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Effect of Surfactants on Carbon Monoxide Fermentations by *Butyribacterium methylotrophicum*

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ABSTRACT

Butyribacterium methylotrophicum has been grown on carbon monoxide as its carbon and energy source in the presence of various surfactants that are capable of forming microbubble dispersions for the screening of surfactants for use in microbubble-sparged synthesis gas fermentations. In the range of 0–3 times the critical micelle concentration, the presence of Tween surfactants was not significantly inhibitory to growth, final cell density, and fermentation stoichiometry, although some of the Brij surfactants caused significant inhibition. As the batch fermentations entered the stationary phase both the pH and the ratio of acetate to butyrate decreased.

Index Entries: *Butyribacterium methylotrophicum*; synthesis gas; microbubbles; aphrons.

INTRODUCTION

The obligate anaerobe *Butyribacterium methylotrophicum* consumes carbon monoxide, CO, to produce acetate, ethanol, butyrate, and butanol *(1)*. Carbon monoxide is a primary component in synthesis gas, which can be obtained from the pyrolysis of biomass *(2)* or the gasification of coal *(3)*. The rate of synthesis gas fermentations is limited by low gas-to-liquid mass-transfer rates arising from the low solubility of CO in aqueous solu-

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tions (4). One technique to enhance gas-to-liquid mass transfer is to use microbubble dispersions (5). Microbubbles are surfactant-stabilized bubbles with diameters on the order of 50 μm. The surfactant layer surrounding a microbubble generates a diffuse electric double layer that acts to repel bubbles and prevent coalescence (6). A microbubble dispersion exhibits colloidal properties and is stable enough to be pumped (7). To be successfully used in synthesis gas fermentations, the surfactant used must be nontoxic to the cells at levels necessary to generate microbubbles. The surfactant should also have no detrimental effect on formation of the desirable products.

This article examines the growth and product formation of *B. methylotrophicum* during CO fermentations in the presence of surfactants that are suitable for generating microbubble dispersions. This information is needed to select surfactants for use in microbubble-sparged synthesis gas fermentations.

METHODS

Culture Techniques

All chemicals and vitamins were obtained from Sigma Chemical Company (St. Louis, MO), except for sodium dodecyl sulfate, which was obtained from Boehringer Manheim (Mannheim, Germany). The nitrogen (N_2) and CO gases were purchased from AGA Gas and Welding (Lansing, MI). *B. methylotrophicum* was obtained from the Michigan Biotechnology Institute (Lansing, MI) and was grown anaerobically at 37°C in a phosphate-buffered (PB), sulfide-reduced medium prepared with 0.5% yeast extract as previously described (8) on 100% carbon monoxide. Resazaurin was used as an oxygen indicator in maintaining stock cultures, but was not used in the experimental fermentations owing to interference with measuring optical density. The medium was dispersed into 125-mL sera bottles (Wheaton, Millville, NJ) at 50 mL/bottle, and sealed with butyl rubber stoppers and aluminum crimps (Wheaton, Millville, NJ). For the experimental runs with surfactant present in the media, the surfactant was added to the media prior to autoclaving. A 1% inoculum of an actively growing culture of *B. methylotrophicum* was used. The cultures were incubated in an Innova 4000 shaker (New Brunswick Scientific, New Brunswick, NJ) at 100 rpm in the dark.

Culture Analysis

Liquid samples (2 mL) were taken throughout the duration of each experiment. Growth was analyzed using a Lambda 3 spectrophotometer (Perkin Elmer, Norwalk, CT) at 660 nm immediately after withdrawing the sample. The sample was then frozen for later product analysis. In experiments in which the pH was monitored, a 1-mL sample of cells was

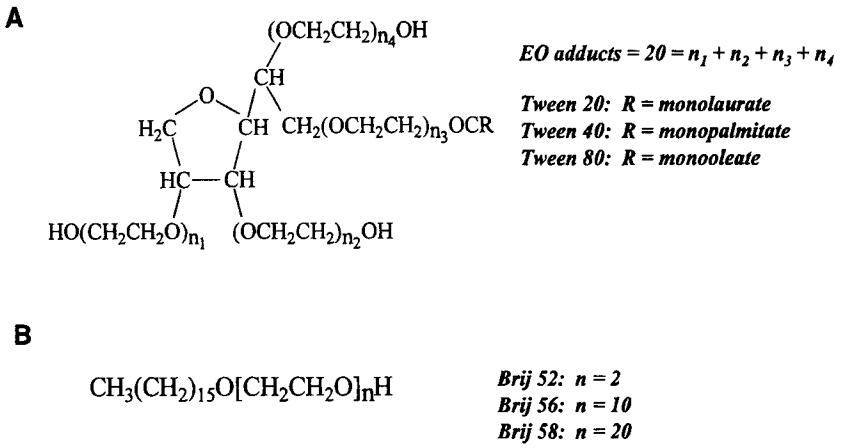

Fig. 1. Chemical structure for Tween and Brij surfactants *(9)*. EO adducts indicates the number of ethylene oxide adducts.

centrifuged in a Fisher Microcentrifuge 235C (Fisher Scientific, Chicago, IL) at 15,000 *g* for 10 min. Phenol red was then added to the sample to 20 μ*M*, and the optical density was measured at 560 nm to determine the pH of the sample. The phenol red assay was calibrated using larger samples with a pH electrode. To prepare samples for product analysis, 10% (v/v) of 1*M* phosphoric acid was added. After a 10 min incubation at 37°C, the samples were centrifuged as before, and then analyzed by gas chromatography for acetate, ethanol, butyrate, and *n*-butanol. The separation was done with a Perkin-Elmer Autosystems GC (Perkin Elmer) with a Hayesep R, 6′ × 1/4″ × 2 mm deactiglass column (Alltech, Waukeegen, WI) and a flame-ionization detector.

The primary surfactants used for this study were Tween (polyoxyethylene sorbitans) and Brij (polyoxyethylene alcohols). The structures of these surfactants are shown in Fig. 1. Dimensionless surfactant concentrations (DSC) are defined as the ratio of the surfactant concentration to its critical micelle concentration. DSC values up to 3 were used because previous studies *(5)* indicated that microbubble dispersions needed DSC values of at least 1 for the formation of stable dispersions. Increasing DSC values above 3 had no effect on the dispersion's stability, presumably because the absorption saturation value of the available gas–liquid interface had been reached. The critical micelle concentrations and aggregation numbers for the surfactants used in these experiments are listed in Table 1 *(9)*.

RESULTS

Figure 2 shows growth curves for *B. methylotrophicum* for the Tween surfactants at different DSC values. Figure 3 shows the growth curves for Brij surfactants at different DSC values. Both the Tween and Brij runs had

Table 1
Surfactant Critical Micelle Concentrations *(8)*

Surfactant	Critical micelle concentration
Brij 52	> 7 μM
Brij 56	7 μM
Brij 58	77 μM
Tween 20	60 mg/L
Tween 40	29 mg/L
Tween 80	13 mg/L

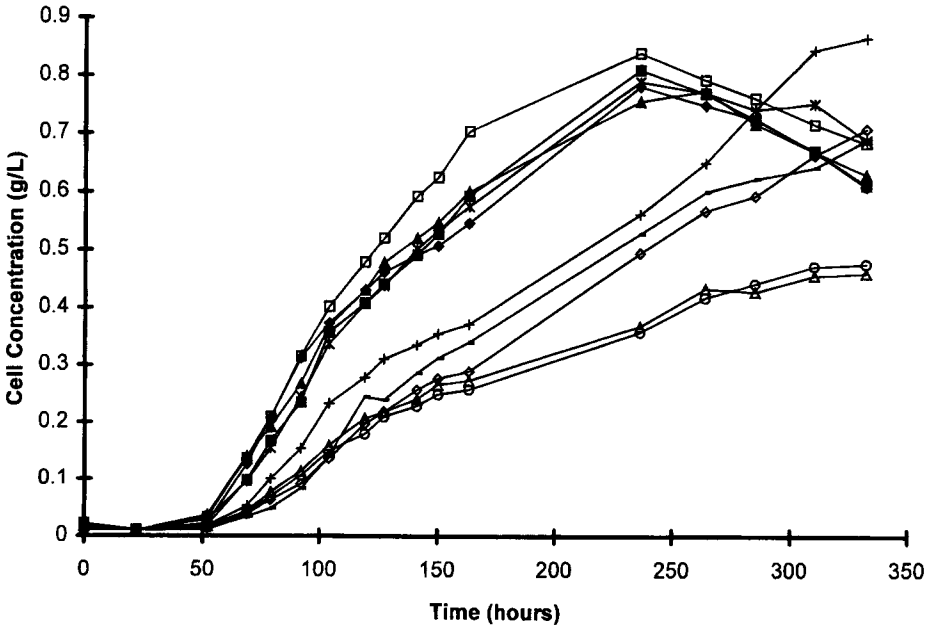

Fig. 2. Growth of *B. methylotrophicum* in batch bottle fermentation with Tween surfactant. -⊟- Control; -◇- Tween 20, DSC = 1; -⊖- Tween 20, DSC = 2; -△- Tween 20, DSC = 3; -✳- Tween 40, DSC= 1; -+- Tween 40, DSC = 2; -—- Tween 40, DSC = 3; -■- Tween 80, DSC = 1; -◆- Tween 80, DSC = 2; -▲- Tween 80, DSC = 3.

individual controls that were prepared and inoculated at the same time and under the same conditions as the data taken. The data in Figs. 2 and 3 are averages of triplicate runs. Figures 4 and 5 show the specific growth rate as a function of surfactant concentration and surfactant chain length for Tween and Brij, respectively.

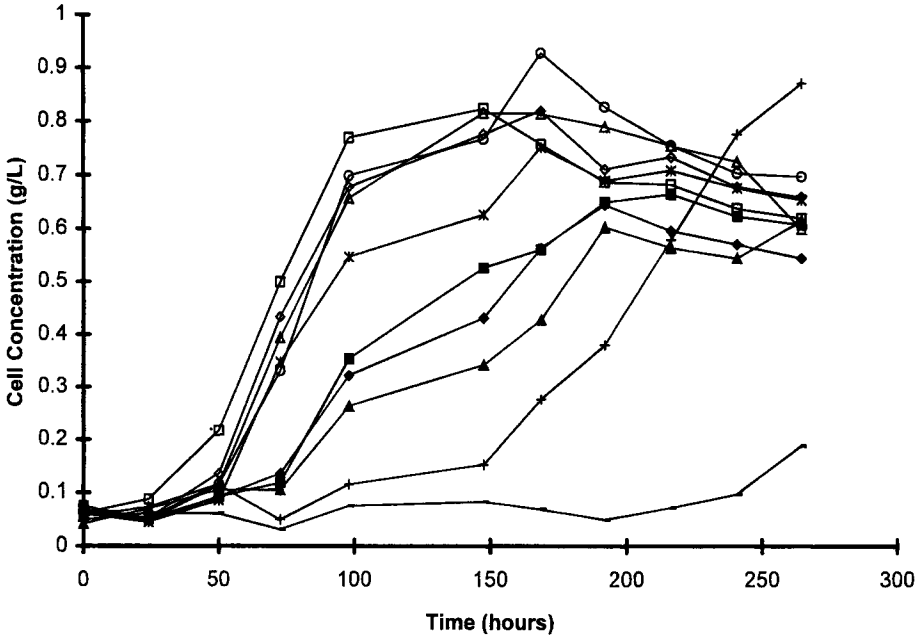

Fig. 3. Growth of *B. methylotrophicum* in batch bottle fermentation with Brij surfactant. -⊟- Control; -◇- Brj 52, DSC = 1; -⊖- Brj 52, DSC = 2; -△- Brj 52, DSC = 3; -✳- Brj 56, DSC = 1; -|- Brj 56, DSC = 2; -— Brj 56, DSC = 3; -■- Brj 58, DSC = 1; -◆- Brj 58, DSC = 2; -▲- Brj 58, DSC = 3;

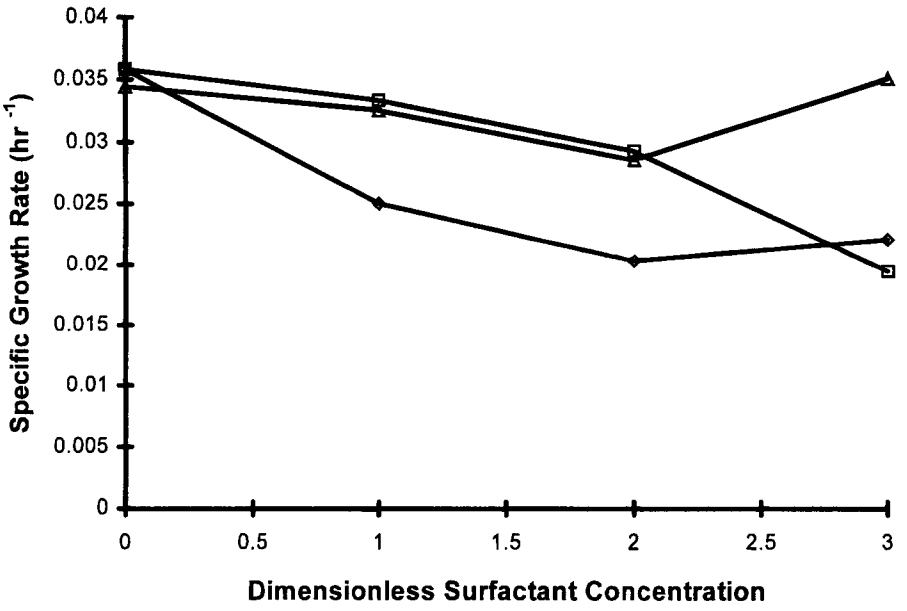

Fig. 4. Specific growth rate of *B. methylotrophicum* determined from data in Fig. 1 for Tween surfactant as a function of surfactant concentration. -◇- Tween 20, -⊟- Tween 40, -△- Tween 80.

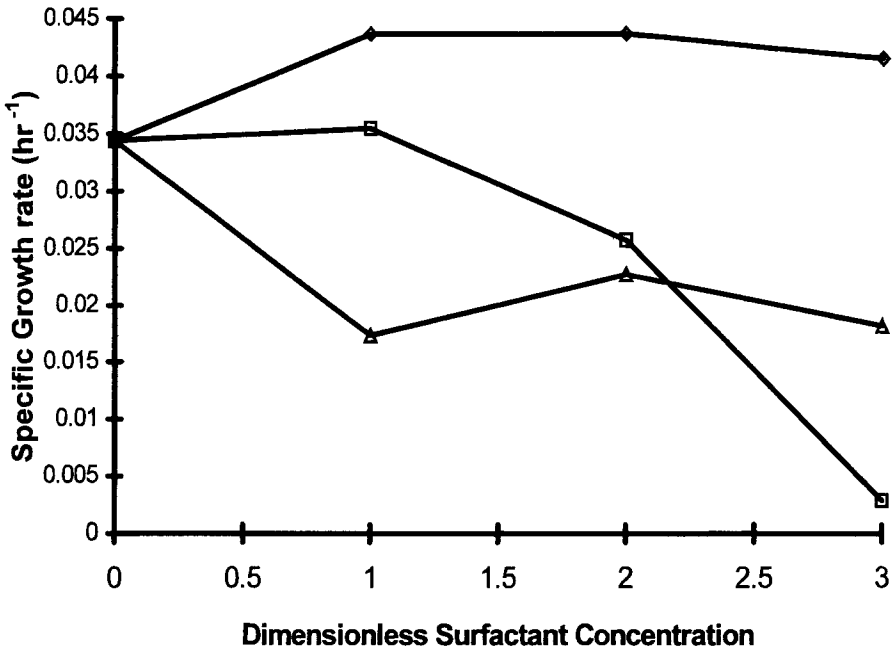

Fig. 5. Specific growth rate of *B. methylotrophicum* determined from data in Fig. 2 for Brij surfactant as a function of surfactant concentration. -◇- Brij 52, -□- Brij 56, -△- Brij 58.

The acetate, ethanol, and butyrate fermentation profiles are shown in Figs. 6, 7, and 8, respectively, as a function of surfactant type and chain length. The remainder of the product concentration data were calculated using carbon and electron balances for each run *(10,11)*. The data shown in Table 2 for Tween surfactants represent the average of triplicate values.

A typical plot of pH as a function of time during the experiment is shown in Fig. 9. The pH profiles for other runs (data not shown) had similar profiles to the ones presented here.

DISCUSSION

None of the Tween surfactants strongly inhibited growth of *B. methylotrophicum* in batch bottle fermentations. However, there did seem to be an effect of the length of the hydrophobic end of the surfactant on the growth of the microorganisms. Tween-20 has a lauric acid group (C12), Tween-40 has a palmitic acid group (C16), and Tween-80 has an oleic acid group (C18-1). In general, the shorter the chain length, the lower the growth rate (*see* Fig. 2). However, for the Tween surfactants, most cultures obtained the same approximate final cell density. It has been suggested that longer chain lengths slow surfactant diffusion into the cellular membrance and thereby result in lower toxicity *(12)*. Tween surfactants have also been found to be nontoxic to other types of culture systems (e.g., soy

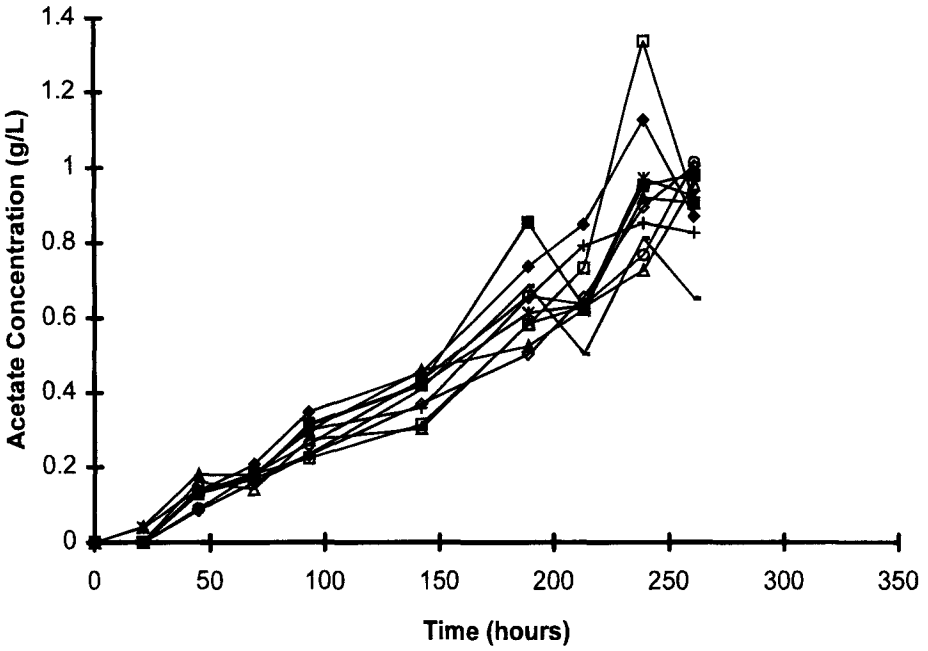

Fig. 6. Acetate production during the Tween surfactant experiments. -⊟- Control; -◇- Tween 20, DSC = 1; -⊖- Tween 20, DSC = 2; -△- Tween 20, DSC = 3; -✳- Tween 40, DSC= 1; -|- Tween 40, DSC = 2; -—- Tween 40, DSC = 3; -■- Tween 80, DSC = 1; -◆- Tween 80, DSC = 2; -▲- Tween 80, DSC = 3.

and carrot suspension cultures), and in some cases, Tween-20 actually enhanced the growth of the culture (Ames, T. T., unpublished data). Higher surfactant concentrations (10–20 DSC) were found to be toxic to plant suspension cultures.

The Brij surfactants did strongly inhibit growth in some cases, but the inhibitory effect again depended on the chain length. The Brij surfactants are polyoxyethylene (polyethylene glycol—PEG) alcohols (*see* Fig. 1) and had the following characteristics (in order of increasing chain length): Brij 52-PEG(2) Cetyl alcohol, Brij 56-PEG(10) Cetyl alcohol, and Brij 58-PEG(20) Cetyl alcohol. As shown in Figs. 2 and 5, the specific growth rate and final cell densities are much lower than those for the control for increasing amounts of the two longer chain surfactants, Brij 56 and Brij 58. In general, the higher the surfactant concentration, the lower the specific growth rate and final cell density. The Brij surfactants are apparently more inhibitory to the growth of *B. methylotrophicum* in concentrations necessary to form microbubbles than are the Tween surfactants.

Growth experiments were also done with a few ionic surfactants (e.g., sodium dodecyl sulfate, cetyl pyridinium choride, and sodium do\decyl benzene sulfonate). The growth of *B. methylotrophicum* on these surfactants was strongly inhibited even at the lowest concentrations tested (0.5 DSC)

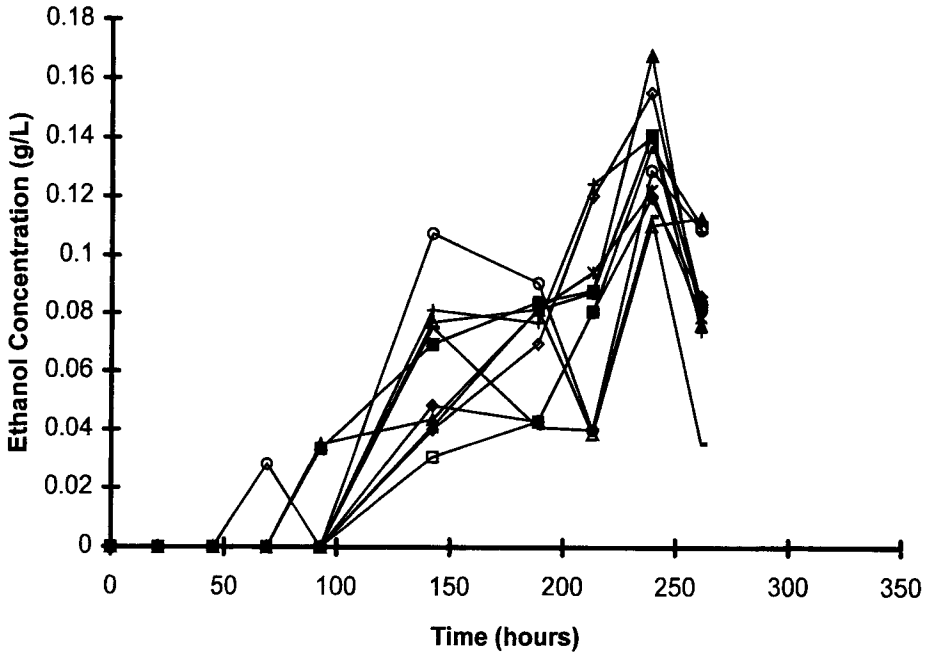

Fig. 7. Ethanol production during the Tween surfactant experiments. -⊟- Control; -◇- Tween 20, DSC = 1; -⊖- Tween 20, DSC = 2; -△- Tween 20, DSC = 3; -✳- Tween 40, DSC= 1; -|- Tween 40, DSC = 2; -▬- Tween 40, DSC = 3; -■- Tween 80, DSC = 1; -◆- Tween 80, DSC = 2; -▲- Tween 80, DSC = 3.

(data not shown). Both anionic and cationic surfactants are prone to bind proteins. The charged groups of the ionic surfactants form relatively strong ionic bonds with charged groups on proteins. Nonionic surfactants, on the other hand, lacking the charged groups bind through much weaker hydrophobic interactions with the protein chain, and thus, are less likely to inactivate or denature enzymes and proteins *(12)*. Nonionic surfactants would thus be expected to have the least influence on cellular metabolism in fermentation.

The specific growth rates were calculated from the exponential portion of the growth curve. There was no inhibition from the products formed at the concentrations seen in this study *(13)*. The deviations from typical exponential growth are because of mass-transfer limitations of the gaseous substrate.

The pH decrease during the fermentation is because of to the production of acetic and butyric acids. The metabolic pathways for acid production have been well characterized *(3)*. A shift in the product ratios for *B. methylotrophicum* has been observed in which less acetate and more butyrate and alcohols are produced at low pH *(13,14)*. Moreover, a pH shift from 6.8 to 6.0 as the cells entered the stationary phase has been shown to induce formation of butyrate as the primary product *(15,16)*.

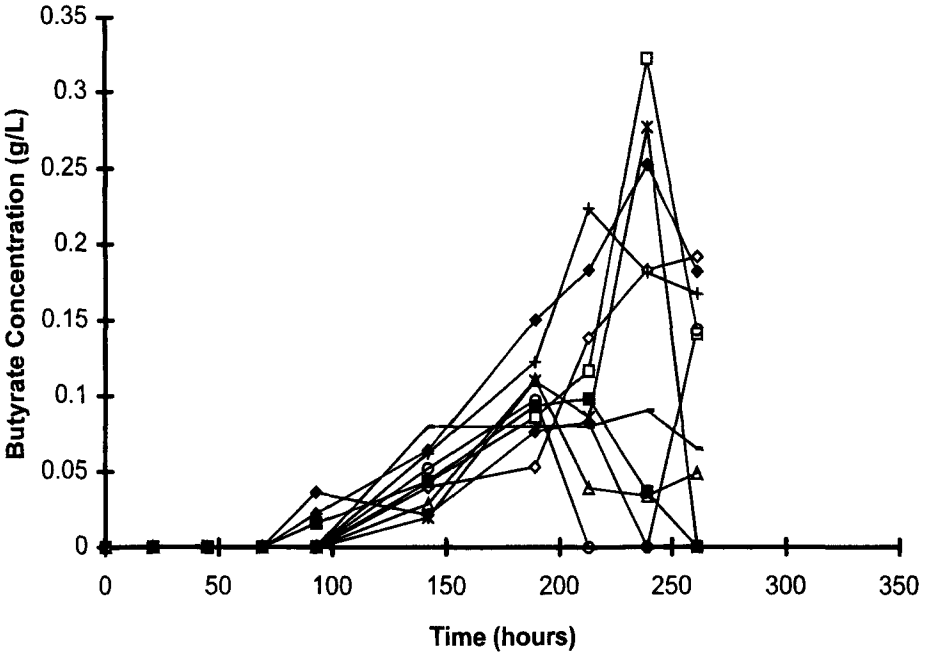

Fig. 8. Butyrate production during the Tween surfactant experiments. -⊟- Control; -◇- Tween 20, DSC = 1; -⊖- Tween 20, DSC = 2; -△- Tween 20, DSC = 3; -✳- Tween 40, DSC= 1; -|- Tween 40, DSC = 2; -— Tween 40, DSC = 3; -■- Tween 80, DSC = 1; -◆- Tween 80, DSC = 2; -▲- Tween 80, DSC = 3.

Table 2
Carbon and Electron Balance Results for Tween Surfactants

Control	4 CO →	0.40 Ace +	0.076 Et +	0.065 Bu +	0.61 CM +	2.17 CO_2
Tween 20 (1 DSC)	4 CO →	0.48 Ace +	0.050 Et +	0.028 Bu +	0.70 CM +	2.12 CO_2
Tween 20 (2 DSC)	4 CO →	0.43 Ace +	0.114 Et +	0.039 Bu +	0.58 CM +	2.18 CO_2
Tween 20 (3 DSC)	4 CO →	0.45 Ace +	0.163 Et +	0.012 Bu +	0.52 CM +	2.20 CO_2
Tween 40 (1 DSC)	4 CO →	0.41 Ace +	0.083 Et +	0.057 Bu +	0.61 CM +	2.17 CO_2
Tween 40 (2 DSC)	4 CO →	0.34 Ace +	0.076 Et +	0.079 Bu +	0.66 CM +	2.19 CO_2
Tween 40 (3 DSC)	4 CO →	0.37 Ace +	0.068 Et +	0.082 Bu +	0.62 CM +	2.18 CO_2
Tween 80 (1 DSC)	4 CO →	0.43 Ace +	0.085 Et +	0.047 Bu +	0.62 CM +	2.16 CO_2
Tween 80 (2 DSC)	4 CO →	0.43 Ace +	0.063 Et +	0.031 Bu +	0.77 CM +	2.13 CO_2
Tween 80 (3 DSC)	4 CO →	0.37 Ace +	0.096 Et +	0.078 Bu +	0.55 CM +	2.20 CO_2

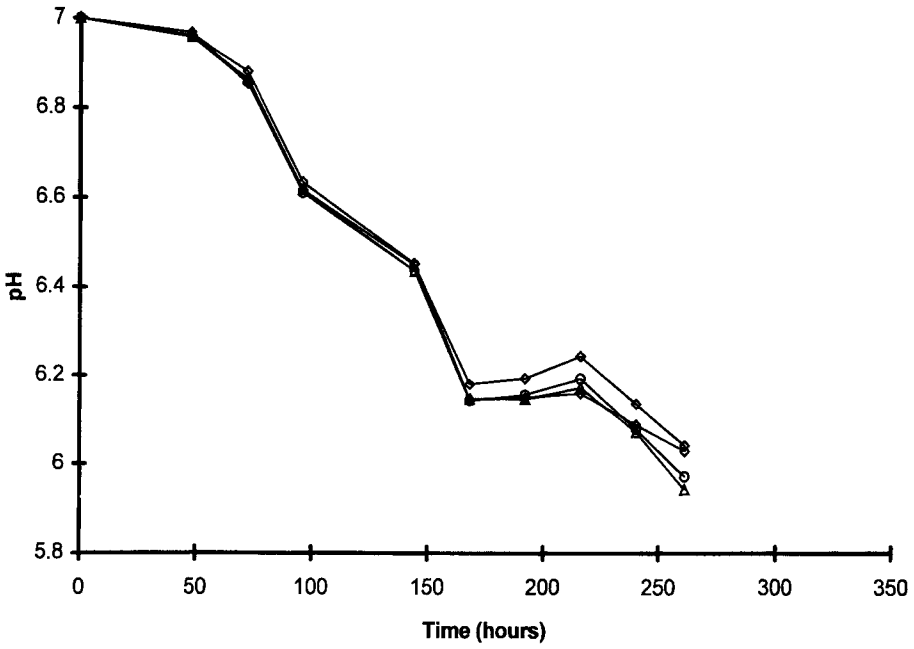

Fig. 9. pH profile of Tween-80 fermentation. -◇- Control; -◇- Tween 20, DSC = 1; -○- Tween 20, DSC = 2; -△- Tween 20, DSC = 3.

Consistent with this trend, Figs. 6 and 8 show that acetate is produced throughout the fermentation, but the butyrate production starts after 70 h. The acetate, ethanol, and butyrate profiles in Figs. 6, 7, and 8 are the averages of triplicate data sets. In the averaged sets of data, the variability was as great as ±25% between bottles. Because of the postautoclave additions done to each bottle and individually inoculating each bottle, these variations are expected.

The carbon and electron balance results for the Tween surfactant experiments given in Table 2 show the ratio of the primary products, acetate, ethanol, and butyrate, was not affected by the presence of the surfactant in the media. This result suggests that the Tween surfactants are a good choice for microbubble-dispersion-sparged CO fermentations.

CONCLUSIONS

The effect of Tween surfactants on growth of *B. methylotrophicum* on CO varied with the chain length. Longer chain lengths had negligible effects on growth of a DSC range of 0–3. Shorter chain lengths slowed growth somewhat, but did not affect the final cell density. None of the Tween surfactants significantly affected the product stoichiometry. Brij surfactants were inhibitory over the same concentration range, in some cases reducing both the growth rate and the final cell density. An

increase in the ratio of butyrate to acetate observed late in the fermentations coincided with a decrease in the fermentation pH and the onset of the stationary phase.

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