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Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*

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SUMMARY

The objective of this work was to optimize butanol formation in the acetone-butanol-ethanol (ABE) fermentation by examining the level of buffering as it affects the dissociation of butyric acid to the less toxic butyrate anion. Experiments were carried out in batch culture using chemically defined (P2) or complex media containing various buffering agents. These included salts of acetate, citrate, phosphate, nitrate, or bicarbonate, representing a range of pK_a values and buffering capacities. Growth in highly buffered medium was found to increase the stationary phase cell density, carbohydrate utilization, and the final butanol concentration. At higher levels of buffering, increased growth and elevated concentrations of butyric acid were required to initiate solventogenesis, suggesting the involvement of a critical threshold level of undissociated butyric acid.

INTRODUCTION

The fermentation pattern of *Clostridium acetobutylicum* has been well documented [9,14]. An initial acidogenic phase gives way to a solventogenic phase at a particular point in the growth cycle. For many years, it was believed that a low pH (ca. 4.5), achieved during the acidogenic phase, was both necessary and responsible for the initiation of solventogenesis [15]. More recent evidence, however, suggests that solvent can be formed at a neutral pH and it is the build-up of a threshold level of organic acids, especially butyric acid, that leads to solventogenesis [7,12]. Although butanol has been shown to inhibit cell growth and cytoplasmic membrane functionality, butyric acid is the fermentation product which is the most inhibitory to *C. acetobutylicum* [3]. Recent evidence also shows that the undissociated forms of organic acids are more inhibitory to various clostridia than are the corresponding ionized species [2,6,17]. These protonated acids act as uncoupling agents upon the cell membrane, causing a collapse of the transmembrane pH gradient [6].

Although it is evident that protonated butyric

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acid is inhibitory to *C. acetobutylicum*, it is important that some accumulation of this acid be allowed to occur. Butyric acid present in the environment re-enters the cell and is reduced to butanol [5]. Furthermore, butyric acid must be in the protonated form to permeate the cell membrane [10].

Based on the above information, it was speculated that increasing the buffering capacity of the growth medium would be a simple method for achieving a high concentration of the less toxic butyrate ion before inhibitory levels of undissociated butyric acid were achieved. This would allow for increased cell growth as well as provide a greater amount of butyrate to serve as a precursor for butanol production. The objective of this study, therefore, was to investigate the effect of increasing buffering capacity in chemically defined and complex growth media on growth and butanol production by *C. acetobutylicum*.

MATERIALS AND METHODS

All experiments were carried out using *C. acetobutylicum* ATCC 824. Batch cultures (200 ml) were grown under anaerobic atmosphere (85% N_2, 10% CO_2, and 5% H_2) in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) at 37°C in chemically defined minimal or complex media. P2 minimal medium (R. Machanoff, personal communication) is a modification of the synthetic medium described by Monot et al. [13]. It contains (per liter of distilled water): 10, 20, 40 or 80 g glucose, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 2.2 g ammonium acetate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g NaCl, 1.0 g *p*-aminobenzoic acid, 0.01 g biotin and 1.0 g thiamine. Unless otherwise indicated, P2 medium contained 2% (w/v) glucose. Media with different buffering capacities were obtained by varying the phosphate concentration as K_2HPO_4 and KH_2PO_4 in equal weight amounts over a range of 6.5 to 65 mM. Media with various effective buffering ranges were obtained by using different ammonium salts to prepare the P2 buffer. The salts were selected on the basis of the pK_a of their corresponding acids. The salts used were: ammonium nitrate (pK_a of nitric acid < 1), ammonium acetate (pK_a of acetic acid $= 4.72$), dibasic ammonium citrate (pK_a of the predominant form of citric acid $= 4.77$) and ammonium bicarbonate (pK_a of carbonic acid $= 10.33$ [$HCO_3^- \rightarrow CO_3^{2-}$]). The salts were added so that the amounts of ammonium were equimolar. The chemically defined media produced in this way were designated nitrate P2, acetate P2, citrate P2 and bicarbonate P2, respectively.

It has been shown that the phosphate concentration of the growth medium affects butanol production by *C. acetobutylicum* [1]. In order to evaluate buffering effects while reducing the effect of phosphate per se, *C. acetobutylicum* was also grown in modified P2 medium containing 1 g/l ammonium nitrate and 1 g/l dibasic ammonium phosphate in lieu of ammonium acetate, KH_2PO_4 and K_2HPO_4 and buffered by three different non-metabolizable 'good' buffers with different effective buffering ranges. Titration against acetic acid indicated that this level of phosphate contributed very little to the buffering capacity of modified P2 medium, while providing phosphate at a level 5-fold above that considered limiting [1]. The buffers used were: 2-[*N*-morpholino]ethanesulfonic acid (MES, pK_a = 6.1), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, $pK_a = 7.5$) and 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES, $pK_a = 9.3$) (Sigma, St. Louis, MO). These buffers were used at concentrations of 15 mM and 45 mM and were adjusted so that the initial pH was at the midpoint of the effective buffering range.

The effect of buffering in TGY [8], soluble starch and corn starch soluble complex media (SSM; CSSM; [11]) was examined by adding both KH_2PO_4 and K_2HPO_4 at either 6.5 or 65 mM.

C. acetobutylicum cells were stored at 4°C in cooked meat medium (CMM; Difco, Detroit, MI) containing 1% glucose. Prior to inoculation, the cells were heat-shocked at 80°C for 10 min. The heat-shocked cells were grown in P2 medium until mid-exponential phase ($A_{600} = 0.4$–0.6). Ten milliliter aliquots of the cell suspension were washed three times with one volume P2 buffer (0.5 g/l KH_2PO_4, 0.5 g/l K_2HPO_4 and 2.2 g/l ammonium

acetate) and the cells were suspended in 5 ml P2 buffer. These 5 ml aliquots served as inocula for the various experiments.

Analytical methods

Growth was monitored by following absorbance at 600 nm using a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, NY) or by determination of cell dry weight. Cells were filtered through a micropore membrane filter (0.45 micron, Millipore, Bedford, MA) and dried at 75°C under vacuum to a constant weight. The pH was measured using a Beckman 45 pH meter (Beckman Instruments, Inc., Irvine, CA). The concentration of butanol was measured by gas-liquid chromatography using a Hewlett-Packard 5700A gas chromatograph equipped with a 1.8 m × 2 mm column packed with 80/100 mesh Carbopack C/0.1% SP-1000 and a flame ionization detector. The concentration of butyric acid was determined in supernatants which had been acidified with formic acid. Two microliter samples were injected into a 1.8 m × 2 mm column packed with 0.3% Carbowax 20 M/0.1% H_3PO_4 on 60/80 mesh Carbopack C (Supelco, Inc., Bellefonte, PA). Analysis of the data was accomplished using a Hewlett-Packard 3390A integrator (Hewlett-Packard, Inc., Avondale, PA). The concentration of undissociated butyric acid was calculated from total butyric acid using the Henderson-Hasselbalch equation and a pK_a value of 4.82 for butyric acid. Glucose levels were determined colorimetrically using the glucose oxidase/peroxidase system (Sigmakit No. 510, Sigma Chemicals).

RESULTS

Effect of buffering in nitrate P2 defined medium

The effect of various concentrations of K_2HPO_4 and KH_2PO_4 added to nitrate P2 medium on the growth response of *C. acetobutylicum* can be seen in Fig. 1. The addition of various levels of phosphate to nitrate P2 medium had a dramatic effect on the growth profile of this microorganism, with the greatest amount of growth being achieved at the highest level of added phosphate. The corresponding fermentation pH profile of *C. acetobutylicum* in nitrate P2 medium containing various levels of added phosphate can be seen in Fig. 2. Although in all cases the pH is seen to decrease over the course of this fermentation, the decrease in pH over time was minimized by increasing the phosphate concentration. This effect is particularly dramatic over the period of time (15–35 h) which corresponds to logarithmic growth. The improved growth response of *C. acetobutylicum* in more highly phos-

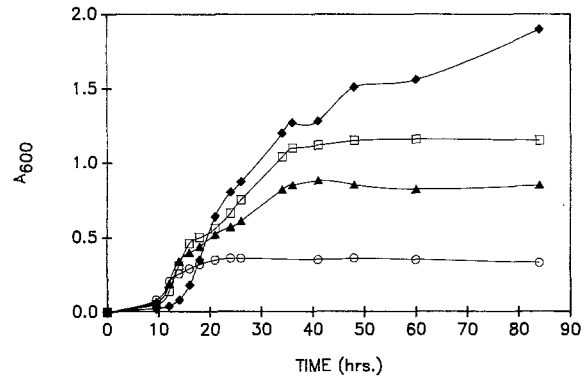

Fig. 1. The effect of various concentrations of phosphate added to nitrate P2 medium on the growth response of *C. acetobutylicum*. Symbols: ○, 6.5 mM; ▲, 13 mM; □, 26 mM; ◆, 65 mM KH_2PO_4 and K_2HPO_4.

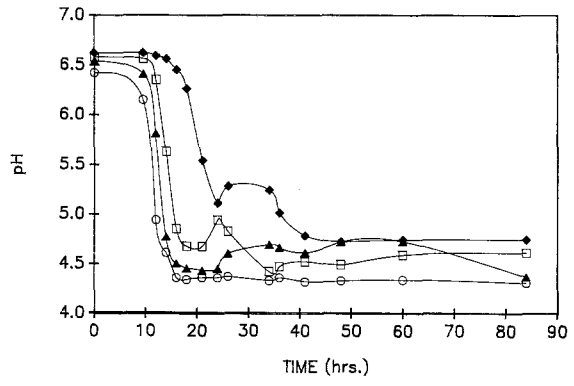

Fig. 2. The pH profile of *C. acetobutylicum* during the course of a small-scale batch fermentation in nitrate P2 medium containing various concentrations of added phosphate. Symbols: ○, 6.5 mM; ▲, 13 mM; □, 26 mM; ◆, 65 mM KH_2PO_4 and K_2HPO_4.

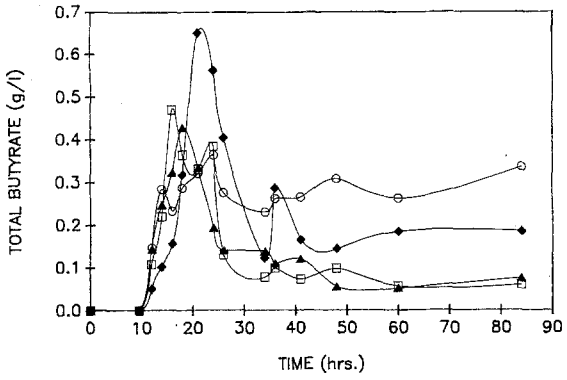

Fig. 3. Total butyrate produced by *C. acetobutylicum* during the course of a small-scale batch fermentation in nitrate P2 medium containing various concentrations of added phosphate. Symbols: ○, 6.5 mM; ▲, 13 mM; □, 26 mM; ◆, 65 mM KH_2PO_4 and K_2HPO_4.

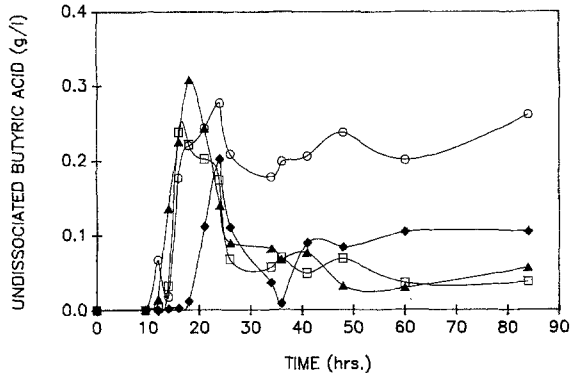

Fig. 5. Calculated levels of undissociated butyric acid present during growth of *C. acetobutylicum* in nitrate P2 medium containing various concentrations of added phosphate. Symbols: ○, 6.5 mM; ▲, 13 mM; □, 26 mM; ◆, 65 mM KH_2PO_4 and K_2HPO_4.

phate-buffered nitrate P2 (Fig. 1) corresponds to increased amounts of butyric acid (up to 0.65 g/l; Fig. 3) and butanol (up to 10.1 g/l; Fig. 4) being produced by this microorganism. The reason for the decrease in the concentration of butanol from a high of 10.1 to ca. 6.5 g/l in 65 mM phosphate-buffered medium is not clear at this time. The calculated levels of undissociated butyric acid in phosphate-buffered nitrate P2 medium over the course of the fermentation are shown in Fig. 5. These data indicate that in nitrate P2 medium containing 6.5 mM phosphate, the equilibrium is maintained toward the more inhibitory protonated form of the acid.

The levels of growth, pH, total butyrate and undissociated butyric acid at initiation of butanol production by *C. acetobutylicum* in nitrate P2 medium

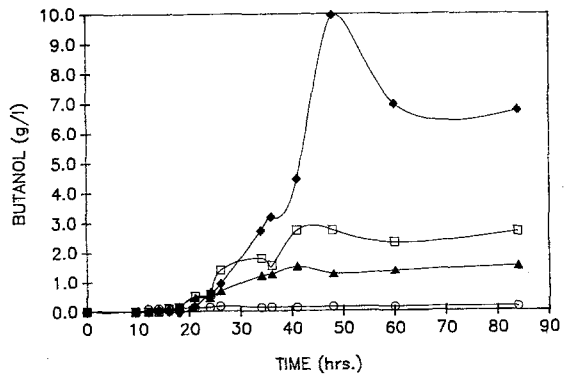

Fig. 4. Butanol production by *C. acetobutylicum* during the course of a small-scale batch fermentation in nitrate P2 medium containing various concentrations of added phosphate. Symbols: ○, 6.5 mM; ▲, 13 mM; □, 26 mM; ◆, 65 mM KH_2PO_4 and K_2HPO_4.

Table 1

Growth response, pH, total butyrate and undissociated butyric acid at initiation of measurable extracellular butanol production by *C. acetobutylicum* in nitrate P2 medium containing various concentrations of phosphate

Phosphate concentration (mM)[a]	Growth[b]	pH	Total butyrate (g/l)	Undissociated butyric acid (g/l)[c]
6.5	0.23	4.78	0.22	0.18
13	0.34	4.77	0.25	0.14
26	0.39	5.24	0.35	0.14
65	0.50	5.90	0.48	0.11

[a] Final phosphate concentration in nitrate P2 medium was achieved by the addition of KH_2PO_4 and K_2HPO_4 in equal weight amounts.

[b] Measured as absorbance at 600 nm.

[c] Calculated using the Henderson-Hasselbalch equation and a pK_a of 4.82 for butyric acid.

containing various concentrations of phosphate can be seen in Table 1. Higher levels of growth and total butyrate are evident at the initiation of solventogenesis in more highly buffered nitrate P2 medium, while the level of undissociated butyric acid is reasonably constant over a range of 0.11–0.18 g/l. These results suggest that a threshold level of undissociated butyric acid may be required for the initiation of butanol production by *C. acetobutylicum*. Similar results for growth, pH, total butyrate and undissociated butyric acid were obtained in acetate, citrate and bicarbonate-based P2 media containing 6.5 or 65 mM phosphate (data not shown).

Effect of buffering on butanol yield

The effect of phosphate level in acetate P2 medium containing various concentrations of glucose on butanol production and the butanol yield by *C. acetobutylicum* can be seen in Table 2. Increasing the buffering capacity of the acetate P2 defined medium to 65 mM resulted in improved growth and more glucose being utilized at all levels of added glucose. Butanol production increased with higher levels of added glucose in acetate P2 medium containing 6.5 mM phosphate. Similar results are seen with more highly buffered acetate P2 medium, up to 4% glucose. A decrease in butanol concentration is seen in the more highly buffered medium containing 8% glucose. A reduction in final butanol concentration at high levels of glucose has also been reported by others [13]. These results are reflected in the corresponding butanol yields which indicate that the highest values are achieved in acetate P2 medium containing 65 mM phosphate at low levels of added glucose (1–4%).

Table 3

Effect of phosphate concentration on growth and butanol production in complex media

Medium[a]	Phosphate concentration (mM)	Cell dry weight (mg/ml)[b]	Butanol concentration (g/l)[b]
TGY	6.5	2.3	7.9
	65	2.7	9.6
SSM	6.5	1.0	1.7
	65	1.9	3.9
CSSM	6.5	2.7	2.0
	65	3.5	3.4

[a] See Materials and Methods for composition.
[b] Determined following 48 h of fermentation.

Table 2

The effect of phosphate level in acetate P2 medium containing various concentrations of glucose on growth, butanol production, glucose utilization and butanol yield by *C. acetobutylicum*

Glucose concentration (% w/v)		Growth[a]		Butanol concentration (g/l)[b]		Glucose utilization (g/100 ml)[c]		Butanol yield[d]	
	Phosphate concentration (mM):	6.5	65	6.5	65	6.5	65	6.5	65
1		2.0	2.3	1.7	2.0	1.0	1.0	0.17	0.20
2		2.0	3.1	2.2	5.2	1.3	2.0	0.17	0.26
4		2.1	3.3	3.8	6.3	2.2	3.3	0.17	0.19
8		2.2	2.3	5.5	5.5	3.6	5.0	0.15	0.11

[a] Maximum growth measured as absorbance at 600 nm.
[b] These values represent maximal concentrations of butanol over the course of the fermentation.
[c] Determined at maximal butanol concentrations.
[d] Determined by dividing the grams of butanol produced by the grams of glucose utilized.

Table 4

Maximum growth, pH change and butanol concentration following growth of *C. acetobutylicum* in modified P2 medium supplemented with various 'good' buffers

Buffer concentration[a]	Growth[b]	Δ pH[c]	Butanol concentration (g/l)[d]
15 mM MES	0.8	2.0	1.2
45 mM MES	1.0	1.7	3.5
15 mM HEPES	0.9	2.5	1.0
45 mM HEPES	0.9	2.1	4.3
15 mM CHES	0.9	2.6	1.1
45 mM CHES	0	0	0

[a] See Materials and Methods for the full names of these buffers.
[b] Maximum growth measured as absorbance at 600 nm.
[c] Represents the maximum change in pH over the course of the fermentation.
[d] Measured following 72 h of fermentation.

Effect of buffering in complex media

The effect of two different levels of phosphate (6.5 and 65 mM) on growth and butanol production in complex media can be seen in Table 3. The data show that increased buffering also had a positive effect on growth and butanol production in all three complex media tested.

Effect of various levels of 'good' buffers on butanol production

When *C. acetobutylicum* was grown in modified P2 medium buffered with non-metabolizable 'good' buffers, higher growth and butanol production was observed in the presence of a higher concentration of buffer, except in the case of P2 medium buffered with 45 mM CHES, where growth was not supported due to the high pH. These results can be seen in Table 4.

DISCUSSION

Data have been presented which indicate that elevating the buffering capacity of the growth medium can lead to an increase in carbohydrate utilization and butanol production by *C. acetobutylicum*. It is thought that the increase in growth and butanol production in either synthetic or complex growth media is primarily due to maintenance of an elevated pH which favors the formation of the less toxic butyrate ion over that of undissociated butyric acid. By preventing the accumulation of undissociated butyric acid, an environment more conducive to growth of the microorganism is maintained. This leads to the accumulation of higher concentrations of butyrate, which may eventually serve as carbon skeleton for the synthesis of butanol.

As stated above, it has been shown that low (growth-limiting) levels of phosphate can lead to increased butanol production [1]. The butyryl-CoA → butyryl-phosphate step is inhibited under these conditions and therefore the direction of the pathway is shifted to the solvent-producing reactions. However, these experiments were carried out in a chemostat using external pH control which would prevent the accumulation of undissociated butyric acid.

It has been suggested by several groups that the shift from acidogenesis to solventogenesis is induced by the accumulation of butyric acid. More recent evidence suggests that it is the concentration of either butyrate [4] or undissociated butyric acid [16] inside the cell that induces the shift. The results obtained from our experiments are in agreement with this model. The shift from acidogenesis to solventogenesis occurs later in the growth cycle (at higher levels of growth) in the more highly buffered media. When the pH of the medium is maintained at a higher level, more butyrate is required to achieve significant amounts of undissociated butyric acid. Since butyric acid must be protonated to permeate the cell membrane, the internal concentration would not reach the level necessary to induce the shift until later in the growth cycle.

It is apparent from this and other reports that control of the pH and the equilibrium between protonated and ionized butyric acid in the acetone-butanol-ethanol fermentation is important in order to optimize butanol production. On a practical basis, we feel that the results derived from this type of

work could be used to develop more efficient, economically feasible batch fermentation processes.

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REFERENCES

1 Bahl, H., W. Andersch and G. Gottschalk. 1982. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Appl. Microbiol. Biotechnol. 15: 201–203.

2 Baronofsky, J.J., W.J.A. Schreurs and E.R. Kashket. 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. 48: 1134–1139.

3 Costa, J.M. and A.R. Moreira. 1983. Growth inhibition kinetics for the acetone-butanol fermentation. In: Foundations of Biochemical Engineering (Blanch, H.W., E.T. Papoutsakis and G. Stephanopoules, eds.), pp. 501–502, American Chemical Society, Washington, DC.

4 Gottwald, M. and G. Gottschalk. 1985. The internal pH of *Clostridium acetobutylicum* and its effects on the shift from acid to solvent formation. Arch. Microbiol. 143: 42–46.

5 Hartmanis, M.G.N., T. Klason and S. Gatenbeck. 1984. Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 20: 66–71.

6 Herrero, A.A., R.F. Gomez, B. Snedecor, C.J. Tolman and M.F. Roberts. 1985. Growth inhibition of *Clostridium thermocellum* by carboxylic acids: a mechanism based on uncoupling by weak acids. Appl. Microbiol. Biotechnol. 22: 53–62.

7 Holt, R.A., G.M. Stephens and J.G. Morris. 1984. Production of solvents by *Clostridium acetobutylicum* cultures maintained at neutral pH. Appl. Environ. Microbiol. 48: 1166–1170.

8 Ionesco, H., A. Wolf and M. Sebald. 1974. Production induite de bactériocine et d'un bactériophage par la souche BP6K-N5 de *Clostridium perfringens*. Ann. Inst. Pasteur (Paris) 125B: 335–346.

9 Jones, D.T. and D.R. Woods. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50: 484–524.

10 Kell, D.B., M.W. Peck, G. Rodger and J.G. Morris. 1981. On the permeability to weak acids and bases of the cytoplasmic membrane of *Clostridium pasteurianum*. Biochem. Biophys. Res. Commun. 99: 81–88.

11 Lin, Y.-L. and H.P. Blaschek. 1983. Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl. Environ. Microbiol. 45: 966–973.

12 Monot, F., J.M. Engasser and H. Petitdemange. 1984. Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch culture of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 19: 422–426.

13 Monot, F., J.R. Martin, H. Petitdemange and R. Gay. 1982. Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. Appl. Environ. Microbiol. 44: 1318–1324.

14 Rogers, P. 1984. Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes. Adv. Appl. Microbiol. 31: 1–60.

15 Rose, A.H. 1961. Acetone-butanol fermentation. In: Industrial Microbiology, pp. 161–166, Butterworths Inc., Washington, DC.

16 Terracciano, J.S. and E.R. Kashket. 1986. Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 52: 86–91.

17 Wang, G. and D.I.C. Wang. 1984. Elucidation of growth inhibition and acetic acid production by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. 47: 294–298.