fermentation products such as succinate and acetate were observed. Strain A10 was also found to utilize (aerobically) a wide range of carbon sources. In addition, auxotrophic mutants were generated and used to demonstrate parasexuality by complementation between auxotrophs and between morphological mutants. The ability of this strain to use a wide variety of carbohydrates (including crystalline cellulose) combined with its minimal nutrient requirements and the availability of a genetic system suggests that the strain merits further investigation of its ability to convert biomass to ethanol.

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Introduction

The production of fuel ethanol from biomass has been considered a laudable goal because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity. In most processes currently under study, cellulosic biomass is first enzymatically hydrolyzed to sugars (often with fungal cellulases), which microorganisms (usually yeast) can then ferment to ethanol. To lower the costs associated with this pretreatment, it is desirable to accomplish the fermentation of cellulose to ethanol in one step. In this process, termed consolidated bioprocessing (CBP) or direct microbial conversion (DMC), all the steps of the conversion are carried out by one microorganism or a consortium of microorganisms in a single bioreactor (Lynd et al. 1999). Most work on CBP has concentrated on the potential of anaerobic bacteria to convert cellulosic biomass to ethanol. However, some filamentous fungi hold promise in this area, and there are some advantages to fungi as the mediators of biomass conversion. They do not require strictly anaerobic conditions and so can be directly inoculated onto cellulosic biomass, and their filamentous growth habit facilitates separation of cell mass from the broth. Also, many fungal strains produce copious numbers of conidiospores (conidia), which could be useful for inoculation at a high level, making the inoculation of non-sterile biomass more practical. Several filamentous fungi have been reported to directly ferment cellulose to ethanol, though on rich, undefined media. These include members of the genera *Aspergillus*, *Rhizopus* (Skory et al. 1997), *Monilia* (Gong et al. 1981), *Neurospora* (Deshpande et al. 1986), and *Fusarium* (Singh and Kumar 1991).

In this study, environmental samples were screened for organisms capable of producing ethanol from cellulose on minimal medium. We have isolated a filamentous fungus capable of this conversion, and have made some progress toward increased ethanol yields and development of a genetic system based on parasexuality.

Materials and methods

Media and growth conditions

Rich medium (yeast-peptone-malt extract; YPM) was as described by Skory et al (1997). Minimal medium (MM) was the yeast nitrogen base medium of Wickerham and Burton (1948), without added vitamins. Unless otherwise noted, the pH of MM was adjusted to 5.0 and carbon sources were added at 5 g l^{-1} for aerobic growth and 50 g l^{-1} for anaerobic fermentation. Sigmacell 20 microcrystalline cellulose (Sigma, St. Louis, Mo., USA) was used as the cellulose source. To periodically check for contamination, cultures were observed by phase-contrast microscopy and streaked onto common bacterial growth media LB agar, M9 medium solidified with agar, and trypticase soy agar (Atlas 1996). Optimal growth temperature was determined by inoculating 5 ml of YPM with conidia and incubating, without shaking, at various temperatures. After 91 h, the mycelial mass was collected by filtration onto a tared glass-fiber filter (GF/D, Gelman Sciences, Ann Arbor, Mich., USA), dried at 105 °C overnight, and weighed. To determine the optimal pH range, flasks containing 50 ml of MM/glucose adjusted to pH 3.8–5.5 were inoculated with conidia (~ 1×10^5), and incubated at 30 °C without shaking. At 50 h the dry weight of the mycelia was determined as above. Potential carbon and energy sources were tested at concentrations of 5 g l^{-1} in 5.0 ml YNB. No attempt was made to control the pH. Tests were performed in 5.0 ml liquid medium and the culture samples were incubated aerobically at 30 °C without shaking. Positive results were scored as clearly visible growth within 5 days after inoculation of conidia.

Fermentation

Anaerobic fermentation of cellulose and other substrates was tested using two growth methods. In the first (sealed-flask) method, medium (20 ml in a 25-ml Erlenmeyer flask) was simply inoculated with conidia (~1×10^5) and the culture vessel was sealed with either a butyl stopper or screw-cap. Thus ~20% of the culture flask volume was left as air space. The O_2 in this space allowed mycelia to develop before the flask became anaerobic and fermentation began. In the second (vented-flask) method, the culture flask was prepared as in the first method but the stopper used to seal the flask was vented through an inserted 26-gauge needle capped with a 3-ml syringe barrel packed tightly with cotton.

To increase the mass of mycelia used to initiate cellulose fermentation, the cultures were allowed to undergo one or two rounds of pre-growth. For this, MM/cellulose (also 20 ml in a 25-ml Erlenmeyer flask) was inoculated with conidia and the culture incubated with a loose-fitting cap without shaking for 3 days. This resulted in heavy growth just up to the time at which conidia were formed. Then, the culture supernatant was aseptically decanted away from the dense, stable mat of mycelia and residual cellulose on the bottom of the flask. New MM broth with no carbon source was then added to a total final volume of 20 ml. This was either repeated for a second round of pre-growth or the flask was fully stoppered and anaerobic conditions were allowed to develop. Ethanol and other fermentation products were analyzed in culture supernatants by HPLC (Weimer et al. 1991).

Mutagenesis

Mutants were generated by UV irradiation of conidia washed (with MM) off the surface of 7-day-old YNB glucose plates. This conidial suspension (2 ml) was transferred to an empty sterile petri dish and placed 5 cm under a 4 W, 256 nm ultraviolet lamp (model UVG-11, UVP, Upland, Calif., USA) for 10 min. This resulted in about 10^1- to 10^3-fold loss in viability as compared with non-irradiated conidia. Irradiated conidia were decimally diluted and plated onto YPM. After 3 days of growth, well-separated colonies from the end of the dilution series were subcultured onto new plates and then onto MM/glucose. The growth requirements of the strains unable to grow on MM were determined by the method of Holliday (1956).

DNA isolation and sequencing

DNA from strain A10 was prepared by the method of Chow and Käfer (1993), originally developed for *Aspergillus*. The internal transcribed spacer (ITS) region was PCR-amplified using the primers ITS4 and ITS5 of White et al. (1990). Amplification reactions were performed in a volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X, ~0.5 µg PCR product template DNA (above), 0.25 mM each dATP, dCTP, dGTP, and dTTP, 5 mM $MgCl_2$, 10 U of *Taq* DNA polymerase (Promega, Madison, Wis., USA), and 50 pmol of each primer (ITS4 and ITS5). Amplification was carried out in an Applied Biosystems GeneAmp PCR system thermocycler programmed for 29 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by one cycle of 72 °C for 5 min and 4 °C for infinity. The PCR product was separated from primers by gel electrophoresis, eluted using spin columns (Ambion, Austin, Tex., USA) and then ethanol-precipitated. The same primers were used for cycle sequencing reactions using the Applied Biosystems PRISM BigDye system and the Applied Biosystems GeneAmp PCR system thermocycler according to the manufacturer's directions, except that the thermocycler was programmed for one cycle of 95 °C for 3 min, 35 cycles of 95 °C for 20 s, 45 °C for 30 s, and 60 °C for 2 min, followed by one cycle of 72 °C for 7 min and 4 °C for infinity. Reactions were resolved on the Applied Biosystems 377XL automated DNA sequencer by the University of Wisconsin Biotechnology Center (Madison, Wis., USA).

Cross-feeding test

Potential cross-feeding between auxotrophic mutants was tested in a U-tube apparatus. The U-tube was formed from two sterile 10-ml syringe barrels held vertically in a ring stand. Two short lengths of sterile tubing connected each barrel to a 0.2-µm syringe filter held at the bottom of the resultant connection. In addition, each syringe barrel was fitted with a stopper pierced with a 20-gauge needle. The top portion of each barrel assembly was kept sterile with a shroud of sterile foil. To fill the apparatus, a third (complete) syringe was used to force minimal medium (10 ml) into one of the syringe barrels through the needle in the stopper. Additional pressure filled both halves of the apparatus approximately equally. As a control, an apparatus was set up in which the syringe filter separating the two halves was replaced by a direct tubing connection. Each syringe barrel was inoculated by addition of an agar plug of actively growing (<48 h) mycelial cultures of two auxotrophs, one into each barrel. This was then incubated for 48 h at room temperature. After this time some small residual growth of mycelia was seen to have accumulated around the agar plug used for inoculation. A syringe was then used to force most of the medium from one syringe barrel to the other and then to flow back again. In this way the medium was mixed and small mycelial fragments could be seen to have fallen to the bottom of the U-tube near the syringe filter or direct connection. The stoppers and needles were then discarded and the tops of the syringe barrels covered with sterile foil. The apparatus was then incubated for an additional 7 days after which growth was evaluated.

Results

Strain isolations

In an effort to obtain novel fungal strains capable of fermenting cellulosic material, 56 strains of fungi were isolated from environments rich in cellulose, such as the

dung of ruminants or rotting wood. These strains were all isolated on MM with either cellobiose or cellulose as the carbon source. When cellobiose was used, chloramphenicol was often added (10 µg ml^{-1}) to suppress bacterial growth. Each strain was either collected from a different source or was determined to be morphologically distinct from all other strains isolated from the same sample. Forty-one of the strains were found to clearly utilize cellulose on MM, and two of these were found to produce measurable amounts of ethanol when incubated anaerobically in screw-capped tubes containing MM/cellulose. One of these, strain A10, originally isolated on cellulose from cow dung, initially produced ~0.4 mg ethanol l^{-1}. Therefore, A10 was chosen for further study, and has been deposited in the NRRL culture collection as NRRL31396. This strain grew well on cellulose in both liquid and solid medium. MM/cellulose plates inoculated with conidia in the center produced mycelia over the entire surface within 5 days, but did not produce any growth when cellulose was omitted. No zone of cellulose clearing could be seen on solid medium, suggesting that large amounts of extracellular cellulase were not secreted.

Taxonomy/physiology

Strain A10 was found to grow rapidly and, when mature, to produce dark green conidia on minimal medium (YNB/glucose), and yellow-green to dark green conidia on rich medium (YPM), often in concentric rings in response to light. Co-incubation of A10 with different isolates on several different media did not result in the appearance of microscopically observable zygospores although exhaustive testing for a sexual phase was not conducted. The conidiophores (conidia-bearing hyphae) can be seen in Fig. 1A. Observation by light and scanning electron microscopy showed that the conidia were smooth-walled (Fig. 1B) and ~3.0 µm in diameter. The morphology of A10 suggests that it is a member of the section Trichoderma of genus *Trichoderma* (Bissett 1991; Gams and Bissett 1998), although identification based solely on morphology can be doubtful (Samuels 1996). To clarify the taxonomy of strain A10, a portion of the rRNA gene cluster useful in species delineation of *Trichoderma* was selected (Gams and Meyer 1998; Dodd et al. 2000). The sequenced region (GenBank accession number AY094141) was 259 nucleotides long and included ITS1 and a portion of the 5.8S ribosomal RNA gene, and was found to be identical to the homologous regions of several strains of *T. aureoviridae* and *T. harzianum*, based on a BLAST search of GenBank (Altschul et al. 1997). Other species of *Trichoderma* were found to be similar but not identical. The morphology of strain A10 is most consistent with *T. harzianum*, and is therefore probably the species of this isolate (Gams and Bissett 1998). Maximum growth was observed at 30–35 °C (Fig. 2A). This strain clearly prefers acidic medium (Fig. 2B), as the best growth occurred at the lowest pH

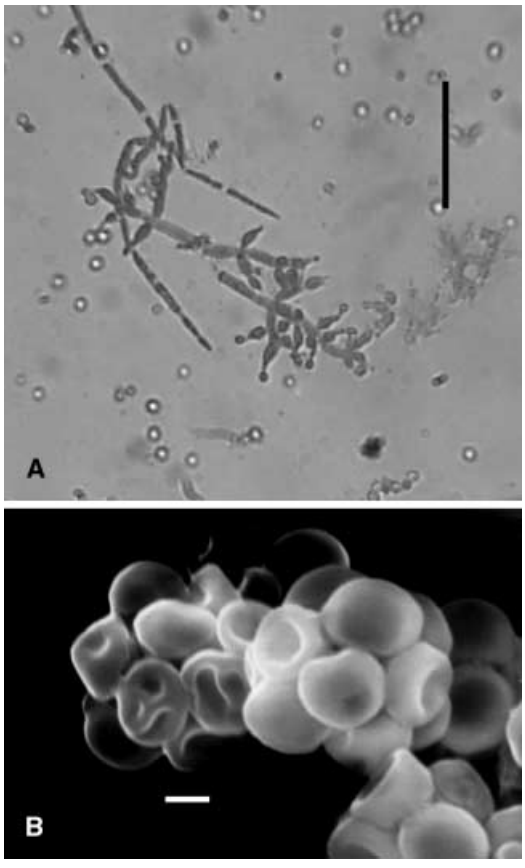

Fig. 1 **A** Conidiophores of isolate A10 showing conidia and phialide (conidia-bearing hyphae) arrangement, from a 3-day-old culture on aerobic solid glucose minimal medium. *Bar* Approximately 50 µm. **B** Scanning electron photomicrograph of non-fixed, air-dried conidia. Samples were Au sputter-coated and visualized with a JEOL 35CF microscope at an accelerating voltage of 25 kV. *Bar* 1.0 µm

range tested, pH 3.2–3.8. The following compounds supported clear growth after 5 days of aerobic incubation: D-galactose, D-mannose, D-xylose, D-arabinose, D-ribose, maltose, cellobiose, sucrose, lactose, trehalose, melibiose, larchwood xylan, tobacco xylan, inulin, polygalacturonic acid, purified citrus pectin, starch, methyl glucoside, ethanol, glycerol, D-sorbitol, Na acetate, and Na citrate. Somewhat poorer growth (observed within 10 days) was supported by: L-rhamnose, L-arabinose, and methanol. Of all the compounds tested, only L-mannose (and a control without any added carbon source) failed to support aerobic growth. No growth was observed under strictly anaerobic conditions on cellulose, cellobiose, glucose or xylose.

Ethanol production

When initially isolated, strain A10 produced only about 0.4 g ethanol l^{-1} when fermentation was by the sealed-flask method in MM with 50 g cellulose l^{-1} added. Ethanol production by filamentous fungi under anaerobic conditions is relatively unusual. Frequent checks for bacterial contami-

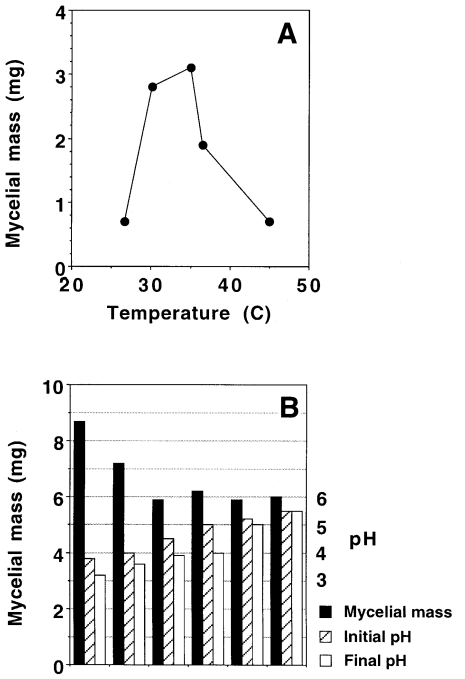

Fig. 2 **A** Mass of strain A10 incubated at various temperatures for 91 h. Results are mean values of duplicate cultures. **B** Growth of strain A10 within various pH ranges. Flasks containing 50 ml of minimal glucose medium were initially pH-adjusted as indicated, inoculated and incubated aerobically at 30 °C for 50 h; the final pH and dry weight of the mycelia were then determined

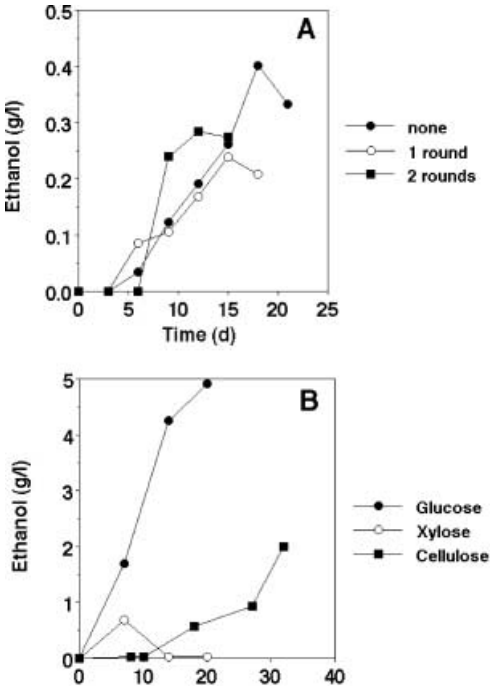

Fig. 3A, B Ethanol production on various carbon sources. **A** Ethanol production from fully anaerobic cultures inoculated with and without pre-growth as described in Materials and methods**. B** Ethanol production from vented cultures grown on different carbon sources as indicated. Each point represents the ethanol yield of a separate culture harvested at the time indicated

nation both by microscopy and plating onto various media consistently failed to reveal bacterial contamination.

Because strain A10 was not found to actively grow under anaerobic conditions, it was thought that ethanol production might be increased by pre-growth to enhance the initial amount of mycelia used in the fermentation. Pre-growth effectively increased the mass of mycelia used to initiate fermentation. As can be seen in Fig. 3A, little difference was observed between cultures simply inoculated, and those started with one 3-day pre-growth cycle. However, two pre-growth cycles (3 days each) resulted in an increase in the rate of ethanol production, although not the final concentration. Thus the size of the inoculum was not deemed a means to significantly increase ethanol production. Substantial amounts of residual carbohydrate remained after all fermentations. To test if CO_2 accumulation might have decreased ethanol production, fermentations were performed using vented flasks. This was found to significantly increase ethanol production from cellulose (Fig. 3B). Fermentations were also carried out with glucose and xylose, using the vented-flask method. The greatest amount of ethanol was observed with glucose as the substrate, and the least with xylose.

Genetic system

In an effort to develop a genetic system for strain A10, auxotrophic and morphological mutants were generated and parasexuality was investigated. Following mutagenesis, three auxotrophic and two morphological mutants were obtained. The auxotrophic strains required arginine (arg$^-$, strain A217), adenine or hypoxanthine (ade$^-$/hpx$^-$, strain A277), or inositol (ino$^-$, strain B429). The two morphological mutants (strains A28 and B462) both produced extremely compact colonies, and the latter strain produced a soluble extracellular yellow pigment. To test for parasexuality, plugs of the arg$^-$ and ade$^-$/hpx$^-$ mutants (strains A217 and A277, respectively) were placed next to each other on YPM, as in Fig. 4A. After 3 days the mycelia had grown together, and three plugs were taken from the area of contact and placed on MM/glucose. Controls were also performed in which each mutant was grown separately. Growth occurred only from mycelia taken from the area of mutant contact, with none from the control (Fig. 4A). Subsequently, nearly identical results were obtained using mutant pairs A277 (ade$^-$/hpx$^-$)+B429 (ino$^-$), and A217 (arg$^-$)+B429 (ino$^-$). This suggests the probable formation of heterokaryons in the manner of parasexuality as first described by Pontecorvo (1956). A region of hyphal contact can be seen in Fig. 4B and probably represents the initiation of anastomosis. The two auxotrophs were grown on YPM and observed microscopically just when the two mycelia began to touch. Heterokaryons thus formed were allowed to form conidia which were then subsequently diluted and plated. It was found that nearly all the resultant conidia germinated into mycelia that had phenotypes like

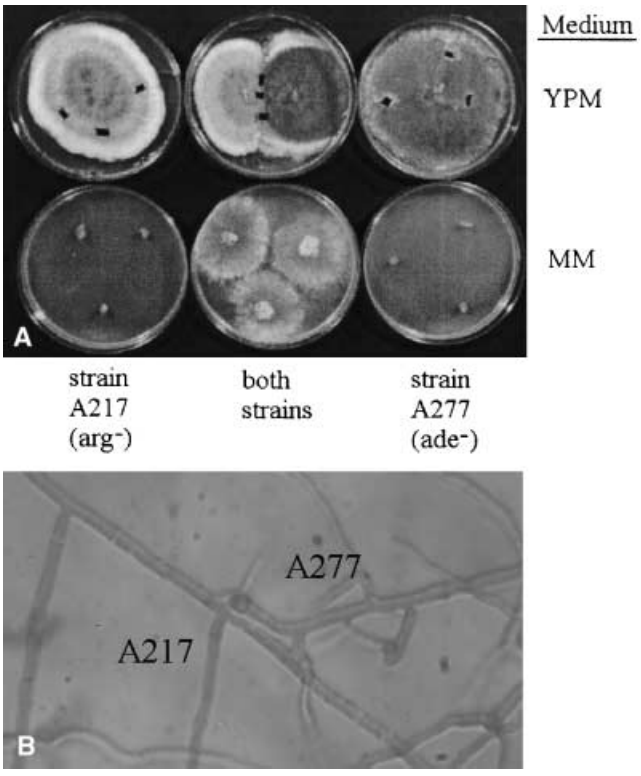

Fig. 4A, B Complementation of auxotrophs of strain A10. **A** *Top row* 3-Day-old plates of rich medium, with an arg⁻ mutant (A217) on the *left* and an ade⁻/hpx⁻ mutant (A277) on the *right*. *Center* Both mutants grew in proximity and can be seen to have grown together. *Bottom row* Plate of minimal medium inoculated with plugs taken from the plates above; growth is only from plugs taken from the region of contact between the two mutants. **B** Photomicrograph of strains A217 (growing in from *left*) and A277 (growing in from *right*). A probable region of hyphal anastomosis can be seen in the *center*. Agar plugs of these strains were deposited approximately 1 cm apart on rich (YPM) medium in a thin layer deposited on a microscope slide and incubated in a moist chamber about 24 h until mycelia were observed to touch. The culture was then stained with crystal violet and photographed

one or the other parental strains. The few prototrophs that were found could be explained as having originated from either mycelial fragments of the original heterokaryon or perhaps as cases of anastomosis occurring between adjacent germinating conidia (these few colonies were always seen at the lowest dilution and therefore the most crowded plates). Only after six to eight rounds of subculturing did strains develop that produced nearly 100% prototrophic conidia (as seen by germination and significant outgrowth under the dissection microscope), indicating that diploidization or recombination had occurred. This procedure was also used to complement two morphological mutants (data not shown), as well as the three crosses of auxotrophs described above.

A U-tube experiment was carried out to test the possibility that cross-feeding was responsible for the complementation between strains A217 (arg⁻) and A277 (ade⁻/hpx⁻). Results showed that complementation occurred only when hyphae came into contact, not when separated by a filter, ruling out cross-feeding as a possibility.

Discussion

Several characteristics of strain A10 are desirable in an organism used for the biological production of ethanol: (1) aerobic growth and anaerobic fermentation both occurred in minimal media; (2) the low pH which this organisms favors (Fig. 2B) reduces the potential for contamination by ethanol-utilizing bacteria; and (3) the organism is amenable to genetic manipulation via parasexuality. The lack of a genetic system has been a major impediment to the use of several species of bacteria for CBP (Lynd et al. 1999). With strain A10, UV mutagenesis produced approximately one auxotrophic mutant for each 50–100 viable conidia (data not shown) indicating that mutant generation was highly practical for this strain. The relative ease with which mutants were obtained from UV-irradiated conidia suggests that the conidia were predominantly uninucleate, as has been observed for other *Trichoderma* strains (Toyama et al. 1984; Stasz et al. 1989). The fact that parasexual fusion occurs readily (Fig. 4) should be of great value in the genetic manipulation of this strain, perhaps to improve the fermentation capabilities or production of extracellular cellulases. For the development of a complete genetic system in fungi it is important that nuclear fusion (karyogamy) or other recombination occur following heterokaryon formation to produce the diploid state. Some strains of *Trichoderma* are very reluctant to do this, and instead maintain the heterokaryon state (Stasz and Harman 1990). When uninuclear conidiophores from such heterokaryons were harvested and germinated, nearly all were of one or the other parental phenotypes. However, a strain of *T. pseudokoningii* was found to exhibit a much higher degree of recombination after hyphal anastomosis (Bagagli et al. 1995). We found that while the frequency of recombination in heterokaryons of strain A10 is very low, it does occur as evidenced by the formation of wild-type mycelia following plating or microscopic observation of conidia after repeated subculturing and subsequent dilution of conidia. Not all crosses produced these prototrophic conidia after the same number of generations, but most occurred, or were observed, after six to eight rounds of subculture.

During the course of this study ethanol yields from strain A10 were significantly increased. While improvements in cultivation and media certainly were partially responsible for this improvement, strain improvement may have also played a part. Each time a fermentation was carried out the strain was re-isolated from the fermentation broth and used for the next fermentation. In this way seven sequential cultivations were carried out – all of which could have selected sub-populations having improved fermentation characteristics. Perhaps the most important factor in the increase of ethanol yield was the use of vented culture as opposed to strictly anaerobic incubation. The mechanism of this improvement is not clear, but it could have been due to the additional O_2 available during initial growth. During the first 3 days or so, a fully stoppered culture was usually found to be un-

der a partial vacuum. Not until incubations exceeded 6 days did the pressure become positive, due to the generation of CO_2. Therefore, vented cultures may have had an advantage in that more O_2 was available in the first few days of incubation. However, increasing biomass density alone played little role in increasing ethanol production, as pre-growth only increased ethanol production over the first few days, and then only when two rounds of pre-growth were used (Fig. 3B). Because concentrations of ethanol produced from glucose were found to be much greater than those from cellulose, ethanol accumulation is unlikely to be responsible for the low ethanol yields on cellulose.

Strain A10 was capable of utilizing numerous compounds as carbon sources when the organism was grown aerobically. This wide range exceeds that described for several other *Trichoderma* strains (Manczinger and Polner 1987). In contrast to glucose and cellulose, very little ethanol was found when xylose was the substrate, although other fermentation products were formed. Many of these products included TCA-cycle intermediates (succinate and fumarate) as well as several unidentified compounds. Interestingly, smaller amounts of these additional fermentation products were from glucose and xylose, and were not observed with cellulose. Thus, the ability of strain A10 to produce ethanol without acidic co-products from cellulose in minimal medium, combined with the potential for genetic manipulation, suggests that further work toward strain improvement is warranted.

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