APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1982, p. 1277-1281
0099-2240/82/121277-05$02.00/0

Vol. 44, No. 6

Autolytic Activity and Butanol Tolerance of *Clostridium acetobutylicum*

ANDRE VAN DER WESTHUIZEN, DAVID T. JONES, AND DAVID R. WOODS*
Department of Microbiology, University of Cape Town, Rondebosch, 7700 South Africa

Received 25 March 1982/Accepted 31 August 1982

The effects of acetone and butanol on the growth of vegetative cells and the stability of swollen-phase bright-stationary-phase cells (clostridial forms) of *Clostridium acetobutylicum* P262 and an autolytic deficient mutant (*lyt-1*) were investigated. There was little difference in the sensitivity of strain P262 and the *lyt-1* mutant vegetative cells and clostridial forms to acetone. The stability of the different morphological stages was unaffected by acetone concentrations far in excess of those encountered in factory fermentations. Butanol concentrations between 7 and 16 g/liter, which are within the range obtained in industrial fermentations, increased the degeneration of strain P262 clostridial forms but had no effect on the stability of *lyt-1* clostridial forms which never underwent autolysis. Vegetative cells of the *lyt-1* mutant were able to grow in higher concentrations of butanol than strain P262 vegetative cells. It was concluded that there is a relationship between butanol tolerance and autolytic activity.

In molasses media *Clostridium acetobutylicum* produces the solvents acetone, butanol, and ethanol in the ratio 3:6:1, respectively (7). Industrial acetone, butanol, and ethanol fermentation is limited by the sensitivity of the bacteria to concentrations of butanol above 13 g/liter (4, 6). The highest butanol concentrations obtained with commercial strains are approximately 13 g/liter (6), and this level of butanol determines the starting concentrations of fermentable sugar (6 to 6.5%, wt/vol).

During preliminary studies on the effect of butanol on *C. acetobutylicum*, it was observed that degeneration and eventual total lysis of the majority of the cells during the later stages of the fermentation occurred when the level of butanol in the culture was high. Previously, the autolysis of *C. acetobutylicum* cultures at a critical stage in the industrial fermentation has been reported (2). This stage also involved the lysis of swollen cigar shaped clostridial forms which were shown to be associated with the production of neutral solvents (3). We therefore investigated the relationship between butanol toxicity and autolytic activity. Our studies were assisted by the use of a pleiotropic autolysis-deficient mutant, *lyt-1*, which has been described previously (1). The *lyt-1* mutant produces less autolysin than the parent strain, and it has an altered cell wall which is more resistant to both its own and parent autolysin.

MATERIALS AND METHODS

Bacterial strains and media. *C. acetobutylicum* P262 was supplied by National Chemical Products Ltd., Germiston, South Africa, and was utilized in previous studies (1-3, 8). The isolation and characterization of the autolysis-deficient *lyt-1* mutant has been described previously (1). The strains were grown at 34°C in the buffered *Clostridium* basal medium described by O'Brien and Morris (5) and the molasses fermentation medium (MFM) of Barber et al. (2). All manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma-Scientific, Marietta, Ohio). The P262 and *lyt-1* strains were maintained at 4°C as clean spore preparations in distilled water. Spores were activated by heat shocking at 75°C for 2 min followed by cooling on ice before inoculation into medium.

Growth measurements. Cell growth was monitored turbidimetrically at 600 nm. Total bacterial counts and clostridial stage counts were carried out with a Thoma counting chamber (Weber Scientific International, Lancing, England) and a Zeiss photomicroscope fitted with phase contrast optics.

Analysis of solvents. Acetone, butanol, and ethanol were determined by gas chromatography as described previously (2).

Cellular autolysis. Cellular autolysis in 0.04 M sodium phosphate buffer (pH 6.3) was measured as described previously by Allcock et al. (1).

Effect of solvents. Oxygen-free acetone and butanol solutions were prepared by placing the prewarmed solvents (60°C) in the anaerobic glove box for 48 h. The solvents were added to early exponential phase or clostridial stage cultures.

RESULTS

Growth and solvent production. Growth, clostridial stage formation, and solvent production by the P262 strain and the *lyt-1* mutant were determined in MFM (Fig. 1). Although minor variations occurred in different experiments, the

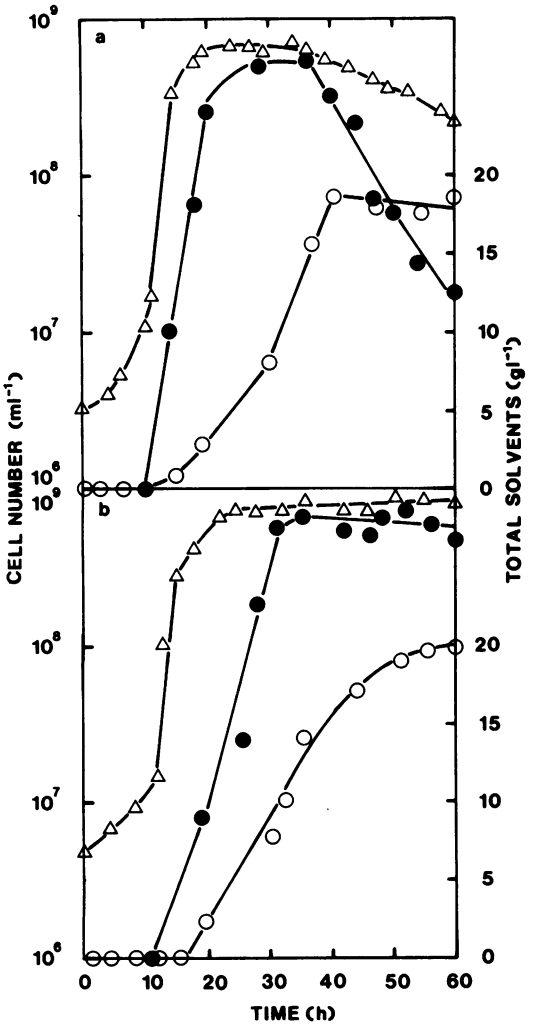

FIG. 1. Growth, clostridial form, and solvent production by *C. acetobutylicum* P262 (a) and *lyt-1* mutant (b) strains in MFM. Symbols: total cell count, △; total clostridial form count, ●; and total solvents, ○.

total cell counts and the number of clostridial forms were very similar (from 0 to 35 h) in the two cultures. After 35 h, the P262 culture began to degenerate and finally to lyse completely so that at 60 h approximately 95% of the clostridial forms had disappeared. In the *lyt-1* mutant culture, the clostridial forms remained intact, and the total cell counts and clostridial form counts remained relatively constant between 35 and 60 h.

The solvent profile for the P262 strain was similar to that reported previously (3). Solvents were detected after 15 h when the culture was in stationary phase, and solvent production correlated with the appearance of clostridial forms. Solvent levels increased between 18 and 40 h before plateauing. The solvent profile for the *lyt-1* mutant was similar to that of the P262 strain between 15 and 40 h. However, with the *lyt-1* mutant the plateau effect was not as marked after 40 h, and a more gradual decrease in solvent production occurred. The *lyt-1* mutant consistently produced slightly higher levels of solvent than the P262 strain. The highest butanol concentrations produced in MFM by the P262 strain and the *lyt-1* mutant were 13.26 and 14.2 g/liter, respectively. At this concentration, solvent production appeared to be inhibited even though substrate exhaustion had not occurred.

Effect of solvents on vegetative cells. The effect of acetone and butanol on the growth of vegetative cells was determined. Acetone (20 g/liter) did not affect the growth of strain P262 and *lyt-1* mutant cells (Fig. 2). Higher concentrations of

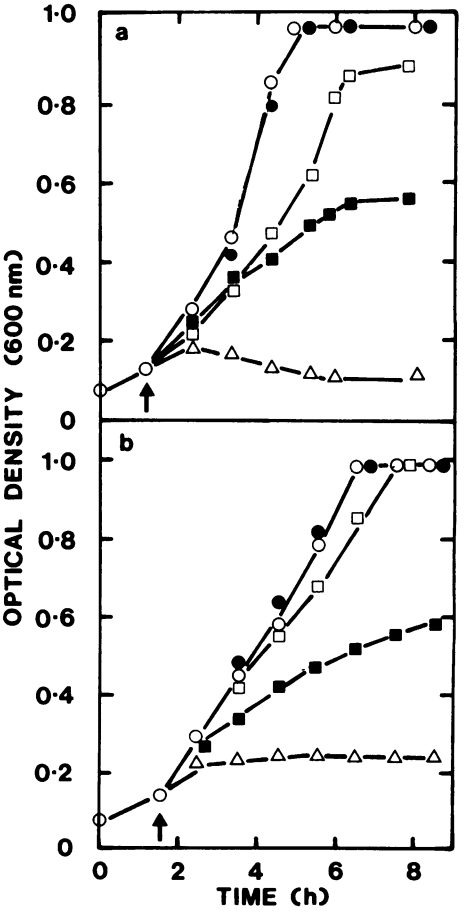

FIG. 2. Effect of acetone on the growth of vegetative cells of *C. acetobutylicum* P262 (a) and the *lyt-1* mutant (b). Acetone concentrations (g/liter); control (no addition), ○; 20, ●; 30, □; 40, ■; and 50, △. Arrow indicates the time of addition of acetone.

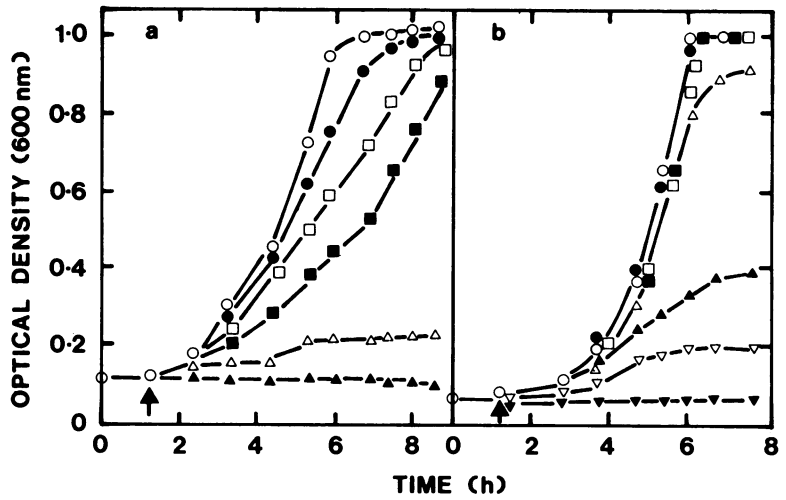

FIG. 3. Effect of butanol on the growth of vegetative cells of *C. acetobutylicum* P262 (a) and the *lyt-1* mutant (b) in CBM. Butanol concentrations (g/liter); control (no addition), ○; 4, ●; 6, □; 8, ■; 10, △; 12, ▲; 15, ▽; and 18, ▼. Arrow indicates the time of addition of butanol.

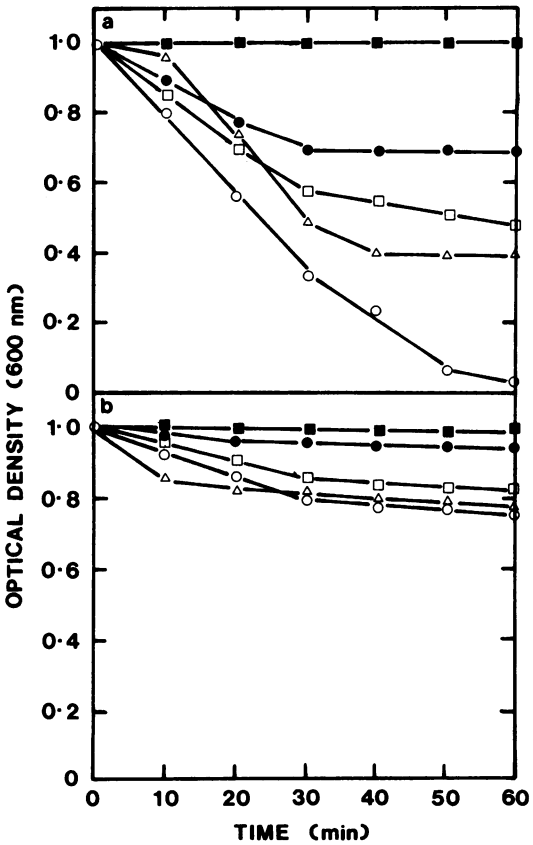

FIG. 4. Effect of butanol on the autolysis of exponential phase cells of *C. acetobutylicum* P262 (a) and the *lyt-1* mutant (b). Exponential cells were harvested and suspended in sodium phosphate buffer (0.04 M, pH 6.3) containing the following concentrations of butanol (g/liter): Control (no butanol added), ○; 10, △; 20, □; 30, ●; and 40, ■.

acetone (30 g/liter) inhibited the rate and total amount of growth of the P262 strain but had little effect on the *lyt-1* mutant. Both strains were inhibited to the same extent by 40 and 50 g of acetone per liter.

Vegetative cells of the P262 strain were more sensitive to butanol than vegetative cells of the *lyt-1* mutant (Fig. 3). The growth rate of the strain P262 cells was progressively reduced by concentrations of butanol between 4 and 8 g/liter, and growth was prevented by 10 g of butanol per liter. The growth of the *lyt-1* mutant was not affected by 8 g of butanol per liter. A butanol concentration of 10 g/liter had little effect on the growth of *lyt-1* cells, which were inhibited by 12 g of butanol per liter.

The effect of butanol on the autolysis of vegetative cells in sodium phosphate buffer (0.04 M, pH 6.3) is shown in Fig. 4. In the absence of butanol, cellular autolysis was markedly less in the *lyt-1* mutant. For both P262 and *lyt-1* cells, the amount of cellular autolysis was reduced by the addition of butanol, and at 40 g of butanol per liter, cellular autolysis was prevented.

Effect of solvents on clostridial forms. Acetone concentrations between 0 and 40 g/liter had no effect on the degeneration of strain P262 and *lyt-1* mutant clostridial forms.

The effect of butanol on clostridial forms was determined by the addition of butanol to stationary phase cultures in which the majority of cells had been converted to clostridial forms. Increasing the concentration of butanol from 7.02 to 16.72 g/liter caused a marked increase in the degeneration of the strain P262 clostridial forms (Fig. 5). Relatively high butanol concentrations (26.03 and 36.59 g/liter) had the opposite effect and 36.59 g of butanol per liter inhibited the

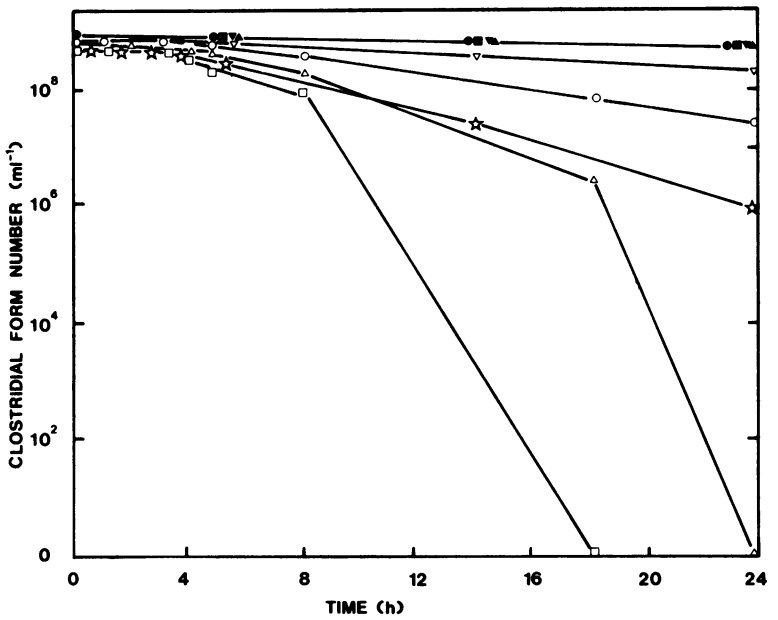

FIG. 5. Effect of butanol on clostridial forms of *C. acetobutylicum* P262 and the *lyt-1* mutant. Butanol was added to clostridial forms to give the following final concentrations (g/liter). Strain P262: control (no extra addition of butanol), 7.02 (○); 11.68 (△); 16.72 (□); 26.03 (☆); and 36.59 (▽). *Lyt-1* mutant: control (no extra addition of butanol) 7.12 (●); 14.27 (▲); 25.64 (■); and 38.19 (▼).

degeneration of strain P262 clostridial forms. *lyt-1* mutant clostridial forms were not affected by concentrations of butanol between 7.12 and 38.19 g/liter, and no degeneration was observed.

DISCUSSION

A previous paper reported that the clostridial forms were responsible for the conversion of acids to neutral solvents and that sporulation mutants which were unable to form clostridial stages did not produce solvents (3). In the acetone, butanol, and ethanol process, butanol toxicity will be most important and apparent during the stationary growth phase when clostridial forms predominate. We therefore determined the effect of butanol on clostridial forms and concluded that there is a relationship between butanol toxicity and clostridial form degeneration and lysis. Clostridial forms from the *lyt-1* mutant were not induced to degenerate by concentrations of butanol which increased the rate of degeneration of strain P262 clostridial forms. It is suggested that the *lyt-1* mutant clostridial forms are more resistant to butanol-induced degeneration and that one of the effects of butanol concentrations between 7 to 16 g/liter could be the induction of autolysis and degeneration of strain P262 clostridial forms. The concentrations of butanol which induce degeneration of clostridial forms were within the concentration ranges obtained in factory fermentations. Higher concentrations of butanol which are lethal to the cells had the reverse effect and prevented degeneration and autolysis. However these high concentrations would not be encountered by the P262 strain in a normal fermentation.

The complete inhibition of solvent production after 40 h observed with the P262 strain in MFM suggests that a threshold level is reached which results in the inhibition of further solvent production. The gradual decrease in solvent production obtained with the *lyt-1* mutant suggests that solvent production in this strain is not subject to inhibition by the same mechanism.

Although not directly important as regards solvent production and butanol toxicity, it is significant that vegetative cells of the *lyt-1* mutant were also more resistant to butanol than the parent P262 strain cells. The mechanism of the increased resistance of *lyt-1* mutant vegetative cells is unclear since butanol did not induce or increase autolysis of the vegetative cells. The increased butanol tolerance shown by *lyt-1* vegetative cells supports our conclusion that there is a relationship between butanol tolerance and autolytic activity.

Although the *lyt-1* clostridial forms did not degenerate and remain phase bright and intact in the presence of concentrations of butanol between 0 and 38 g/liter, the ability of the clostridial forms to produce solvents at high butanol

concentrations was inhibited. Nevertheless the pleiotropic *lyt-1* mutant is butanol tolerant and its potential to produce higher solvent levels is being investigated.

ACKNOWLEDGMENT

This work was supported by a grant to D. R. Woods from Sentrachem Limited, South Africa.

LITERATURE CITED

1. **Allcock, E. R., S. J. Reid, D. T. Jones, and D. R. Woods.** 1981. Autolytic activity and an autolysis-deficient mutant of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **42**:929–935.
2. **Barber, J. M., F. T. Robb, J. R. Webster, and D. R. Woods.** 1979. Bacteriocin production by *Clostridium acetobutylicum* in an industrial fermentation process. Appl. Environ. Microbiol. **37**:433–437.
3. **Jones, D. T., A. Van Der Westhuizen, S. Long, E. R. Allcock, S. J. Reid, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43**:1434–1439.
4. **Mc Cutchan, W. N., and R. J. Hickey.** 1954. The butanol-acetone fermentations, p. 347–388. *In* L. A. Onderkofler and R. J. Hickey (ed.), Industrial fermentations, vol. 1. Chemical Publishing Co., New York.
5. **O'Brien, R. W., and J. G. Morris.** 1971. Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. J. Gen. Microbiol. **68**:307–318.
6. **Ryden, R.** 1958. Development of anaerobic fermentation processes: acetone-butanol, p. 125–148. *In* R. Steel (ed.), Biochemical engineering. Heywood Books, London.
7. **Spivey, M. J.** 1978. The acetone/butanol/ethanol fermentation. Proc. Biochem. **13**:2–5.
8. **Webster, J. R., S. J. Reid, D. T. Jones, and D. R. Woods.** 1981. Purification and characterization of an autolysin from *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **41**:371–374.