

# Rumen Microbiology

## The Bacteriocins of Ruminal Bacteria and Their Potential as an Alternative to Antibiotics

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### Introduction

Simple stomached animals lack enzymes that can degrade cellulose or hemicellulose, and fibrous materials are poorly utilized. Ruminant animals do not synthesize fiber digesting enzymes, but they have formed a symbiotic relationship with ruminal microorganisms that can. The ruminant provides the microorganisms with a habitat for their growth, the rumen, and microorganisms supply the animal with fermentation acids, microbial protein and vitamins. However, ruminal fermentation also produces methane and ammonia, and these end-products are a loss of energy and nitrogen, respectively. When methane is inhibited, acetate production declines, the fermentation is diverted towards propionate, and energy retention increases. If proteins can be protected from ruminal deamination, ammonia declines and the animal has more amino acids for its nutrition. Some ruminal bacteria produce lactic acid at a rapid rate, and this acid can cause pronounced declines in ruminal pH, founder, and in severe cases, even death of the animal. Ruminant nutritionists, farmers and ranchers have used ionophores and other antibiotics to modify ruminal fermentation and increase the efficiency of feed digestion. However, there has been an increased perception that antibiotics should not be routinely used as feed additives. Some bacteria produce small peptides (bacteriocins) that inhibit gram-positive bacteria, and the bacteriocin, nisin, had effects on ruminal fermentation that were similar to the ionophore, monensin. However, preliminary results, indicated that mixed ruminal bacteria degraded nisin and became resistant. A variety of ruminal bacteria produce bacteriocins, but the effect of these peptides on ruminal fermentation had not been examined.

### Materials and Methods

Ruminal fluid (diluted 1 to 10 or 1 to 100 in basal medium) was streaked onto agar-plates and incubated anaerobically at 39°C. Colonies that developed after 18 h were picked, transferred to broth. Isolates (n = 90) were then spotted onto basal agar and the plates were incubated at 39°C. After 24 h of incubation, the plates were overlaid with soft agar seeded with approximately 10<sup>5</sup> *S. bovis* JB1 cells ml<sup>-1</sup>. The plates were re-incubated for another 24 h at 39°C, and each isolate was scored for its ability to create a distinct zone of clearing (≥ 3 mm) in the agar overlay. HC5 (the isolate that produced the largest zone of clearing, see above) was grown overnight in basal medium. Samples were diluted 10-fold and subjected to a heat treatment to lyse the cells. 16S rDNA was amplified using universal 27F and 1492R eubacterial primers. PCR products were cloned. Clones containing the 16S rDNA insert were sequenced. Sequences were assembled and aligned to known *Streptococcus* 16S rRNA sequences using ClustalX.

Stationary phase *S. bovis* JB1 cells were harvested by centrifugation, washed anaerobically in basal medium lacking ammonia, and re-suspended in 10 ml of the same medium. The washed cell suspensions were energized with glucose and some suspensions were treated with either nisin or partially

purified *S. bovis* HC5 bacteriocin. Samples were centrifuged through silicon oil. The microcentrifuge tubes were frozen, and the bottom of the tubes containing the cell pellets were removed with a pair of dog nail clippers. Potassium in cell pellets was determined with a flame photometer.

Stationary phase *S. bovis* HC5 cells were re-suspended in acidic sodium chloride (100 mM, pH 2.0, 2 h, 4 °C). The cell suspensions were re-centrifuged to remove cells, and the cell-free supernatant was lyophilized. The lyophilized material was re-suspended in sterile water. The bacteriocin extract was then applied to an SP Sepharose column. The final purification of the active peptide was completed by re-injecting the active fractions onto a C16 column using ethanol as a carrier solvent. The active fractions were then collected and lyophilized. Purified bacteriocin from *S. bovis* HC5 was subjected to Edman degradation analysis.

## Results

A freshly isolated *S. bovis* strain (HC5) had antimicrobial activity against a variety of *S. bovis* strains and other of gram-positive ruminal bacteria. This activity was primarily associated with the cells, but it could be removed with acidic NaCl. The activity was purified by HPLC. The purified peptide had an N-terminal that was VG-RYAS-PG-SWKYV-F. Amino acid residues that did not correspond to amino acids commonly found in proteins had approximately the same position as dehydroalanines found in some lantibiotics. The N-terminal amino acid sequence of bovicin HC5 showed similarity to a lantibiotic precursor of *S. pyogenes* SF370, but the identity was only 55%. Further work will be needed to locate the gene, but bovicin HC5 appears to be a novel bacteriocin. Because the purified extract caused potassium efflux from glycolyzing *S. bovis* JB1 cells, it appears to be a pore forming bacteriocin.

## Discussion

Because some ruminal bacteria can produce bacteriocins, there were speculations that these compounds might provide “effective alternatives to ionophore antibiotics as feed supplements.” Because the rumen is a highly diverse bacterial ecosystem that is inhabited by many different species (and strains within a species) and bacterial competition is very intense, inoculation would not necessarily increase the amount of a bacteriocin in the rumen. However, cattle are often fed silages, and silage fermentation is a batch culture system that favors rapidly growing lactic acid bacteria. When inoculated silages, “*S. bovis* grew faster than any of the commercial species tested and resulted in the most homolactic fermentation.” Given the observation that some *S. bovis* produce very potent bacteriocins, silage fermentation could be a vehicle for delivering bacteriocins to the rumen..

## **In Vitro Fermentation of Polydextrose by Bovine Ruminal Microorganisms**

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### **Introduction**

Polydextrose (PD) is a water-soluble condensation polymer of dextrose (D-glucose) that is used as a bulking and texturizing agent for the manufacture of low-calorie foods. This use is based on the fact that PD provides physical and flavor characteristics of fat, yet it is only partially metabolized in monogastric animals. Unsold commercial PD is occasionally available as a ruminant feed. However, no data are available on the ruminal utilization of PD, and nutritionists have no information on which to evaluate informal claims that PD can serve as a starch substitute in ruminant rations. This study was carried out to compare PD with two starches and two fiber sources in terms of fermentation kinetics and product formation by mixed ruminal microbes *in vitro*, and by pure cultures of carbohydrate-fermenting ruminal bacteria.

### **Methods**

PD was obtained from Bower's Feed and Grain, (Wrightsville, WI), which obtained the material as a manufacturer's overrun. Purified corn starch (CS), potato starch (PS) and Sigmacell 50 microcrystalline cellulose (SD50) were obtained from Sigma. Barley (BH; field-grown, first cut, 55.5% NDF, 35.8% ADF); was air-dried and then ground through a Wiley mill having a 1-mm screen.

*In vitro* fermentations were conducted using a gas pressure transducer-based measurement system having 40 experimental channels, plus 4 channels dedicated to relative barometers and 2 channels dedicated to absolute barometers. Two separate fermentations runs were conducted in Goering-Van Soest buffer. Each experiment was carried out using an inoculum from a different fistulated, lactating Holstein cow fed a total mixed ration (corn silage, corn grain, alfalfa hay, soybean meal, and supplemental vitamins and minerals). For each run, fermentations of each substrate were conducted in quadruplicate in serum vials (nominal 60 ml). Vials contained 80 mg of dry matter (PD, CS, PS, SC50, BH, all weighed to 0.1 mg). Blank vials, prepared in the same manner but lacking added substrate, were also run to permit subsequent calculation of net gas production from experimental samples. End products of the fermentation were determined by HPLC.

Growth experiments with pure cultures were performed in a modified Dehority medium in microtiter plates inside an anaerobic glovebag (5% H<sub>2</sub>/95% CO<sub>2</sub>), with eight replicate wells per culture/substrate combination. Culture turbidity was determined using a microtiter plate reader contained within the glovebag. Plates were incubated in the instrument's heated chamber at 39 °C, and were automatically subjected to gyratory shaking at maximal speed for 30 s prior to each optical density (600 nm) reading, taken at 15-30 min intervals. Growth rates were calculated from the slope of the linear region of plots of ln OD<sub>600</sub> versus time.

Model coefficients, and the data from fermentation, microbial growth and enzymatic hydrolysis experiments were analyzed using the ANOVA protocol of the GLM procedure of the SAS statistical software package (SAS Institute 1986), and comparisons among treatment means were conducted using Duncan's Multiple Range test at a significance level of  $P < 0.05$ .

## Results and Discussion

The monosaccharide composition (molar basis) of the the PD sample, analyzed following hydrolysis (2 N trifluoroacetic acid, 120 °C, 2h), was: glucose, 0.961; mannose, 0.017; xylose, 0.014; arabinose, 0.007. PD did not contain detectable NDF or measurable levels of N when assayed by combustion analysis. In general, PD was hydrolyzed by starch-degrading enzymes to an extent similar to that of potato starch, but less than that of corn starch.

Time courses of gas production from the polysaccharides are shown in Figure 1. PD was incompletely fermented by the mixed ruminal microbial culture. The fermentation proceeded without a significant lag, even in cultures not previously exposed to PD, suggesting that PD is readily depolymerized by hydrolytic enzymes produced by the indigenous microflora (Fig. 1). Gas production from PS or CS was adequately described by a single-pool exponential model (Table 1), with lag times of about 2 h, and first-order rate constants that averaged 0.43 h<sup>-1</sup> and 0.17 h<sup>-1</sup> respectively. By contrast, gas production from PD, SC50, or barley hay was best described by a two-component digestion model. The relatively small, more rapidly digesting pools of these substrates displayed little or no lag, while the larger and more slowly digesting pool displayed lag times of 2 – 10 h. For PD, one pool, which contained 20-47 per cent of the substrate, was fermented without a lag period and at rates similar to that of purified PS or CS. The second pool was fermented considerably more slowly than was cellulose. Both the total amount of gas produced and the concentrations of VFA after 48 h were about one-third lower from PD than from starch. The molar ratios of acetate to propionate ranged from 2.7 to 3.5 and were generally higher for PD than from the other purified polysaccharides

The growth rate of *Selenomonas ruminantium* D was higher on PD than on starches, while the reverse was true for *Streptococcus bovis* JB1, *Prevotella ruminicola* B<sub>1</sub>4, and *Butyrivibrio fibrisolvens* H17c (Table 2). For all four strains, the low final culture densities obtained suggest that only a fraction of PD was utilized. Neither PD nor either of the starches supported growth of the fiber-digesting strain *Fibrobacter succinogenes* S85. Separate experiments in tube cultures (rather than in microtiter plates) revealed that PD did not support the growth of two other fiber-digesting strains, *Ruminococcus albus* 7 and *Ruminococcus flavefaciens* FD-1.

PD is produced by thermal processing of starch under vacuum in the presence of certain reactive cosubstrates, resulting in a product having a monosaccharide composition similar to that of starch, but with altered physical properties and all possible glycosidic linkages, with  $\alpha$ -1,6-linkages predominating. The altered properties of PD are reflected in its reportedly poor digestibility in nonruminant animals. While the postruminal degradability of PD has not been examined, experiments with nonruminant animals suggests minimal digestibility. On the whole, PD appears to be unsuitable as a starch substitute for ruminants under production conditions.

## Conclusions

The fermentation properties of PD suggest that this material, even if available at competitive prices, is a not an effective substitute for starch in ruminant feeds.

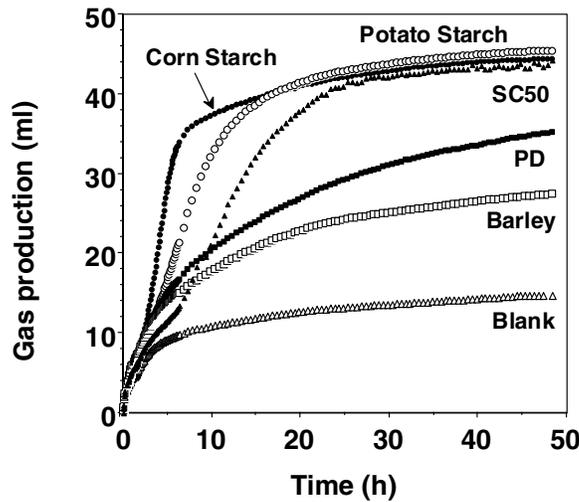


Fig. 1. Time course of in vitro gas production by mixed ruminal microorganisms fed polydextrose (PD), corn starch, potato starch, cellulose, or barley hay. Blank vials contained inoculum and carryover substrates, but no other fermentable substrate

Table 1. Kinetic constants for gas production from rapidly and slowly digesting pools of substrate during in vitro fermentations by mixed ruminal microflora.<sup>a</sup>

Substrate	Model <sup>b</sup>	A		$k_1$		$L_1$		B		$k_2$		$L_2$	
		(ml gas/g substrate)		(h <sup>-1</sup> )		(h)		(ml gas/g substrate)		(h <sup>-1</sup> )		(h)	
		Expt 1	Expt 2	1	2	1	2	1	2	1	2	1	2
Polydextrose	2	153.0 <sup>c</sup>	62.6 <sup>d</sup>	0.110 <sup>f</sup>	0.387 <sup>c</sup>	0.09 <sup>c</sup>	0 <sup>d</sup>	167.3 <sup>d</sup>	245.6 <sup>d</sup>	0.021 <sup>c</sup>	0.034	9.58 <sup>c</sup>	2.62 <sup>d</sup>
Corn starch	1	362.0 <sup>d</sup>	341.8 <sup>c</sup>	0.384 <sup>c</sup>	0.481 <sup>d</sup>	2.21 <sup>c</sup>	0.86 <sup>c</sup>	-	-	-	-	-	-
Potato starch	1	429.6 <sup>c</sup>	361.6 <sup>c</sup>	0.141 <sup>c</sup>	0.193 <sup>f</sup>	2.64 <sup>c</sup>	1.17 <sup>c</sup>	-	-	-	-	-	-
Cellulose	2	74.9 <sup>f</sup>	38.3 <sup>d</sup>	0.137 <sup>ef</sup>	0.087 <sup>g</sup>	0.68 <sup>d</sup>	0 <sup>d</sup>	271.4 <sup>c</sup>	337.0 <sup>c</sup>	0.125 <sup>c</sup>	0.112 <sup>c</sup>	7.85 <sup>c</sup>	8.14 <sup>c</sup>
Barley hay	2	117.8 <sup>e</sup>	64.3 <sup>d</sup>	0.225 <sup>d</sup>	1.82 <sup>c</sup>	0 <sup>e</sup>	0 <sup>d</sup>	107.0 <sup>e</sup>	180.0 <sup>e</sup>	0.052 <sup>d</sup>	0.060 <sup>d</sup>	8.03 <sup>c</sup>	2.15 <sup>d</sup>

<sup>a</sup> Kinetic parameters: A and B, gas production from rapidly and slowly digesting pools, respectively;  $k_1$  and  $k_2$ , first-order rate constants for rapidly and slowly digesting pools, respectively;  $L_1$  and  $L_2$ , lag time prior to initiation of gas production in rapidly and slowly digesting pools, respectively. Results are mean values of four replicates for each sample.

<sup>b</sup> Kinetic model: 1, single pool; 2, dual pool. For single pool model, only coefficients A,  $k_1$  and  $L_1$  are used. Values for goodness of fit of data to the models, calculated as in Weimer et al. (2000) and averaged within treatments for a given experiment, ranged from 0.9909 to 0.9991.

<sup>cdefg</sup> Means within a column having different superscripts differ ( $P < 0.05$ )

Table 2. Maximum specific growth rate constants and optical density increases during growth of pure cultures of ruminal bacteria on glucose, maltose, potato starch and polydextrose. Results are mean values from eight replicate cultures grown in microtiter plates.

<u>Bacterial strain</u>	$\mu_{\max}$ ( $\text{h}^{-1}$ )				OD <sub>600</sub> increase			
	<u>Glucose</u>	<u>Maltose</u>	<u>Potato Starch</u>	<u>PD</u>	<u>Glucose</u>	<u>Maltose</u>	<u>Potato Starch</u>	<u>PD</u>
<i>Butyrivibrio fibrisolvens</i> H17c	0.26 <sup>b</sup>	0.24 <sup>b</sup>	0.35 <sup>a</sup>	0.13 <sup>c</sup>	0.93 <sup>b</sup>	1.43 <sup>a</sup>	1.46 <sup>a</sup>	0.19 <sup>c</sup>
<i>Fibrobacter succinogenes</i> S85	0.23 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	1.38 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0
<i>Lachnospira multipara</i> 40	0.66 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	1.87 <sup>a</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0
<i>Prevotella ruminicola</i> B <sub>1</sub> 4	0.59 <sup>a</sup>	0.47 <sup>b</sup>	0.36 <sup>c</sup>	0.18 <sup>d</sup>	1.39 <sup>a</sup>	1.14 <sup>c</sup>	1.21 <sup>b</sup>	0.20 <sup>d</sup>
<i>Selenomonas ruminantium</i> D	0.61 <sup>a</sup>	0.56 <sup>b</sup>	0.12 <sup>d</sup>	0.25 <sup>c</sup>	0.59 <sup>b</sup>	1.11 <sup>a</sup>	0.12 <sup>d</sup>	0.30 <sup>c</sup>
<i>Streptococcus bovis</i> JB-1	1.27 <sup>a</sup>	1.02 <sup>b</sup>	0.92 <sup>c</sup>	0.43 <sup>d</sup>	1.11 <sup>b</sup>	1.23 <sup>a</sup>	1.19 <sup>a</sup>	0.30 <sup>c</sup>

<sup>abcd</sup> Means within a row having different superscripts for a each parameter ( $\mu_{\max}$  or OD<sub>600</sub> increase) differ ( $P < 0.05$ ).

## Growth of Ruminal Bacteria in Culture Media Reduced by Photocatalytic Interaction of Resazurin and Cysteine

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### Introduction

Many ruminal bacteria are strict anaerobes that require pre-reduced culture media for growth in the laboratory. Removal of oxygen and establishment of reducing conditions requires gassing with CO<sub>2</sub> or other O free gas, and addition of a chemical reducing agent. Cysteine (Cys) is a preferred reducing agent because of its low toxicity to the bacteria, but reduction of the medium by Cys is very slow, requiring preparation of the medium and addition of Cys well in advance of the medium's use. We have developed a simple means of accelerating the reduction by using light to exploit the photocatalytic reactivity of resazurin, a dye normally added to anaerobic culture media to assess oxygen status.

### Material and Methods

Modified Dehority Medium (MDM) containing the standard concentration (2 mg/L) of resazurin was prepared under a CO<sub>2</sub> gas phase, without the evacuation and flushing of the gas phase that was normally performed to decrease the O<sub>2</sub> concentration in the medium. Cys was typically added at 1.25 g/l, and the tubes were exposed to ordinary laboratory illumination (10  $\mu\text{E}/\text{cm}^2/\text{s}$ ) or to quartz/tungsten light source (360  $\mu\text{E}/\text{cm}^2/\text{s}$ ). For uninoculated culture media, bleaching of resazurin was measured spectrophotometrically at 540 nm. For growth experiments, media were inoculated with the microorganisms as soon as RNO was reduced ( $A_{540} < 0.05$ ), media. Microbial growth was measured turbidimetrically (540 nm) with a Milton-Roy Spectronic 21, at various intervals following inoculation (2% v/v) with pure culture of ruminal bacteria. The effect of light intensity on O<sub>2</sub> removal in medium solutions was determined with a Clark-type O<sub>2</sub> electrode.

### Results and Discussion

The rate of reduction of MDM, as measured by bleaching of the redox dye resazurin, was enhanced by light in an intensity-dependent manner (Fig.1), and the time required for medium reduction was decreased from several hours under normal light, to a few minutes under high intensity light. O<sub>2</sub> electrode experiments (Fig.2) revealed that the photoinduced bleaching of resazurin was related to light-enhanced removal of O<sub>2</sub> from the medium (i.e., was not due to a reaction of RNO unrelated to its status as a redox dye), although partial removal of O<sub>2</sub> prior to Cys addition was required. Media that had been rapidly reduced by photocatalysis supported growth of five strains of ruminal bacteria in an identical fashion to the same media reduced with Cys over a period of hours under typical laboratory illumination (Table 1). Similar results were obtained with four strains of *Clostridium* and one strain of *Thermoanaerobacter* in another culture medium (CM5) under a N<sub>2</sub> gas phase (data not shown).

## Conclusion

Photocatalytic reaction between cysteine and RNO accelerates the reduction of anaerobic culture media, permitting almost immediate use of the medium for cultivation of ruminal bacteria and other strict anaerobes. Medium reduced photocatalytically supports growth of ruminal bacteria in a manner quantitatively identical to that of media reduced more slowly at low or zero light.

Table 1. Growth parameters for five strains of ruminal bacteria in media reduced at low or high light intensity<sup>a</sup>

Culture	Substrate <sup>b</sup>	Lag time (h)		Growth rate (h <sup>-1</sup> )		MaximumOD <sub>540</sub>	
		Low	High	Low	High	Low	High
<i>Lachnospira multipara</i> 40	Glucose	3.25 ± 0.47	3.04 ± 1.20	0.49 ± 0.06	0.46 ± 0.06	1.18 ± 0.05	1.17 ± 0.03
<i>Prevotella ruminicola</i> B <sub>14</sub>	Glucose	0.08 ± 0.03	0.25 ± 0.30	0.56 ± 0.02	0.56 ± 0.03	1.41 ± 0.01	1.42 ± 0.03
<i>Ruminococcus flavefaciens</i> FD-1	Cellobiose	1.76 ± 0.93	0.93 ± 0.72	0.27 ± 0.01	0.27 ± 0.01	0.70 ± 0.04	0.79 ± 0.06
<i>Selenomonas ruminantium</i> D	Glucose	1.22 ± 0.24	1.09 ± 0.06	0.61 ± 0.01	0.61 ± 0.03	1.19 ± 0.03	1.21 ± 0.04
<i>Streptococcus bovis</i> JB1	Glucose	1.57 ± 0.18	1.33 ± 0.16	1.23 ± 0.03	1.23 ± 0.03	1.41 ± 0.04	1.30 ± 0.03

<sup>a</sup> Media were reduced at low (10 □E/cm<sup>2</sup>/s) or high (360 □E/cm<sup>2</sup>/s) light intensity. Results are mean values of four replicate cultures ± S.E.M.

<sup>b</sup> Cultures were grown in MDM + 1 g of yeast extract /l, plus glucose (10 g/l) or cellobiose (4 g/l), under a CO<sub>2</sub> gas phase.

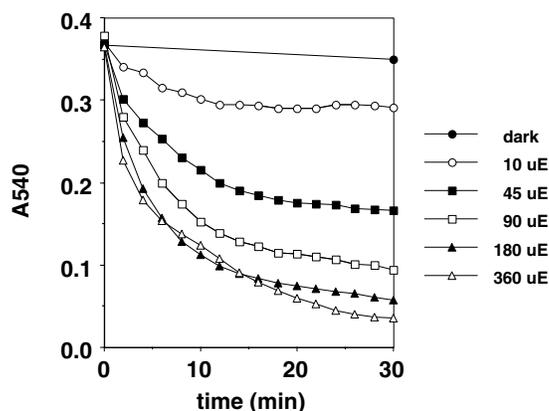


Fig.1. Effect of light intensity on bleaching of RNO in MDM.

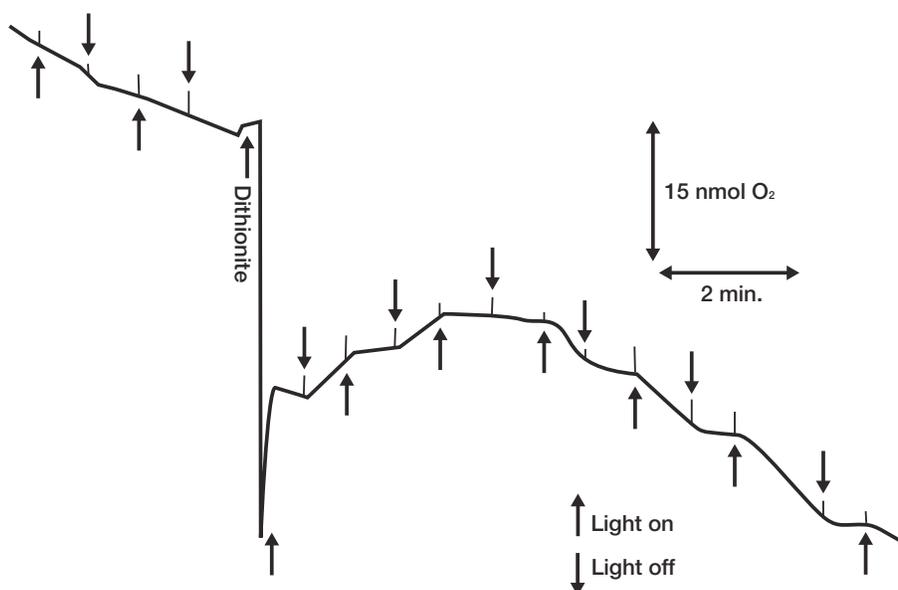


Fig.2. Effect of illumination (arrows) on  $O_2$  removal from MDM containing RNO (2 mg/l) and Cys (1.25 g/l). RNO-photocatalyzed  $O_2$  removal required prior removal of air, achieved in the air-saturated  $O_2$  electrode chamber by addition of dithionite. In experiments performed in culture tubes (see text), air was removed by gassing tubes with  $CO_2$  prior to Cys addition and illumination, and no dithionite was used in the medium. Control incubations lacked RNO or Cys, and did not result in  $O_2$  removal (data not shown).

## Isolation and Characterization of a *Trichoderma* Strain Capable of Fermenting Cellulose to Ethanol

D. M. Stevenson and P.J. Weimer

### Introduction

For several decades 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 of the processes currently under investigation, cellulosic biomass is first enzymatically broken down into sugars (often utilizing fungal cellulases) which microorganisms (usually yeast) then ferment to ethanol. To lower capital and operating costs, it is desirable to accomplish the fermentation of cellulose to ethanol in one step. Few species of filamentous fungi are capable of this conversion, and all require rich (and thus expensive) culture media. In this study environmental samples were screened for organisms capable of producing ethanol from cellulose on minimal medium. We isolated a filamentous fungus capable of this conversion, and developed for this organism a genetic system based on parasexuality.

### Methods

Rich medium (yeast-peptone-malt extract; YPM) consisted of (per l) 3.0 g yeast extract, 5.0 g peptone, 0.3 g malt extract, and 5.0 g glucose. Minimal medium (MM) was yeast nitrogen base

(YNB) without added vitamins. Unless otherwise noted, the pH of MM was adjusted to 5.0 and carbon sources were added at 5 g/l for aerobic growth and 50 g/l for anaerobic fermentation. Sigmacell 20 microcrystalline cellulose was used as the cellulose source. Growth on soluble substrates was measured as dry weight after filtration onto tared glass fiber filters.

Anaerobic fermentation of cellulose and other substrates was tested using two growth methods. In the first (sealed flask) method, MM (20 ml in a 25 ml Erlenmeyer flask) was simply inoculated with conidia ( $\sim 1 \times 10^5$ ) and the culture vessel was sealed with either a butyl stopper or screw-cap. Thus approximately 20% of the flask volume was left as air space. The oxygen 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. Ethanol and other fermentation products were analyzed in culture supernatants by HPLC.

Mutants were generated by exposure of conidia (washed off the surface of 7 d-old YNB glucose plates) to UV irradiation (5 cm from a 256 nm, 4W lamp for 10 min). Potential cross-feeding between auxotrophic mutants was tested in a U-tube apparatus constructed from two 10 ml syringe barrels, separated by a short length of tubing with or without an intervening 0.2  $\mu\text{m}$  membrane filter disc. Each of syringe barrel contained MM, and was inoculated with an agar plug of an individual mutant strain. After 48h incubation, syringe pressure was used to force liquid (and, in the absence of the membrane filter, mycelial fragments) back and forth across the tubing, and growth was allowed to continue for an additional 7 d.

## Results and Discussion

Fifty-six strains of fungi were isolated from environments rich in cellulose, such as the dung of ruminants, rotting wood, etc. 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 mg/ml) 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 use cellulose aerobically on MM, and two of these were found to produce measurable amounts of ethanol upon anaerobic incubation in MM plus cellulose. The more active strain, originally isolated from cow dung on cellulose medium, was selected for further study and was designated strain A10. This strain grew well on cellulose in both liquid and solid medium.

Strain A10 was found to grow rapidly and, when mature, to produce dark green conidia on MM plus glucose, and yellow-green to dark green conidia on rich medium (YPM), often in concentric rings as a response to light. The conidiophores (conidia bearing hyphae, Figure 1A) bore bottle-shaped cells (phialides) from which the conidia arose singly, and phialides were generally paired along the conidiophores. Little accumulation of conidia was seen on the conidiophores, except in very old cultures. Under light microscopy, conidia formed on solid medium were smooth-walled and nearly spherical with a mean diameter of 3.0  $\mu\text{m}$  (Figure 1B) (Figure 1A). These observations suggest that A10 is a strain of *Trichoderma*. Sequencing of the intergenic spacer region ITS1 (GenBank Accession No. AY094141) and subsequent BLAST search indicated 100% sequence identity to several strains of *T. harzianum*. The culture has been deposited at the National Center for Agricultural Utilization Research as strain NRRL 31396. .

Maximum growth was observed at around 30-35°C, and the best growth occurred at the lowest pH range tested, pH 3.2 to 3.8. The strain grew well on cellulose in both liquid and solid medium, although no zone of cellulose clearing could be seen on solid medium suggesting that large amounts of extracellular cellulase were not secreted by this strain. Numerous carbohydrates were shown to support growth in YNB within 5 d under aerobic conditions. Growth under strict anaerobic conditions was tested using. No growth was observed on cellulose, cellobiose, glucose or xylose under anaerobic conditions; other carbohydrates were not tested.

When initially isolated, strain A10 produced only about 0.4 g ethanol/l when fermentation was by the sealed flask method in MM with 50 g added cellulose/l. Ethanol accumulation was eventually improved, by selection and the use of a vented fermentation flask, to 2 g/l when the fermentation was carried out in submerged culture. The highest levels of ethanol, >5.0 g/l, 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. Increasing the size of the inoculum for anaerobic incubations by several stages of pregrowth (obtained by removing spent medium from the aerobic culture and refilling with fresh media) did not increase the final concentration of ethanol.

Following mutagenesis, three auxotrophic and two morphological mutants were obtained. The auxotrophic strains required arginine (*arg<sup>-</sup>*, strain A217), adenine or hypoxanthine (*ade/hpx<sup>-</sup>*, strain A277), or inositol (*ino<sup>-</sup>*, 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<sup>-</sup>* and *ade/hpx<sup>-</sup>* mutants (strains A217 and A277, respectively) were placed next to each other on rich medium (YPM), as in Figure 2. After three 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, and none from the control (Figure 2A). Subsequently, nearly identical results were obtained using mutants A277 (*ade/hpx<sup>-</sup>*) and B429 (*ino<sup>-</sup>*), and also strains A217 (*arg<sup>-</sup>*) and B429 (*ino<sup>-</sup>*). This suggests the probable formation of heterokaryons in the manner of parasexuality. 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 one or the other parental strains. Only a few prototrophs were found, and these 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 6-8 rounds of subculturing did strains develop that produced nearly 100% prototrophic conidia, 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 (see Methods) was carried out to test the possibility that cross-feeding was responsible for the complementation between strains A217 (*arg<sup>-</sup>*) and A277 (*ade/hpx<sup>-</sup>*). Complementation occurred only when hyphae came into contact, ruling out cross-feeding as a possibility.

## Conclusions

The ability of this new fungal 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.

## Acknowledgments

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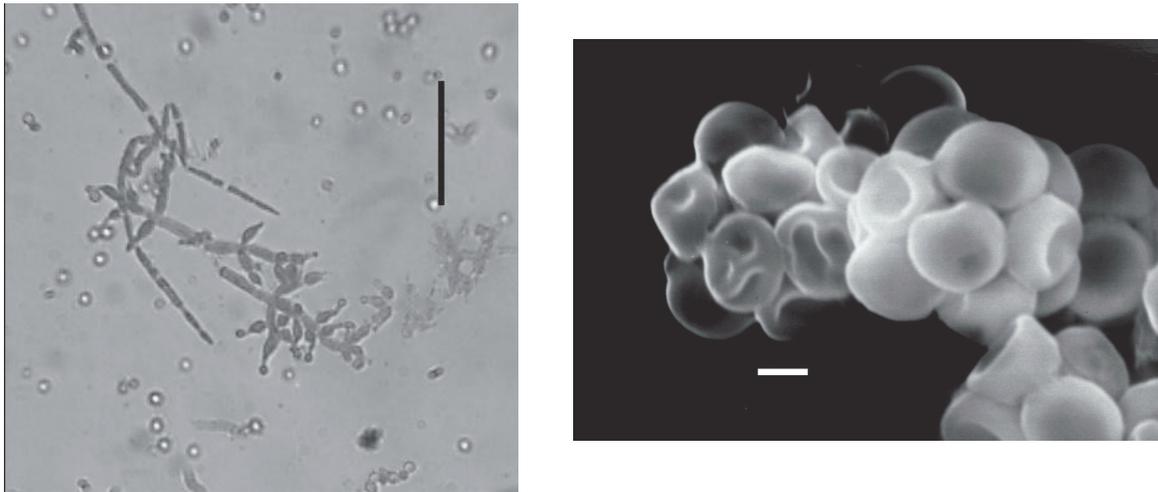


Fig. 1. Left panel: Conidiophores of isolate A10 showing arrangement of conidia and phialides (conidia bearing hyphae) from a 3 d old culture on aerobic solid glucose minimal medium. The bar represents approximately 50  $\mu\text{m}$ . Right panel: SEM on non-fixed, air-dried conidia. The bar represents 1.0  $\mu\text{m}$ .

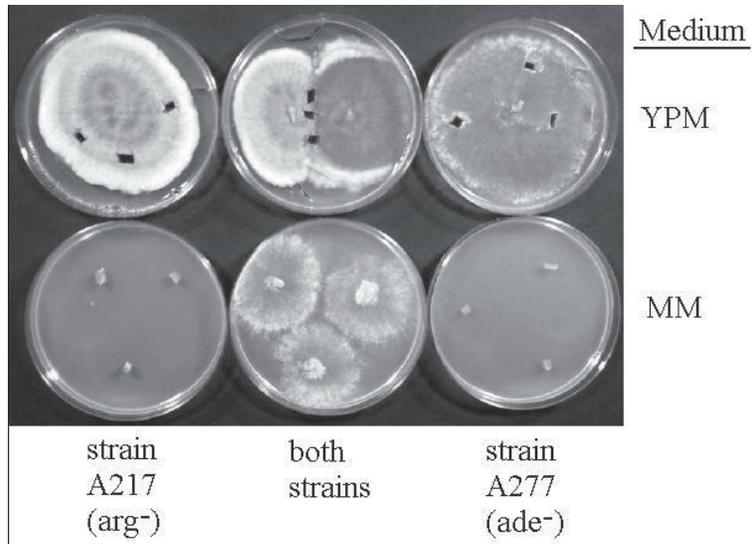


Fig. 2. Complementation of auxotrophs of A10. Top row shows 3 day old plates of rich medium, with an *arg*<sup>-</sup> mutant (A217) on the left and an *ade*<sup>-</sup>/*hpx*<sup>-</sup> mutant (A277) on the right. In the center both mutants grew in proximity and can be seen to have grown together. The bottom row shows plate of minimal medium inoculated with plugs taken from the plates above, and clearly shows growth only from plugs taken from the region of contact between the two mutants.