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Reactor Comparisons for the Biodegradation of Thiodiglycol, a Product of Mustard Gas Hydrolysis

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ABSTRACT

An environmentally benign method for the mineralization of sulfur mustard has been proposed involving chemical hydrolysis of sulfur mustard to thiodiglycol, and then the biological degradation of thiodiglycol to generate biomass and gaseous carbon dioxide. *Alcaligenes xylosoxidans* (SH91) was isolated based on its ability to utilize thiodiglycol as a sole carbon source. This article compares different biological reactor designs and experimentally assesses their relative effectiveness in degrading thiodiglycol using pure cultures of SH91. The reactor configurations studied are batch, continuous stirred-tank reactor (CSTR), and CSTR with cell recycle. From the results, it is clear that the CSTR with cell recycle offers superior performance for a given residence time or volume. These pure culture data are necessary for accurate design of a pilot-scale system where mixed cultures will be employed because of a possible incomplete chemical hydrolysis step.

Index Entries: Biodegradation; chemical warfare agents; sulfur mustard; thiodiglycol.

INTRODUCTION

As an alternative to incineration, a method to mineralize sulfur mustard involving chemical hydrolysis followed by biodegradation of the hydrolysis products was proposed to the National Research Council *(1)*. As a result, this paper examines the exsitu bioremediation of thiodiglycol (TDG), a product of sulfur mustard hydrolysis. *Alcaligenes xylosoxidans* (SH91) *(2)*, an obligate aerobic, gram-negative bacterium, was isolated specifically for its unique ability to degrade TDG. SH91 exhibits substrate-inhibited growth in batch cultures where a notable decrease

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in cell growth rate occurs above 80 m*M* TDG *(3)*. Consequently, biological degradation of TDG in a batch bioreactor is not feasible when the initial TDG concentration exceeds 80 m*M*, which is likely. Instead, several continuous and semicontinuous bioreactor operating strategies have been proposed *(3,4)* that would enable stable operation at effluent concentrations below the toxic limit while maintaining an acceptable degradation rate. This article describes experimental results of batch, continuous stirred-tank reactor (CSTR), and CSTR with cell recycle reactors for degrading TDG. These data when coupled with design criteria and optimization techniques can be utilized to develop a pilot-scale bioremediation system. In carrying out this work, we noted physiological behavior (an apparent maximum optical density) that adds an additional constraint to reactor operation. Our limited analysis suggests this is the result of the intracellular accumulation of a dead-end metabolic byproduct, TDG sulfoxide. Practical considerations for the eventual use of these reactor systems are also discussed.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

A. xylosoxidans (SH91) was isolated at Aberdeen Proving Ground, MD. *A. xylosoxidans* an obligate aerobic, gram-negative bacteria that utilizes TDG as its sole carbon source. *A. xylosoxidans* grows in media with TDG as the sole carbon source. The media composition (per liter) of the salt media is as follows: 1 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, and 10 mL Modified Wolin Salts Solution (WS42). The WS42 solution contains (per liter): 3 g nitrilotriacetic acid, 6 g $MgSO_4 \cdot H_2O$, 1 g NaCl, 1 g $MnSO_4 \cdot H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.02 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.01 g $CuS0_4$. TDG is added to the salt media at the desired concentration before sterilization. During fermentation, the temperature and pH are maintained at 30°C and 8.0, respectively.

Batch fermentations were performed in a 5-L BioFlow III fermenter (New Brunswick Scientific) with pH and temperature controlled at 8.0 and 30°C, respectively (pH is controlled by the addition of NaOH to the fermenter). The agitation rate was kept at 250 rpm, and the aeration rate was 2.5 liter/min. Five-milliliter samples were withdrawn from the fermentor periodically to measure optical density (OD) and to assay for TDG. The same reactor was used in a CSTR at the identical pH, temperature, agitation, and aeration rates. The influent media was pumped into the fermenter from a media bottle via a calibrated peristaltic pump. The reactor volume was kept constant with an overflow line. The CSTR with cell recycle system (Fig. 1) was similar to the CSTR; however, the cells were concentrated and recycled using a 22-μm hollow-fiber membrane filter from A. G. Technology Corporation (Model: UFP-10-D-4, type: 10,000 NMWC). The filter concentrated cells by rejecting clear fluid as the fermentation broth flows through the filter module. The flow rates were adjusted so that the recycle ratio, α, and the concentration factor, β, average 1.0 and 1.2, respectively. The relationship between a and β is given by the following equation at steady-state operations *(4)*:

$$\alpha = [\mu V - F/F\,(1 - \beta)] \tag{1}$$

where μ is the specific growth rate (h^{-1}), V is the reactor's volume (L), and *F* is the volumetric feed rate (L/h).

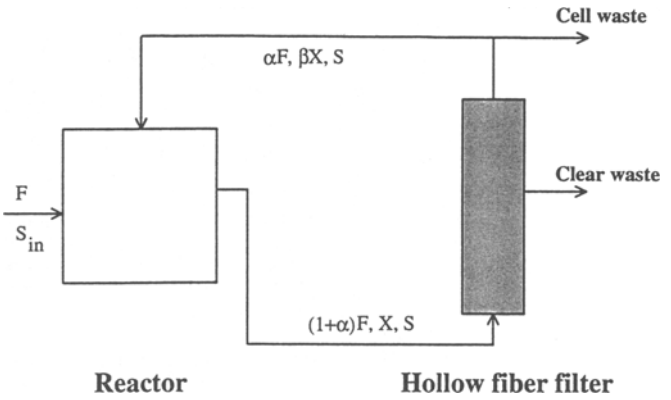

Fig. 1. Schematic diagram of the CSTR with cell recycle.

Optical Density

Optical density (OD) was used to quantify cell mass density. The OD was measured against a blank of deionized distilled water at 590 nm. All samples above the OD of 0.25 were diluted to within the linear range with deionized water.

TDG Assay

TDG concentration was determined using a Waters Chromatography high-performance liquid chromatography (HPLC) system with absorbance detector at 214 nm. The filtered fermentation broth was eluded through a Waters Chromatography FAM-PAK column (7.8 mm × 15 cm) with 3 m*M* phosphoric acid at 65°C. The residence time for thiodiglycol was approx 6.5 min. A correlation between peak area and concentration was linear between 0 and 10 m*M*. All samples above 10 m*M* were diluted into the linear range.

RESULTS AND DISCUSSION

Batch Reactors

Results of three batch experiments with 40, 50, and 100 m*M* initial TDG are depicted in Fig. 2. At 40 m*M* initial thiodiglycol, the stationary phase is reached at an OD of 0.65 after approximately 80 hours. The TDG concentration decreased rapidly to 10 m*M* during the exponential phase and continued to decrease during the stationary phase, but at a much slower rate. At the termination of the experiment, the final thiodiglycol concentration was 5 m*M*. At 50 mM initial TDG, the maximum OD was 0.75, and 25 m*M* TDG remained at the onset of the stationary phase. Again, during the stationary phase, the TDG concentration decreased slowly, reaching 13 m*M* by the end of the experiment. Finally, at 100 m*M*, initial TDG, the stationary phase was reached at an OD of 0.7, and TDG concentration was 78 m*M* at the end of the experiment. During the stationary phase, the TDG concentration also decreased slowly to 74 m*M* by the end of the experiment.

In all three batches, the TDG concentration decreased rapidly during the exponential phase and was degraded more slowly during the stationary phase. This

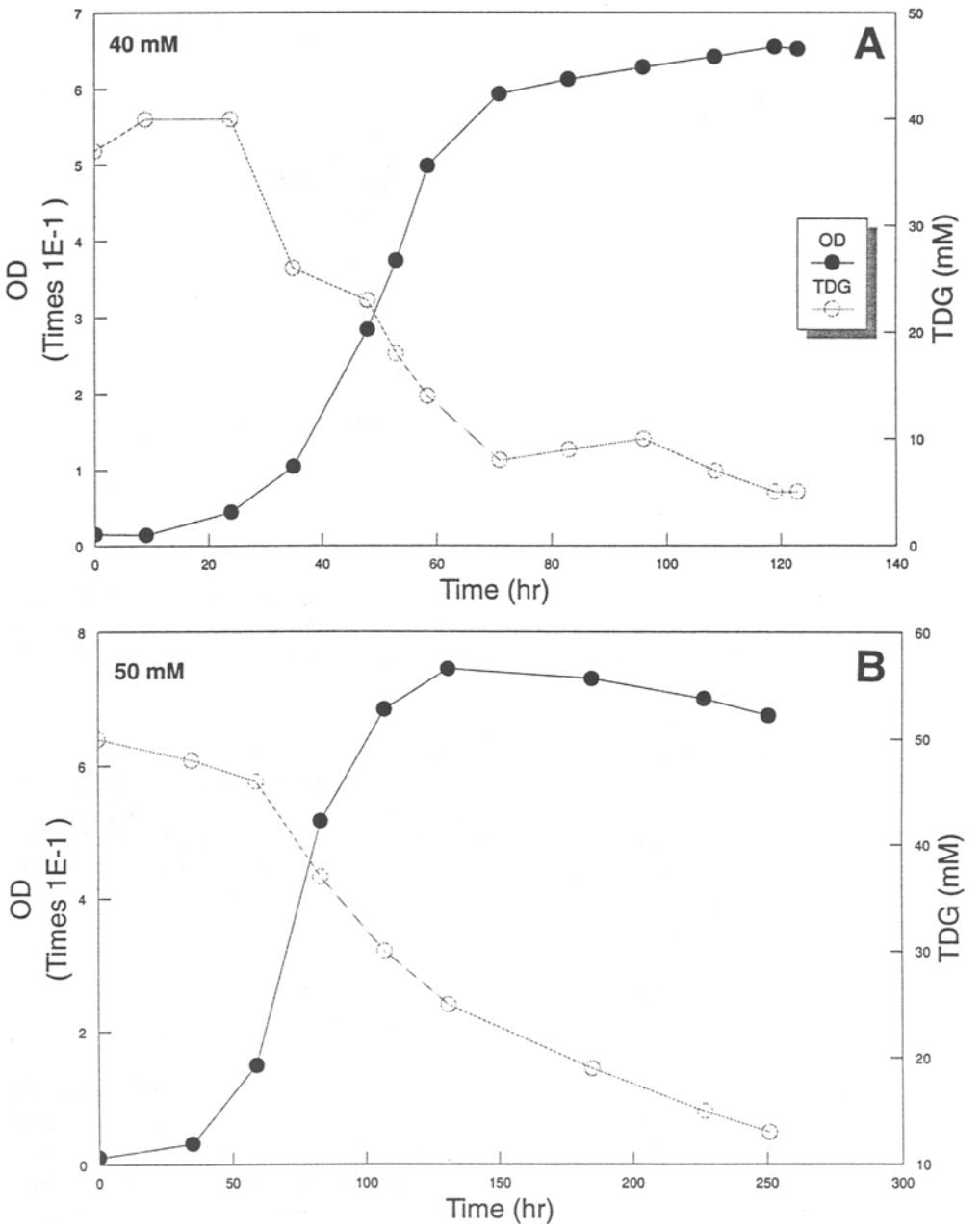

Fig. 2. Batch fermentations at **(A)** 40 m*M* initial TDG; **(B)** 50 m*M* initial TDG; and **(C)** 100 m*M* initial TDG.

is consistent with the increased demands for growth during the exponential phase. While in the stationary phase, the bacteria require little energy to maintain a constant cell mass.

Also, a significant extracellular fermentation product was detected by HPLC analysis at a residence time of 5.5 min (Fig. 3). Mass spectrophotometer and NMR

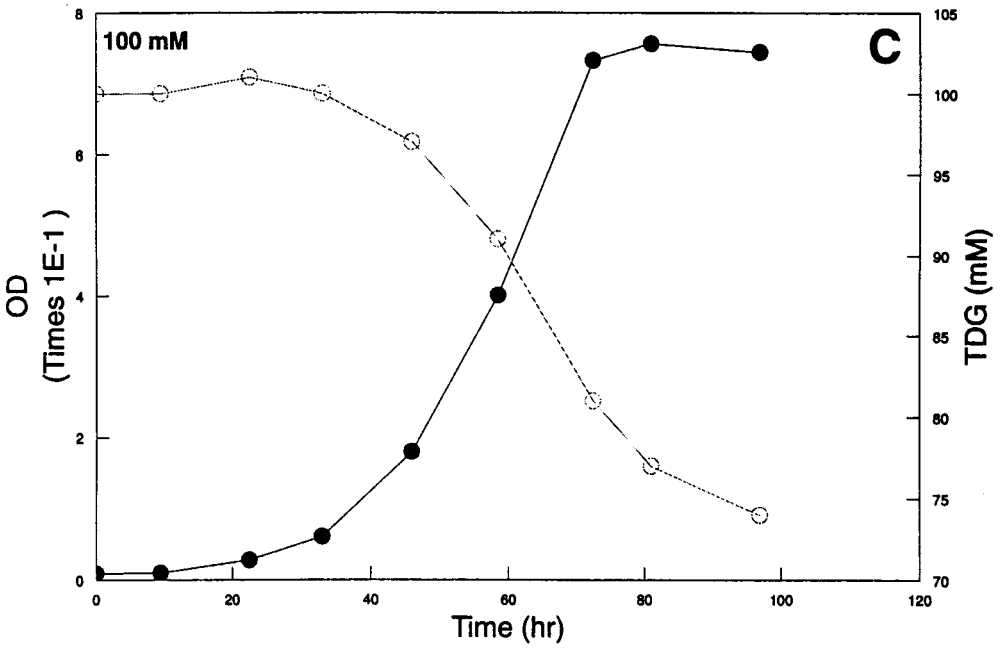

Fig. 2C

analysis performed at the US Army ERDEC suggest that the compound is TDG sulfoxide. Figure 3 also depicts the sulfoxide peak area vs TDG concentration during a batch fermentation (100 m*M* initial TDG). Since this plot yields a straight line, it indicates that the unknown compound is a direct consequence of TDG degradation.

Another interesting phenomena observed from the batch fermentations was that bacterial growth repeatedly reached stationary phase after about 25 m*M* of TDG were degraded. This observation is in contrast to the common paradigm that stationary phase is reached when growth-limiting substrate is totally depleted (TDG is the sole carbon source and limiting substrate in our case). Since cell growth stopped with abundant TDG it was initially hypothesized that cell growth was halted owing to product inhibition or another nutrient limitation; however, the experiment described in Fig. 4 suggests otherwise. Figure 4 shows the results of an experiment where a batch reactor (100 m*M* initial TDG) was grown to stationary-phase (75 m*M* remaining). The fermentation broth was centrifuged to separate the cells from the liquid media. The cells were resuspended with fresh media of 100 m*M* TDG to the OD of the original stationary-phase OD and allowed to grow. The supernatant (spent media) was also incubated in a separate shake flask at 30°C. In Fig. 4, it was evident that cells grew in the supernatant precluding a limitation from other nutrients, whereas, no growth was observed in flask containing the resuspended cells. Instead, the optical density actually decreased. These observations clearly indicated that there was no soluble product inhibition. On the other hand, these results are consistent with the hypothesis that an inhibition product was accumulated intracellularly and not released at toxic levels into the media. Perhaps the extracellular byproduct indicated not only accumulates intracellularly, where there is a toxic effect, but is also released into the media. This is presently being investigated.

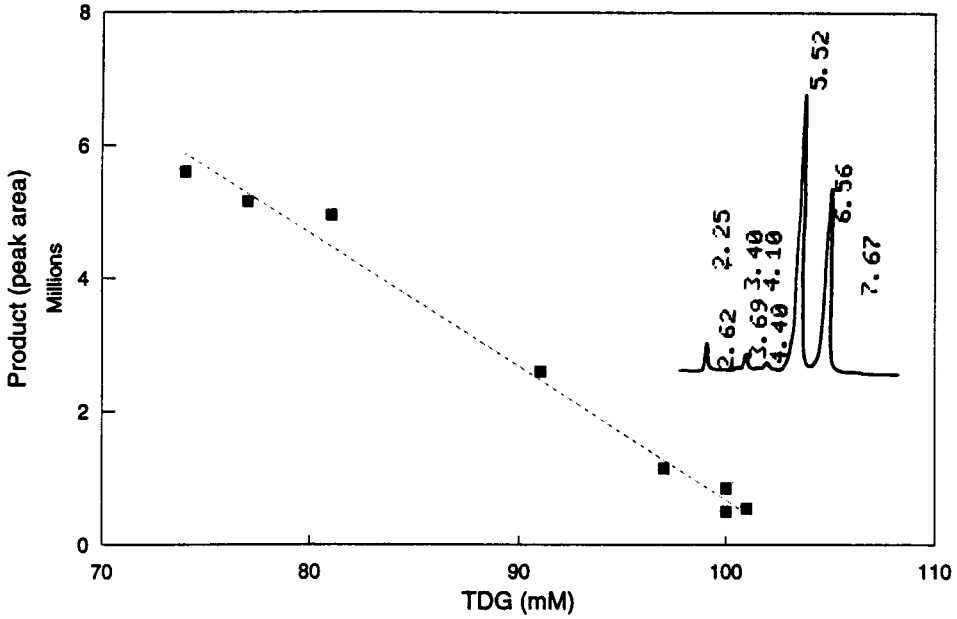

Fig. 3. Product concentration vs TDG concentration for 100 m*M* batch fermentation (slope = $-2.0 \times 10^5 \pm 8.1 \times 10^3$, *y*-intercept = $2.1 \times 10^7 \pm 2.5 \times 10^5$); and HPLC analysis of fermentation broth (product peak is at 5.5 min, TDG peak is at 6.5 min).

CSTRs

Although batch reactors can be used to biodegrade TDG, it is highly labor intensive and according to our previous results, demonstrating significant substrate inhibition, requires low initial concentration; thus, CSTRs were investigated as an alternative owing to their low maintenance and simple operation. Furthermore, CSTRs may alleviate the buildup of toxic byproducts since their production may be a function of cell growth rate, which can be manipulated independently. A CSTR was run at 50 m*M* influent TDG at several dilution rates (D): 0.014/h, 0.030/h, 0.046/h, and 0.065/h. The results are shown in Fig. 5A. In Fig. 5A where the results are similar to those found in batch cultures, the total consumption varied from 25–30 m*M* TDG. Note also that on changing the dilution rate, the reactor OD quickly arrived at a new steady state. Figure 5B summarizes the relationship between steady-state OD and TDG concentration as a function of dilution rate. At a low dilution rate (between 0.014/h and $0.030/h^{-1}$), the steady-state OD rises with an increase in dilution rate. This observation implies that cell death is important at low dilution rates, since the growth rate is not high enough to mask the death rate. The death rate for *A. xylosoxidans* (SH91) was reported by Lee *(5)* to be between 0.007/h and 0.008/h.

Also in Fig. 5A, during the 0.046 h dilution rate, we noticed a shift in the steady state. At first, the OD was steady at approx 0.8, and then near 1000 h the OD increased to 1.3 and TDG decreased from approx 22 to 16 m*M*, and remained steady until the next dilution rate. There was no experimental perturbation to have caused this shift, and we are in the process of examining this in more detail. One possible explanation that can be offered is the existence of multiple steady states. During, substrate inhibited growth, for each dilution rate other than that coinciding with

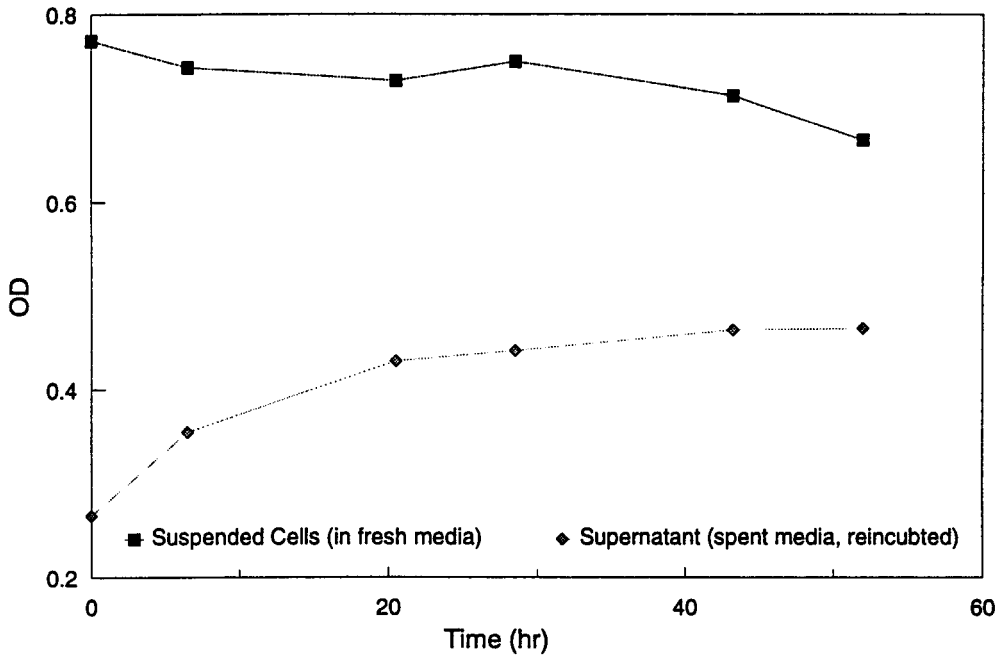

Fig. 4. Growth in supernatant after cell removal by centrifugation.

the maximum growth rate, there are two steady states, one stable and one unstable *(6)*. Additionally, more steady states are possible for cells exhibiting a variable yield coefficients *(6)*. In Fig. 6, the calculated apparent yield coefficient is depicted as a function of dilution rate. This shape does not correspond to the typical equation:

$$Y_{X/S} = (\mu Y_m / \mu + k_d) \quad (2)$$

as described by Bailey and Ollis *(7)*. We are currently investigating the impact of variable yield on bioreactor behavior and design. In the present study, however, both steady states depict degradation of TDG, but neither consumed TDG to a significant level. Consequently, efforts to enhance the performance of the CSTR were attempted first.

CSTR with Cell Recycle

By providing cell recycle, we can increase the cell mass available for degradation in the reactor. This may improve the efficiency of a CSTR even though there maybe buildup of intracellular byproducts. In addition, it may circumvent the maximum OD phenomena found in the batch cultures. A CSTR with cell recycle was operated at 50 m*M* influent TDG at dilution rates of 0.014/h, 0.030/h, 0.046/h, and 0.065/h. Figure 7A summarizes the results for the cell recycle experiment. Note that the time required to reach a steady state was significantly longer in this reactor scheme, and also that the effluent TDG was significantly lower. The relationships between steady-state OD, TDG concentration, and dilution rate are outlined in Fig. 7B. As was the case with the CSTR, at low dilution rates (between 0.014/h and 0.030/h), the OD increased with increasing dilution rate. This observation further supports the fact that cell death plays an important role at low growth rate.

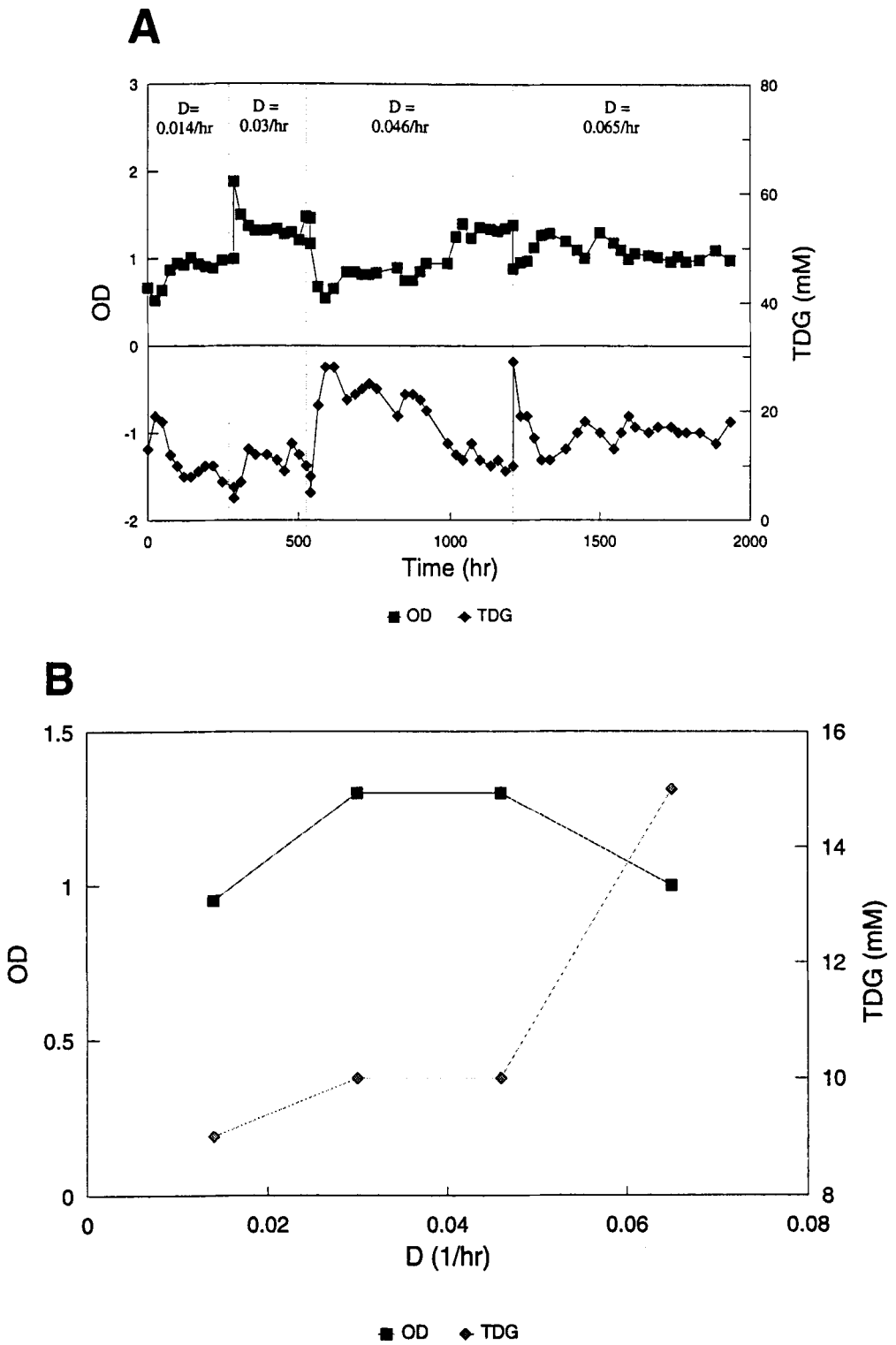

Fig. 5. **(A)** CSTR results and **(B)** Steady-state OD and TDG as a function of dilution rate for 50 m*M* influent CSTR.

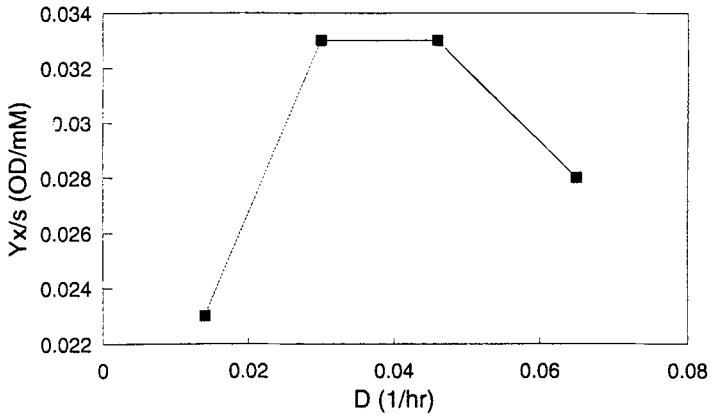

Fig. 6. Yield as a function of dilution rate for 50*M* CSTR.

When comparing the CSTR to the CSTR with cell recycle and batch reactors, it was evident that the recycle system offered superior TDG degradation. For the cases presented, lower effluent TDG was observed at dilution rates of 0.014/h, 0.030/h, and 0.046/h (the steady-state ODs and TDG concentrations for these dilution rates were 1.8 and 4 m*M*, 1.9 and 7 m*M*, and 1.8 and 9 m*M*, respectively, for the CSTR with cell recycle; and 0.95 and 9 m*M*, 1.3 and 10 m*M*, and 1.3 and 10 m*M*, respectively, for the CSTR); however, no significant difference was discernible at the higher dilution rate of 0.065/h (the steady-state ODs and TDG concentrations were 1.1 and 17 m*M* for the CSTR with cell recycle, and 1.0 and 16 mM for the CSTR). Typically, compared with a CSTR, a cell recycle system allows operation at higher dilution rates, i.e., washout occurs at a higher dilution rate in a cell recycle system than in a CSTR; however, maximum dilution rate was not extended in our recycle system. This was likely owing to the inefficiency of the filter module in concentrating cells at high dilution rates. As an alternative, a settling tank may be more effective than a filter in cell concentration. Augmenting the concentration factor will increase the maximum permissible dilution rate of the CSTR with cell recycle. Overall, at 50 m*M* influent TDG and similar residence time, the CSTR with cell recycle offered greater TDG degradation than the batch reactor and the CSTR.

CONCLUSION

Experiments were performed on batch, CSTR, and CSTR with cell recycle reactors. From the experimental data, it was evident that for 50 m*M* hydrolysis effluent (bioreactor feed concentration), CSTR with cell recycle offered superior performance over batch reactors and CSTRs. Other interesting phenomena were also elucidated from our experiments. First, a metabolic byproduct of TDG was found. Second, it was observed in batch fermentations that the bacteria reached stationary phase long before TDG was depleted. We believe this is owing to a dead-end intracellular metabolic byproduct. Its existence in the media either suggests cell lysis or secretion. Finally, the CSTR data showed that the yield was not constant, but was a strong function of growth rate. Further, multiple steady states may be occurring, which will require further theoretical and experimental analysis.

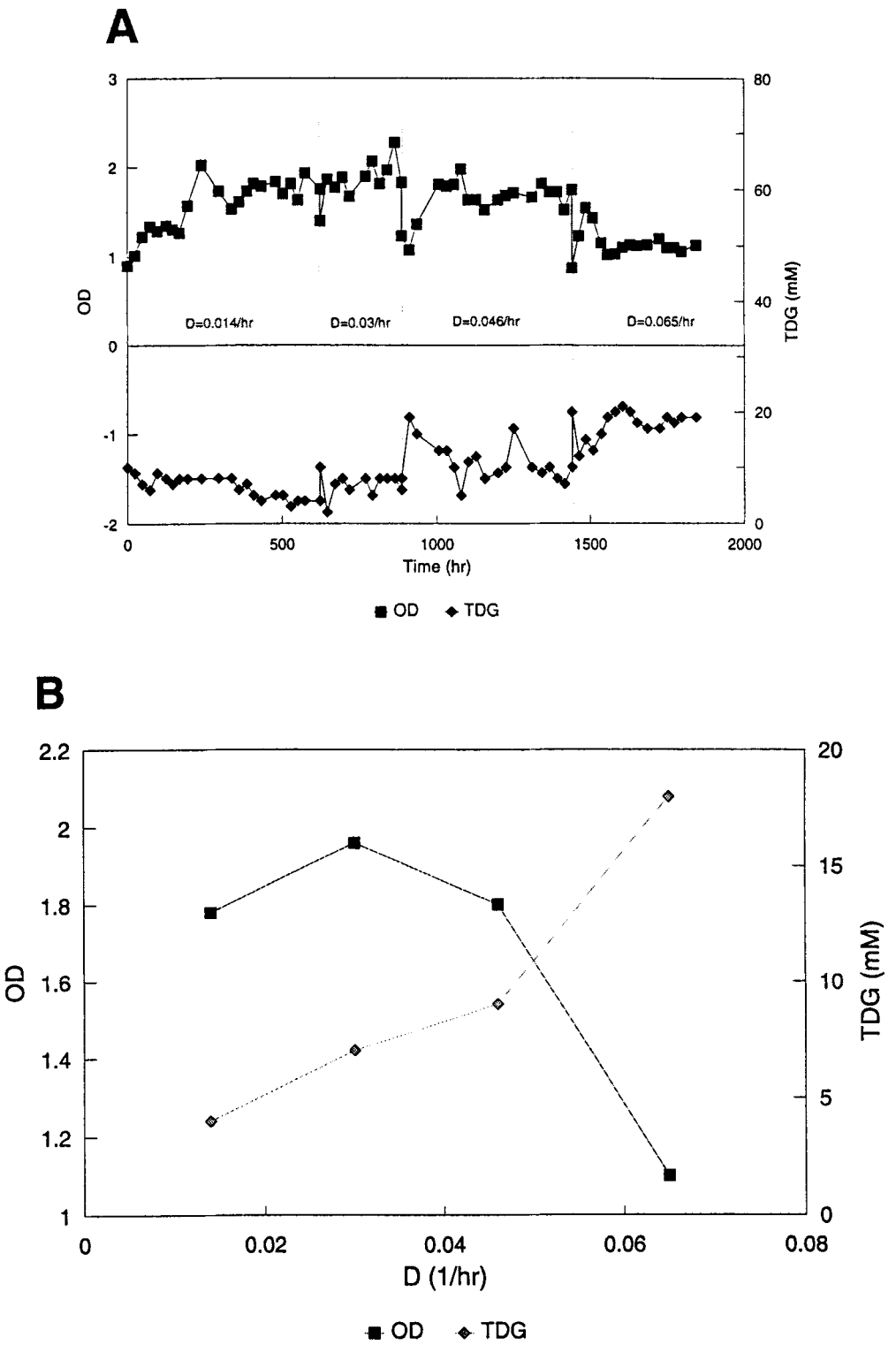

Fig. 7. **(A)** CSTR with cell recycle results; and **(B)** Steady-state OD and TDG as a function of dilution rate for 50 m*M* influent CSTR with cell recycle.

REFERENCES

1. Bentley, W. E., Bunnett, J. F., DeFrank, J. J., von Fahnestock, M., Haley, M. V., Harvey, S. P., Issacson, L., Kilbane, J. J., Kolakowski, J. E., Labare, M. P., Ontiveros, J.-L., and Wild, J. R. (1994), *Proceedings of the 1993 Scientific Conference on Chemical Defense Research*, Aberdeen Proving Ground, MD, pp. 1133–1138.
2. Harvey, S. and DeFrank, J. J. (1993), in *Army Science: The New Frontiers, Military and Civilian Applications*, Kamely, D. and Sasmor, R., eds., Borg Biomedical Services, Richmond, VA, pp. 141–151.
3. Lee, T., Weigand, W. A., Bentley, W. E., and Harvey, S. P. (1996), Bioreactor strategies for the degradation of thiodiglycol, the main hydrolysis product of sulfur mustard. (submitted).
4. Sines, B. J., Teather, E. W., Harvey, S. P., and Weigand, W. A. (1994), *Appl. Biochem. Biotech.* **45/46,** 881–895.
5. Lee, T. (1994), Using *Alcaligenes xylosoxidans* (SH91) to degrade thiodiglycol. MS Thesis, University of Maryland, College Park, MD.
6. Yano, T. and Koga, S. (1969), *Biotechnol. Bioeng.* **11,** 139–153.
7. Bailey, J. E. and Ollis, D. F. (1977), *Biochemical Engineering Fundamentals*, MacGraw-Hill, New York.