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Regulation and Butanol Inhibition of D-Xylose and D-Glucose Uptake in *Clostridium acetobutylicum*

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Clostridium acetobutylicum **exhibited diauxie growth in the presence of mixtures of glucose and xylose. Both glucose- and xylose-grown cells had a glucose uptake activity. On the other hand, growth on xylose was associated with the induction of a xylose permease activity, which was repressed by glucose in xylose-induced cells. The rate of sugar uptake with increasing sugar concentrations showed saturation kinetics with an apparent K_m of 1.25×10^{-5} M for glucose and 5×10^{-3} M for xylose. Concomitant with the production of solvents, the activities of the glucose and xylose transport systems decreased. Among the main products of fermentation, butanol was shown to be a potent inhibitor of the growth of the organism and of the rate of sugar uptake as well as of sugar incorporation into cell materials. These inhibitory effects of butanol were more pronounced in xylose-grown cells than in glucose-grown cells. Butanol completely inhibited growth at a concentration of 14 g/liter for cultures growing on glucose and 8 g/liter for cultures growing on xylose. Concentrations of 7 and 10.5 g/liter of butanol caused a 50% inhibition of the xylose and glucose incorporations into cell materials. These inhibitory levels of butanol were found in typical glucose or xylose fermentation.**

In the hydrolysates of lignocellulosic compounds, glucose and xylose are the major sugars. Although glucose serves as a suitable substrate for microbial activity, the general inability of microorganisms to ferment pentoses is well known (3, 4, 11). Among the yeasts, only *Candida tropicalis*, *Candida utilis*, and *Pachysolen tannophilus* are able to grow slightly in anaerobic conditions on xylose, with the production of low levels of ethanol (12). Some yeasts can ferment xylulose obtained by isomerization of xylose by using bacterial isomerases (15). Many mycelial fungi are known to metabolize pentoses, but the rate of ethanol production is much slower than that observed in bacteria (13). Recently it was shown that *Clostridium acetobutylicum* was a microorganism of great interest in the fermentation of pentoses (5, 6, 10, 15). We have reported (10) that xylose is converted to solvents with a yield of 28%, which is close to the maximal value of 32% obtained with glucose. The two fermentations differ in growth rate, which is higher on glucose than on xylose, and in the limitation of sugar consumption. Although 60 to 65 g of glucose per liter was fermented, concentrations of about 50 g of xylose per liter are the highest substrate concentrations metabolized. The present report is an attempt to understand the limitation of pentose fermentation by studying the metabolism of *C. acetobutylicum* on glucose and xylose and to understand the inhibitions of the bacterial metabolisms by the end products of the fermentation.

MATERIALS AND METHODS

Organism and culture conditions. The *C. acetobutylicum* strain used in this study was obtained from the American Type Culture Collection (ATCC 824). Cultures were grown anaerobically at 35°C in the synthetic medium described by Monot et al. (8), containing the following components per liter of distilled water: ammonium acetate, 2.2 g; KH_2PO_4, 0.5 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; $MnSO_4 \cdot H_2O$, 0.01 g; NaCl, 0.01 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; *p*-aminobenzoic acid, 1 mg; biotin, 0.01 mg; and carbon source.

Growth and fermentation analysis. Growth was determined by measuring the optical density of the cultures at 600 nm with a Beckman model 25 spectrophotometer (10-mm light path). The correlation between optical densities and bacterial densities was established by the usual technique of dry weight determination. Other analyses were made on supernatant fluids of culture samples centrifuged at 12,000 rpm for 10 min. Residual sugars were determined by the method of Miller et al. (7). Xylose in glucose-xylose-grown cultures was measured as the difference between the total carbohydrate determined with the dinitrosalicylic acid reagent and the glucose measured by the glucose oxidase (EC 1.1.3.4) obtained from Sigma Chemical Co. (kit no. 510). Concentrations of solvents (ethanol, acetone, and butanol) and acids (acetic and butyric) were determined by injecting acidified supernatants into an Intersmat IGC 121 FL gas chromatograph equipped with a flame ionization detector. Separation was done in a glass column (2 m long by 2 mm in diameter) packed with Porapak Q, 80/100 mesh. N_2 was used as carrier gas. Injector and detector temperatures were 220°C, and the column temperature was programmed from 160 to 200°C. The analysis of chromatographic data were carried out with an Intersmat ICR 1B integrator.

Sugar uptake. Cells were harvested by centrifugation at $5,000 \times g$ for 15 min at 2°C and anaerobically washed with the synthetic medium lacking sugar but containing 0.1% β-mercaptoethanol, recentrifuged at $5,000 \times g$ for 15 min, and resuspended in the same medium to a density of about 600 μg of cells (dry weight) per ml grown on glucose and 1,200 μg of cells per ml grown on xylose. Cell suspension (5.3 ml each) was transferred anaerobically to a tube (diameter, 0.6 cm) continuously flushed with argon. After the cells were conditioned for 10 min at 36°C, uptake was initiated by adding 0.7 ml of a mixture containing the chloramphenicol (50 μg per ml) and the radioactively labeled sugar. D-[*U*-^{14}C]glucose was used at a final specific activity of 1.4

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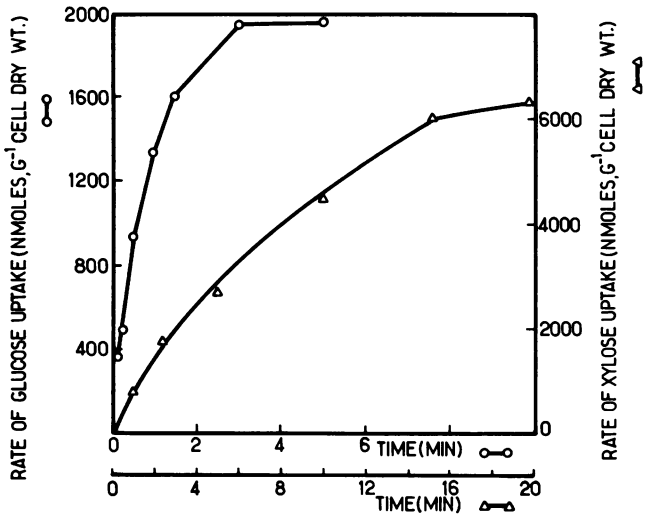

FIG. 1. Time course of sugar uptake by exponentially growing cells. Uptake of glucose by glucose-grown cells (○) and of xylose by xylose-grown cells (△).

μCi/μmol and a final concentration of 2.5×10^{-5} M, and D-[*U*-^{14}C]xylose was used at a final specific activity of 0.1 μCi/μmol and a final concentration of 10^{-3} M. Radiochemicals, glucose (300 mCi/mmol), and xylose (165 mCi/mmol) were obtained from the Commissariat à l'Energie Atomique, Gif-sur-Yvette. Portions of the cell suspension (1 ml) were removed at different times, filtered immediately through a Sartorius cellulose acetate membrane filter (pore size, 0.45 μm), and washed three times with 1.0 ml of the synthetic medium (36°C). The filters were placed in scintillation vials and dried at 50°C, and radioactivity was counted in 15 ml of scintillation fluid (Scintran, Cocktail T, BDH, Poole, England) in an Intertechnique model SL 4000 scintillation spectrometer.

Incorporation of ^{14}C into whole cells. Cells in the exponential phase were harvested by centrifugation from the synthetic medium and resuspended in the same medium to a density of about 1.0. The nonradioactive sugar concentration was 5 g/liter. After 30 min at 36°C, 0.5 μCi of D-[*U*-^{14}C]glucose or 0.4 μCi of D-[*U*-^{14}C]xylose was added per ml. After 6 h, 2.5 ml of the cell suspension was removed and filtered through a cellulose acetate membrane filter (pore size, 0.45 μm) and washed three times with 1.0 ml of the synthetic medium (36°C). The filters were dried and counted as described above for uptake studies.

Effect of butanol on cell growth and on glucose and xylose utilization. Different amounts of butanol were added to culture growing in the exponential phase to study the inhibitory effects of this compound on the growth of *C. acetobutylicum* cells. Growth was determined by measuring the optical density at 600 nm. Sugar uptake and incorporation of ^{14}C into whole cells were determined as described above, but the reactions were initiated by adding the sugar and various concentrations of butanol.

RESULTS

Glucose and xylose accumulations in *C. acetobutylicum*. The time courses of D-glucose and D-xylose uptake by *C. acetobutylicum* are shown in Fig. 1. As calculated from the plot of $1/V$ versus $1/S$, the V_{max} values of glucose and xylose uptake were 2,080 and 2,850 nmol/g per min, respectively, and the apparent K_m of 5×10^{-3} M for xylose was considerably greater than that observed for glucose (1.25×10^{-5} M). The low affinity of the xylose uptake system explains why the external concentration of xylose was 10^{-3} M, but the external concentration of glucose was 2.5×10^{-5} M (Fig. 1). The K_m values correlated with growth variations obtained for different glucose and xylose concentrations (Fig. 2). With a K_m of 0.750 g/liter, no growth was observed on xylose for sugar concentrations of 1 g/liter, whereas with a K_m of 2.25 mg/liter, initial glucose concentrations of 0.5 and 1 g/liter resulted in an increase in the optical density. A study of the activities of the glucose and xylose transport systems at various stages of culture growth showed that there were considerable variations (Table 1). A six- to sevenfold increase in the rate of sugar uptake coincided with the end of the logarithmic phase.

Effect of glucose on the xylose utilization. Fermentation was performed with glucose and xylose present as substrates and inoculated with cells grown on glucose. The results are shown in Fig. 3. The density of the culture increased until shortly after the glucose was exhausted; the culture then went through a diauxic lag of more than 2 h and then resumed growth during xylose dissimilation. Only cell suspensions of xylose-grown *C. acetobutylicum* incorporated xylose, whereas glucose was incorporated by cells grown on either substrate. When mixture of sugars were inoculated

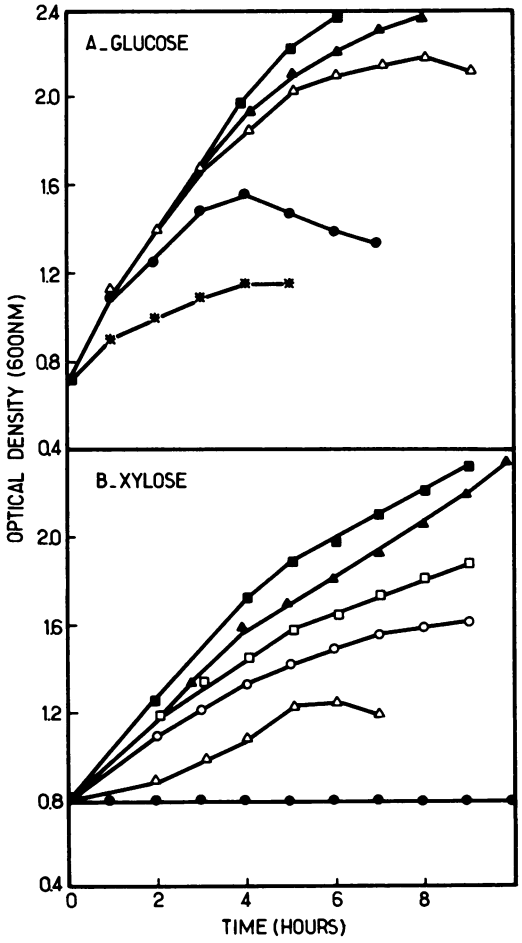

FIG. 2. Influence of the glucose and xylose concentrations on cell growth. The initial sugar concentrations (in grams per liter) used were 0.5 (*); 1 (●); 2 (△); 4 (○); 5 (□); 10 (▲); and 20 (■).

TABLE 1. Sugar transport activity during glucose and xylose fermentation by *C. acetobutylicum*

Substrate	Fermentation time (h)	pH	Growth[a]	Amt of sugar degraded (g/liter)	Amt of product formation (g/liter):					Rate of uptake (nmol/min per g)
					Acetone	Butanol	Ethanol	Acetic acid	Butyric acid	
Glucose	0	6.6	0.2	0						
	8	5.0	2.2	6.5	0.1	0.4	0.1	2.2	2.1	310
	14	4.4	2.4	12	0.2	0.9	0.1	2.6	3.0	1,090
	38	4.2	3.2	37	3.1	6.4	1.1	2.5	1.0	1,920
	62	4.0	4.2	51	3.6	11.4	1.7	2.5	0.5	400
	86	4.5	3.2	66	4.3	13.6	2.4	2.3	0.7	150
Xylose	0	6.4	0.4	0						
	15	4.6	1.3	8	0.1	0.6	0.10	2.8	2.2	370
	32	4.5	2.0	22	0.3	3.0	0.2	2.3	3.3	1,030
	52	4.4	2.9	34	1.2	5.9	0.6	1.5	0.5	1,760
	63	4.5	3.5	42	2.2	7.5	0.7	1.4	0.7	760
	87	4.5	4.0	48	3.3	8.9	0.9	1.3	1.5	0
	100	4.5	1.9	48	3.3	9	0.9	1.3	1.5	0

[a] Growth was estimated by measurement of the optical density.

with cells grown on xylose, formation of the xylose system uptake was repressed, and the activity was subsequently diluted until the glucose was nearly exhausted from the medium (Table 2).

Effects of butanol concentrations on growth of *C. acetobutylicum*. Butanol and acetone were the main products at the end of the fermentation (Table 1). Inhibition of cell growth was studied by challenging cultures with various concentrations of each product. No inhibitory effect of acetone and ethanol was observed on the growth of *C. acetobutylicum*; for example, a concentration of 20 g of acetone per liter or 20 g of ethanol per liter, concentrations never attained during a fermentation (Table 1), did not cause any growth inhibition. On the other hand, the inhibitory effect of butanol was most pronounced when growth occurred on xylose rather than on glucose (Fig. 4). Butanol completely inhibited the growth of *C. acetobutylicum* at a concentration of 14 g/liter for cultures growing on glucose and at 8 g/liter for cultures growing on xylose.

Effects of butanol on the initial uptake of sugar and on the incorporation of ^{14}C into the whole cells. The initial rate of transport of glucose and xylose was studied as a function of the butanol concentration. The results clearly show that the transport process decreases as the butanol concentration is increased (Fig. 5). The initial rate of xylose transport was inhibited by 50% at a butanol concentration of 4.5 g/liter, whereas the initial rate of glucose transport was inhibited by 50% at 7 g/liter. A similar decrease of the ^{14}C incorporation was noted when challenged with increasing butanol concentration. Xylose and glucose incorporation into cell materials was inhibited by 50% at butanol concentrations of 7 and 10.5 g/liter, respectively. The levels of butanol that cause inhibi-

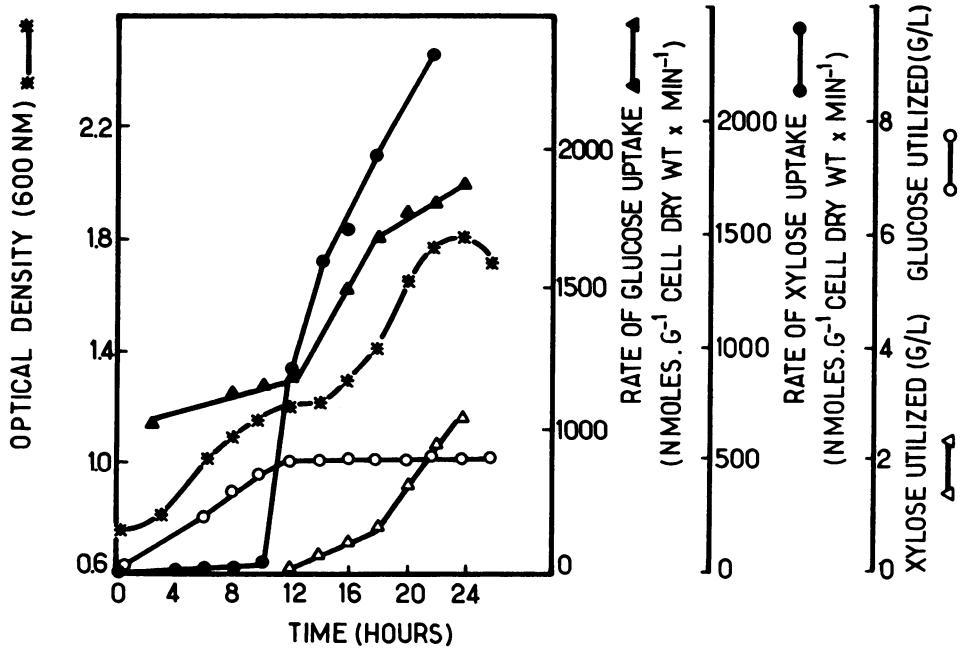

FIG. 3. Combined-substrate metabolism on glucose and xylose. Synthetic medium with 0.2% glucose and 0.4% xylose was inoculated with cells grown on glucose. Sugar uptake activities were determined as described in the text.

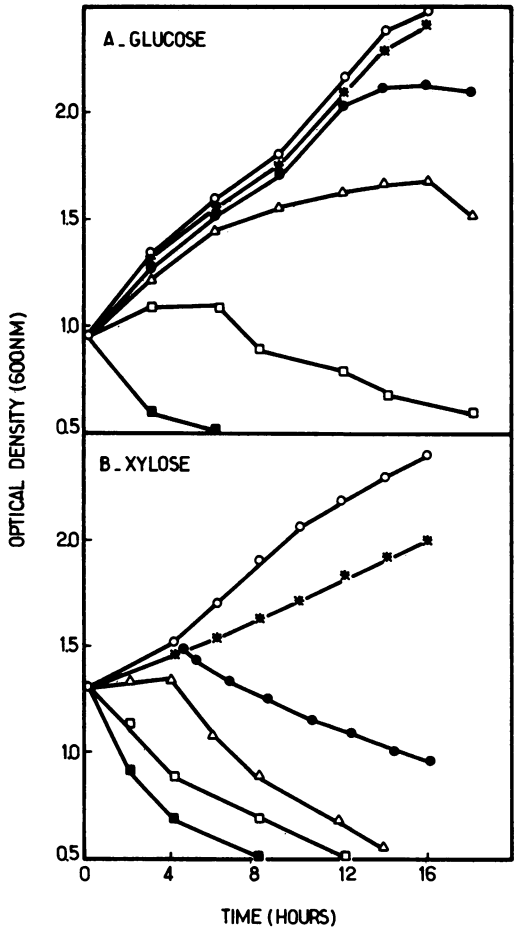

FIG. 4. Growth on *C. acetobutylicum* on glucose (1%) and xylose (1%) at the following butanol concentrations (in grams per liter): *, 3; ●, 8; △, 10; □, 14; ■, 20. ○, Control.

tion of growth and sugar transport as well as sugar incorporation are comparable to that obtained during a typical fermentation (Table 1).

DISCUSSION

Fermentation of glucose and xylose by *C. acetobutylicum* was examined. The growth of *C. acetobutylicum* on xylose is preceded by the induction of a xylose entry system. Acquisition of this enzyme allows the organism to channel the carbon of xylose into the Embden-Meyerhof-Parnas pathway via the transketolase-transaldolase sequence (1, 2). The induced synthesis of the transport system is repressed by inclusion of glucose in the growth medium. This fact explains why, regardless of the inoculum energy source (either glucose or xylose), a more rapid depletion of glucose was observed when *C. acetobutylicum* was grown on the sugar mixture (10). In comparison with traditional hexose sugar fermentation, the uptake system has a much lower affinity for xylose than it does for glucose, but the use of the culture conditions described previously (10) allows good yields of solvents to be obtained with either glucose or xylose. In agreement with the K_m values determined for sugar uptake at nonsaturating initial values, residual concentrations of glucose measured are below the limits of detection, whereas residual xylose concentrations are about 1 g/liter.

The early work on D-xylose fermentation indicated that less xylose than glucose is used as substrate (6, 10, 16). A possible explanation for this observation is that *C. acetobutylicum* grown on xylose is more strongly inhibited by solvent production than is *C. acetobutylicum* grown on glucose. Among the end products of the fermentation, butanol is the most potent inhibitor. It also has been reported that butanol, and not acetone, is the primary toxic substance in the acetone-butanol fermentation (9). The results obtained in this study indicate that growth inhibition on both substrates is correlated with the inhibitory effects of butanol on the sugar transports, which are most pronounced

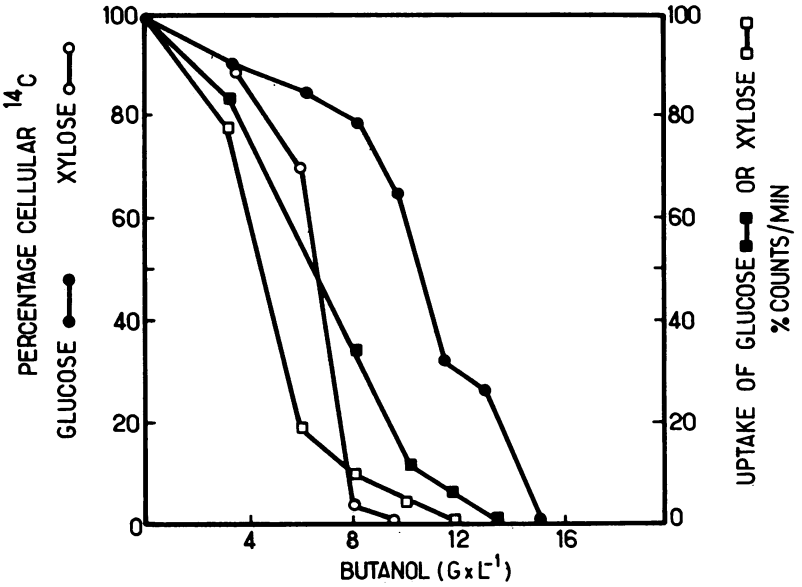

FIG. 5. Initial uptake of sugar and incorporation of ^{14}C into whole cells as a function of butanol concentration. Symbols: ■, glucose uptake; □, xylose uptake; ●, incorporation of [^{14}C]glucose; ○, incorporation of [^{14}C]xylose.

when growth occurs on xylose rather than on glucose. These data indicate an inhibition of the membrane functionality at concentrations similar to those formed during xylose and glucose fermentation. The final concentration of butanol in the broth is shown to play an important role in the ability of *C. acetobutylicum* to utilize either glucose or xylose. This concentration is found to vary with the nature of the sugar, i.e., about 14 g/liter for the glucose-grown cells and 9 g/liter for the xylose-grown cells. Therefore, to improve this process, microorganisms with extended tolerance to butanol must be found (14). Nevertheless, for a sum of glucose and xylose which does not exceed 60 g/liter and for a ratio near to 1, the mixed carbon source is nearly exhausted from the medium and converted to solvents at a rate of 30% (10). This is an important positive factor in considering the use of a single organism such as *C. acetobutylicum* for the conversion of both hexose and pentose to solvents.

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TABLE 2. Induction and repression of the xylose permease

Substrate	Fermentation time (h)	pH	Growth[a]	Amt of sugar degraded (g/liter)		Rate of sugar uptake (nmol/min per g)	
				Glucose	Xylose	Glucose	Xylose
A[b]	0	5.7	0.3	0	0	1,700	0
	5	5.2	0.4	2	0.35	800	0
	9	4.4	1.3	8	0.35	1,800	340
	24	4.3	2.0	11.8	2.4	1,800	800
	32	4.4	2.4	12	4.0	2,000	1,900
	62	4.3	2.4	12	7.0	1,800	1,400
B[c]	0	6.0	0.2	0	0	1,900	1,400
	4	5.6	0.3	1	0	1,340	540
	8	5.0	1.0	2	0	1,680	390
	10	4.6	1.3	4	0	1,400	390
	22	4.7	2.6	7.8	1.2	1,500	200
	28	4.7	3.0	9.6	2	1,600	740
	48	4.6	2.0	10	5.7	2,000	920
	72	4.5	1.5	10	10	1,700	900

[a] Growth was estimated by measurement of the optical density.
[b] Combined substrate (glucose [12 g/liter] plus xylose [10 g/liter]) inoculated with cells grown on glucose.
[c] Combined substrate (glucose [10 g/liter] plus xylose [12 g/liter]) inoculated with cells grown on xylose.

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