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Concurrent Production and Consumption of Ethanol by Cultures of *Pachysolen tannophilus* Growing on D-Xylose†

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Growing cultures of *Pachysolen tannophilus* concurrently consumed and produced ethanol in the presence of substantial concentrations of D-xylose. Ethanol was also assimilated in the presence of other sugars, the amount depending on the sugar. Less ethanol assimilation occurred with D-glucose than with D-xylose. The rate of ethanol consumption decreased as the concentration of glucose was increased, but some consumption still occurred when 2% glucose was present. The rate increased with the amount of oxygen available to the culture when D-xylose or ethanol was the carbon source. In most instances, estimates of consumption were based on the extent of incorporation of ^{14}C from [1-^{14}C]ethanol into trichloroacetic acid-insoluble material. The results are pertinent to the use of *P. tannophilus* for the production of ethanol from D-xylose.

Recently, it was shown that the yeast *Pachysolen tannophilus* can convert D-xylose into ethanol (10, 11). The conversion occurs aerobically as well as anaerobically, but cell growth requires oxygen (9–11). It had previously been held that yeasts do not ferment pentoses (1, 12). Because the organism can also ferment D-glucose, it is potentially useful for the production of ethanol from hydrolyzed polysaccharides which contain appreciable amounts of D-xylose. Other D-xylose-fermenting yeasts have also been identified (5, 6, 8).

During aerobic fermentation of D-xylose by *P. tannophilus*, ethanol yields range downward from 78% of a theoretical value (9–11). The variation is associated, in part, with differences in aeration. The yield of ethanol from D-xylose has also been shown to depend on the extent of aeration of *Candida tropicalis* cultures (6). To improve yield, factors which limit or control ethanol concentration during D-xylose fermentation have been under investigation. The present study shows that cultures of *P. tannophilus* can concurrently consume and produce ethanol from D-xylose under aerobic conditions. Consumption was assayed in most instances by determining the extent to which cells incorporated [^{14}C]ethanol added to the growth medium.

MATERIALS AND METHODS

Microorganism. *P. tannophilus* NRRL Y-2460 was used throughout. It was maintained on YEPD slants (1% yeast extract, 1% peptone [Difco Laboratories, Detroit, Mich.], 2% glucose, 1.5% agar).

Media and cultures. The cells used as inocula were grown in Erlenmeyer flasks (250 ml), each of which contained 50 ml of 0.67% Difco yeast-nitrogen base (YNB) with 179 to 325 μmol of ethanol per ml (0.83 to 1.5%), 2% D-glucose, or 2% D-xylose as the sole carbon source, as specified below. The cultures were incubated for 48 h at 30°C and 150 rpm on a gyratory shaker. They were then harvested by centrifugation at 5°C and 10,000 × *g* for 10 min, washed twice with 0.67% YNB, and then resuspended in 0.67% YNB plus the carbon source to the required optical density at 600 nm (OD_{600}).

Concurrent ethanol production and consumption during growth. The physical conditions employed for testing ethanol production and consumption were similar to those referred to previously as "semi-aerobic" (10). We added 9 ml of culture to a loosely screw-capped test tube (16 ml), which we rotated at ~100 rpm about its long axis, which was kept 30° from horizontal. The culture was inoculated to an OD_{600} of 0.85 with cells grown on 217 μmol of ethanol per ml (1%). We used 2% D-xylose as the carbon source. Growth was monitored by measuring the OD_{600} (model 275 Coleman spectrophotometer), and the measurements were converted to dry weights, using a previously determined calibration curve. The initial OD_{600} value used, 0.85, corresponds to a dry weight of 2.75 mg/ml. Radiolabeled ethanol was added immediately after inoculation to bring the initial concentration of ethanol in the medium to 14 μmol/ml (0.065%). The concentration of ethanol in the medium was followed by gas chromatography (6 ft by 0.25 in. [182.88 by 0.63 cm] Chromosorb 102 column; 170°C; internal standard, methanol). The concentration of D-xylose in the medium was measured by gas chromatography of the silylated oxime (13) (4 ft by 0.25 in. [121.92 by 0.63 cm] column filled with 3% OV-17 on Chromosorb W-HP;

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temperature programmed from 140 to 250°C).

Incorporation of [^{14}C]ethanol was followed by taking 200-μl aliquots of the culture at intervals and filtering them through filters (pore size, 0.45 μm). Each filter was washed once with 5 ml of 0.67% YNB, twice with 5 ml of 10% trichloroacetic acid, and then once with 5 ml of water. The filters were dried under an infrared lamp and then placed in scintillation vials (20 ml), each of which contained 5 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.). After 45 min in the dark, they were counted in a scintillation counter to obtain 0.5% errors.

The amount of ethanol consumed was computed from the ^{14}C activity associated with the trichloroacetic acid-insoluble material remaining on the filters. Because of this procedure, all values for ethanol consumption represent underestimates. During the course of the experiment, the specific activity of the [^{14}C]ethanol in the medium changed, owing to the production of ethanol from D-xylose. Such changes were taken into account by measuring the concentration of ethanol in the medium. However, we did not take into account the production of ^{14}C compounds other than ethanol which appeared in the medium (e.g., CO_2). The presence of such components would make the value for the specific activity of [^{14}C]ethanol erroneously high. In addition, we did not take into account cell-localized radiolabeled products which were not retained on the filters after treatment with trichloroacetic acid. The initial specific activity of the [^{14}C]ethanol used was 2.7 to 4.7 μCi/nmol.

Effects of different sugars on ethanol consumption. The physical conditions used to determine the effects of sugar on ethanol consumption were those described above. However, each tube contained 1 ml of culture and was inoculated to an OD_{600} of 0.3 to 0.4 (dry weight, 0.075 to 0.085 mg/ml) with cells in 217 μmol of ethanol per ml plus 1% of the sugar to be studied. The cells had been grown in 325 μmol of ethanol per ml. Incorporation and consumption of [^{14}C]ethanol after 6 or 8 h were evaluated as described above.

Effect of oxygen availability on ethanol consumption. The degree to which oxygen was limited in the cultures was varied by using different volumes of medium in the rotated tubes. The effectiveness of this technique was monitored by measuring generation time. The cells used were grown initially on 217 μmol of ethanol per ml, and the cultures were inoculated to an OD_{600} of 0.06 with cells in 1% D-xylose and, in separate experiments, with 179 μmol of ethanol per ml. Growth was followed by measuring the OD_{600}.

Ethanol consumption under different degrees of oxygen availability when D-xylose was the carbon source was evaluated by monitoring the extent of incorporation of ^{14}C from radiolabeled ethanol as described above. The cells used had been grown on 217 μmol of ethanol per ml, and the cultures were inoculated to an OD_{600} of 0.825 with cells in 2% D-xylose. When ethanol was used as the sole carbon source, its disappearance from the medium was followed by gas chromatography. The cells used had been grown on 217 μmol of ethanol per ml, and the cultures were inoculated to an OD_{600} of 0.6 with cells in 179 μmol of ethanol per ml.

Chemicals. The D-xylulose used was described previously (13). The other sugars used (Sigma Chemical Co., St. Louis, Mo.) were of the purest grade available. [1-^{14}C]ethanol (21 μCi/nmol) was from New England Nuclear.

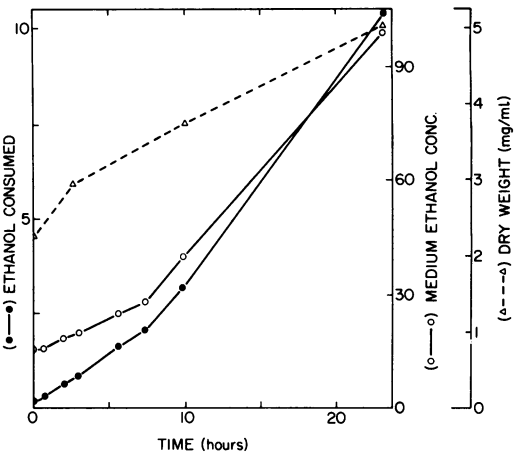

FIG. 1. Concurrent consumption and production (both expressed as micromoles per milliliter) of ethanol by a culture of *P. tannophilus* growing on D-xylose (initial concentration, 2%; final concentration, 1.1%).

RESULTS

Concurrent production and consumption of ethanol. Data from a typical experiment demonstrating the ability of a culture in which D-xylose was the sole sugar to grow and to simultaneously produce and consume ethanol are presented in Fig. 1. The initial D-xylose concentration was 2%, and the final concentration was 1.1%. Within a 23-h period, the dry weight increased by a factor of 2.2, and the ethanol concentration in the medium increased by a factor of 7.6 (14 to 107 μmol/ml). During the same interval, the amount of ethanol consumed increased by a factor of 105 (0.1 μmol/ml after 1 min to 10.5 μmol/ml after 23 h). The amount of ethanol consumed during the experiment was computed to be 9.8% of that produced. All values relating to ethanol consumption are considered to be underestimates, as indicated in Materials and Methods.

The data shown in Fig. 1 were obtained for cells grown initially on ethanol. Similar data were obtained for cells grown initially on 2% D-xylose.

Effects of different sugars. To determine if incorporation occurred also in the presence of other sugars, we first grew the cells under identical conditions with ethanol as the sole carbon source and then suspended the cells in 217 μmol of ethanol per ml plus 1% sugar. Figure 2 shows the effects of D-glucose in some detail. This hexose inhibited ethanol consumption rapidly: even at 0.1%, it inhibited consumption within 1 h. The extent of inhibition increased with sugar

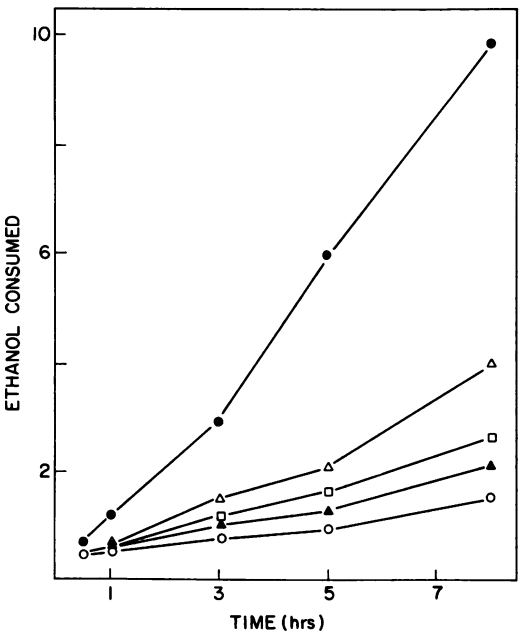

FIG. 2. Degrees to which various glucose concentrations inhibited ethanol consumption (expressed as micromoles per milligram; initial concentration, 217 μmol/ml) by *P. tannophilus*. Symbols: ●, ethanol alone; △, ethanol plus 0.1% glucose; □, ethanol plus 0.5% glucose; ▲, ethanol plus 1% glucose; ○, ethanol plus 2% glucose.

concentration, but ethanol was still consumed in the presence of 2% glucose. The amounts of ethanol consumed in 8 h corresponded to 9.2, 3.7 and 1.4% of the ethanol concentrations present initially with 0, 0.1, and 2% D-glucose, respectively. The amounts consumed in the presence of 0.1 and 2% D-glucose were 40 and 15%, respectively, of the amounts consumed in the absence of the sugars.

The results for all of the sugars tested are summarized in Table 1. *P. tannophilus* grew on all of these compounds. Although *P. tannophilus* is classified taxonomically as an organism that does not assimilate xylitol (2), slow growth did occur. All of the compounds tested inhibited consumption but did so to different extents. To simplify comparison, we computed the percentage of ethanol consumed in the presence of a sugar by considering ethanol consumption in the absence of sugar to be 100%. Glucose was the best inhibitor tested: the amount of ethanol consumed in its presence was 23% of the amount consumed in its absence. The corresponding value for D-xylose was 37%.

The different extents of consumption in the presence of D-glucose and D-xylose were not due to the initial growth of the cells on ethanol. Similar results were obtained when the cells were grown initially on a 2% solution of the test sugar.

TABLE 1. Degrees to which various sugars inhibited ethanol consumption by *P. tannophilus*

Substrate	Ethanol consumed (μmol/ml per 6 h)	% Consumed[a]
Ethanol (217 μmol/ml)	5.0	100
Ethanol +		
D-Glucose	1.15	23
D-Mannose	1.40	28
D-Xylose	1.85	37
D-Cellobiose	2.50	50
Glycerol	3.60	72
D-Xylulose	4.25	85
D-Galactose	4.75	95
L-Arabinose	4.85	97
Xylitol	4.90	98

[a] Relative to the percentage consumed in the absence of sugar as 100%.

Effect of oxygen availability. The effectiveness of varying the volumes of medium in the rotated tubes to vary oxygen availability was demonstrated by the relationship between medium volume and generation time: as medium volume was decreased, generation time decreased. Examples of such variations observed when ethanol was the carbon source are shown in Table 2. When 1% D-xylose was the carbon source, the generation times were 5.4, 4.6, 4.2, 3.7, and 3.2 h for medium volumes of 10, 8, 6, 3, and 1 ml, respectively.

As oxygen availability increased (and generation times became shorter), the amount of ethanol consumption increased (Table 2). Loss of ethanol from the medium by volatilization was negligible: controls indicated that such losses amounted to less than 5% of the amount initially present. Typical data illustrating the phenomenon when D-xylose was the carbon source for cells initially grown on ethanol are shown in Table 3. Similar effects occurred when the cells were grown initially on 2% D-xylose.

The relationship between the loss of ethanol due to metabolism and the availability of oxygen was also observed for nongrowing cells. The nongrowing state was attained by nitrogen deprivation. Cells grown on 217 μmol of ethanol per ml were transferred to 245 μmol of ethanol per ml in 0.17% YNB without amino acids or ammonium sulfate. With medium volumes of 9, 5, and 2 ml, the percentages of the original ethanol concentration remaining after 45 h were 80, 49, and 22, respectively.

Ethanol consumption requires the presence of oxygen. When ethanol (217 μmol/ml) was the sole carbon source, no consumption occurred in 8 h under the anaerobic conditions achieved by

TABLE 2. Variations in generation time and ethanol consumption with oxygen availability when 179 μmol of ethanol per ml was the sole carbon source

Medium vol (ml)	Generation time (h)	Medium ethanol concn after 66 h (μmol/ml)	% of original ethanol concn consumed after 66 h
10	4.5	131	21
6	4.0	81	55
3	3.6	0.65	99
1	3.3	ND[a]	100

[a] ND, Not detectable.

sealing the tubes immediately after inoculation (OD_{600}, 0.6; cells were grown initially on 325 μmol of ethanol per ml or on 2% D-xylose, and consumption was assayed with [^{14}C]ethanol).

DISCUSSION

In cultures of *P. tannophilus*, appreciable losses of ethanol occurred aerobically as the result of metabolism while D-xylose was present. Such losses also occurred in the presence of other sugars. In one set of experiments in which ethanol was produced from D-xylose, the amount of ethanol lost was computed to be equivalent to at least 9.8% of the amount of ethanol produced. Therefore, for optimization of yield when the organism is used to ferment D-xylose, process design will have to take such a potential source of loss into consideration.

In substrates expected to be used in practice, D-xylose is present, along with other sugars. In wood hydrolysates, these include D-glucose and D-mannose. Although both of these hexoses were more effective than D-xylose in inhibiting ethanol consumption, it still occurred. In addition, when sugar mixtures were tested, D-glucose and D-mannose were utilized first (unpublished data); therefore, the extent of ethanol consumption inhibition would be eventually controlled by D-xylose.

Less ethanol was consumed when the amount of oxygen available to the culture was decreased. Although ethanol can be produced anaerobically from D-xylose, results to date from small-scale experiments indicate that within a given fermentation time, yields obtained from anaerobic cultures are lower than those obtained from aerobic cultures (9, 11). Thus, a process which includes a low degree of aeration may ultimately prove to be optimal.

The molecular basis for the effects of sugars on ethanol consumption by *P. tannophilus* has still to be established. One possibility is that the Crabtree effect, which refers to the inhibition of yeast respiration by sugars (4), is involved. In *Saccharomyces cerevisiae* growing on D-glucose in the presence of air, this effect is thought to be responsible for the initial conversion of D-glucose to ethanol and the oxidation by ethanol which occurs after the glucose has fallen to a low level. In *S. cerevisiae*, the extent of respiration inhibition varies when other sugars are substituted for D-glucose (4). In *P. tannophilus*, the different degrees to which the various sugars inhibited ethanol consumption might reflect different extents to which the sugars cause the manifestation of the Crabtree effect.

TABLE 3. Variations in ethanol consumption with oxygen availability when 2% D-xylose was the sole carbon source

Medium vol (ml)	Ethanol consumed (μmol/ml) after:			
	2 h	5 h	7 h	9 h
9	0.27	0.80	0.92	1.52
2	1.29	2.12	2.59	3.78

An additional factor which could be involved is repression of synthesis of alcohol dehydrogenase activity to various extents by different sugars. In *S. cerevisiae*, glucose represses two of the three distinguishable dehydrogenases (3, 7).

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