0273-2289/88/1800-0315/$02.00

Toxicity of Organic Solvents to *Clostridium acetobutylicum* for Extractive ABE Fermentation

Scientific Note

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Index Entries: Toxicity; solvent; *Clostridium*; fermentation.

INTRODUCTION

Efficient separation and recovery of butanol, acetone, and so on in the acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is of considerable importance to an economic operation of this fermentation process. These fermentation products may be separated *in situ* by dispersion-free solvent extraction. A novel microporous hollow fiber membrane based fermentation-extraction technique for separating ethanol *in situ* in a *Saccharomyces cerevisiae* fermentation system without dispersing the solvent in the broth or vice versa (*1,2*) is especially relevant.

In this system, the solvent is continuously passed through the fiber lumen and fermentation proceeds on the outside of the fibers (shell side). An immobilized cell system is used to facilitate fermentation. The organic solvent wets the fibers as soon as it comes into contact with them. To prevent this solvent from dispersing into the shell side, the shell side pressure is kept higher than that in the fiber lumen. The organic solvent stays in the pores and the aqueous-organic interface is immobilized at the pore mouth on the shell side (*1,3–5*). Ethanol in the broth comes in contact with solvent at the pore mouth and is extracted into the solvent in the pores and thence to the bulk solvent.

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A similar configuration is being used by us for the ABE fermentation process. We have selected solvents, e.g., 1-octanol and 2-ethyl-1-hexanol because of their attractive characteristics for extraction of acetone, butanol, and ethanol. These solvents have beneficial distribution coefficients, high separation factors, high boiling points, and low solubility in water (*6*). In such a direct solvent extraction process from broth, the bulk solvent does not come into direct contact with the immobilized cells in the shell side. However, there is a low but finite concentration of the extracting solvent in the broth. Thus, it is necessary to study the toxicity of these solvents to *C. acetobutylicum* cells.

Bar and Gainer (*7*) have studied the toxicity of organic solvents to *L. delbrueckii*, *A. niger*, and *A. aceti*. They have studied toxicities at two levels, i.e., molecular and phase levels. Since in our system there will not be any bulk organic phase, our investigation is mainly carried out below saturation level in the medium. The present work also includes excess solvent present as a separate phase. The product concentration and cell density are monitored. The varying concentration of these with solvent levels will define the extent of toxicity. Some of these results are presented and discussed herein.

MATERIALS AND METHODS

Microorganism

The microorganism used was *Clostridium acetobutylicum* (NRRL B-592). The organism was received in the freeze dried state. This was reconstituted into 20 mL of cooked meat medium (Difco, Detroit, MI). This medium also contained glucose (50 g/L). The cooked medium with freeze dried cells was incubated at 30°C. Soon after gassing ceased, 5 mL of this culture was inoculated into 90 mL of cooked meat medium containing glucose (50 g/L), heat shocked at 75°C for one min and incubated at 30°C. After sporulation, the culture was stored at 4°C and used as the master culture (*8*).

Medium

The composition of fermentation medium in one L of distilled water was as follows (*9*).

Glucose: 20 g/L, KH_2PO_4: .5 g/L, $K_2HPO_4 \cdot 3H_2O$: .5 g/L, $MgSO_4 \cdot 7H_2O$: .2 g/L, $MnSO_4 \cdot H_2O$: .01 g/L, $FeSO_4 \cdot 7H_2O$: .01 g/L, NaCl: .01 g/L, ammonium acetate: 2.2 g/L, P-aminobenzoic acid: .001 g/L, biotin: .00001 g/L.

This medium was autoclaved and used for fermentation. The inoculum level was about 3%.

Analysis

The cell growth was estimated by the measurement of optical density at 576.9 nm using Bausch and Lomb spectrophotometer model 1001. The cells were centrifuged, washed, and dried overnight at 60°C for dry wt calculation. The concentrations of acetone, butanol, ethanol, acetic acid, and butyric acid in the broth were measured by gas chromatography using a Hewlett Packard Gas Chromatograph model 5890 fitted with a Porapak Q (80/100) column and FID detector. The samples were filtered through .22 μm filter paper before injection. The glucose concentration was analyzed by a glucose analyzer (YSI model 27).

Solvents

Solvents 1-octanol and 2-ethyl-1-hexanol were procured from Fisher Scientific Co. These solvents were pure and used without autoclaving. The properties of these solvents are given in Table 1 (Dadgar and Foutch, 1985).

Experimental Procedure

Fifty mL of each solvent was mixed with the equal volume of medium for 12 h and then allowed to settle for 24 h. The solvent concentration in the medium was determined by GC. This is assumed to be the saturation level concentration of solvent in the medium.

Butanol, acetone, and ethanol solutions of 2%, .7%, and .3% (v/v), respectively, were prepared. Fifty mL of each of these were mixed with 50 mL of 1-octanol for 24 h and then allowed to settle. The concentrations of butanol, acetone, and ethanol in the aqueous and organic phases were

Table 1
Properties of Solvents

Properties		Solvents	
		1-Octanol	2-Ethyl-1-hexanol
Distribution coefficient	E[a]	.53 (.67)	.47 (.48)
	A[b]	.52 (.56)	.58 (.62)
	B[c]	5.60 (4.78)	6.09 (5.98)
Separation factor	E	11.27	21.36
	A	11.06	26.36
	B	119.15	276.70
Solubility in water by wt %		.538	Insoluble
Boiling point,°C		194.4	185.0

[a]E = ethanol.
[b]A = acetone.
[c]B = butanol.

determined. The same procedure was repeated with 2-ethyl-1-hexanol. The distribution coefficients, thus determined, are shown in parenthesis in Table 1.

Two hundred mL medium was transferred into each of the three flasks. The medium was sparged with nitrogen. One flask was used as control flask and into the other two flasks, the required volume of each solvent was transferred. These were inoculated and incubated at 37°C. The samples were collected at different time intervals and stored for analysis. The same procedure was repeated with other solvent/medium ratios.

RESULTS AND DISCUSSION

Following the procedure described, the solvent concentrations in saturated aqueous phase was found to be .66% and .7% (v/v) for 2-ethyl-1-hexanol and 1-octanol respectively. The solvent concentrations of .2%, .4%, .6%, 1.0%, and 5.0% (all vol %) were investigated for toxicity studies. The first three concentration levels are below the saturation level, while the last two are above the saturation level.

Five sets of experiments were carried out with the above concentration levels of solvents. In each set, three flasks containing equal volume of medium were inoculated with previously grown *C. acetobutylicum* cells. One flask was used as a control flask, and into the other two the required amount of solvents were transferred. The same procedure was repeated with other concentration levels of the solvents. The pH was not controlled in any of these experiments. The starting pH was 6.4.

The results for the control experiment, i.e., fermentation carried out without solvents, are shown in Fig. 1. The concentrations of products are plotted against time. In this plot the cell density is also shown. It can be seen that the exponential phase starts after 15 h and ends at about 40 h. Maximum concentrations of 6.6 g/L, 2 g/L, and .6 g/L are obtained, respectively, for butanol, acetone, and ethanol. Overall maximum solents concentration achieved is 8.2 g/L. All of the glucose is consumed roughly in 100 h.

The effects of different levels of 2-ethyl-1-hexanol and 1-octanol in the medium on glucose consumption are plotted against time in Figs. 2 and 3. The initial glucose concentration was 20 g/L. From Fig. 2, we observe that a higher amount of glucose is consumed at lower solvent levels. It is also clear that beyond the saturation level, a much smaller amount of glucose is consumed. The same trend is observed in Fig. 3, where the extracting solvent is 1-octanol. Comparison of the results of Figs. 2 and 3 suggests that 2-ethyl-1-hexanol is less toxic than 1-octanol, because a smaller amount of glucose is consumed in the presence of 1-octanol.

The total neutral solvent (acetone, butanol, and ethanol) concentrations are plotted against time in Figs. 4 and 5 at various concentration

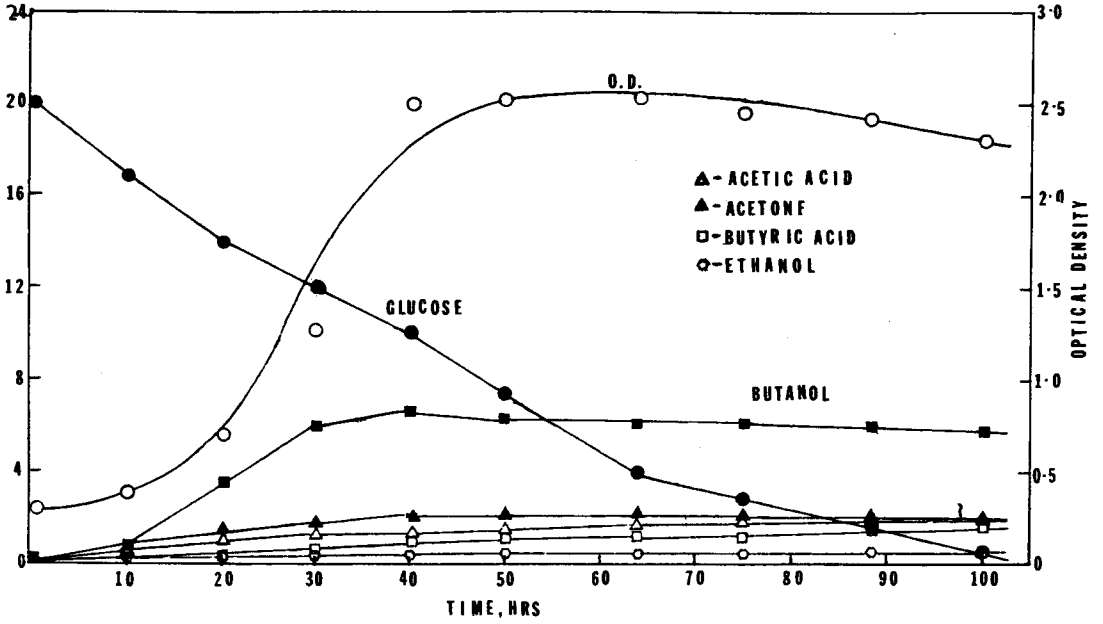

Fig. 1. Production of solvents and acids in presence of *Clostridium acetobutylicum* without organic solvent. ○ - optical density; ● - glucose; □ - butyric acid; ■ - butanol; △ - acetic acid; ⬡ - ethanol; ▲ - acetone.

levels of extracting solvents. Larger amounts of neutral solvents form at lower levels of 2-ethyl-1-hexanol and 1-octanol. Since total neutral solvent level reaches a steady value, the latter condition suggests that no viable cells are present to carry out fermentation although glucose is present. This is clear at a 5% concentration level of solvent.

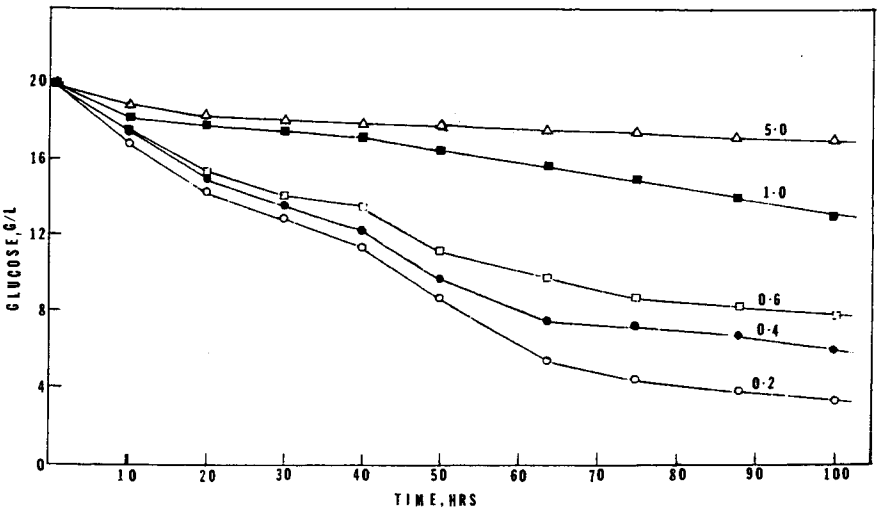

Fig. 2. Effects of 2-ethyl-1-hexanol in medium on glucose consumption. Solvent concentration is shown in volume percent. ○ - .2, ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

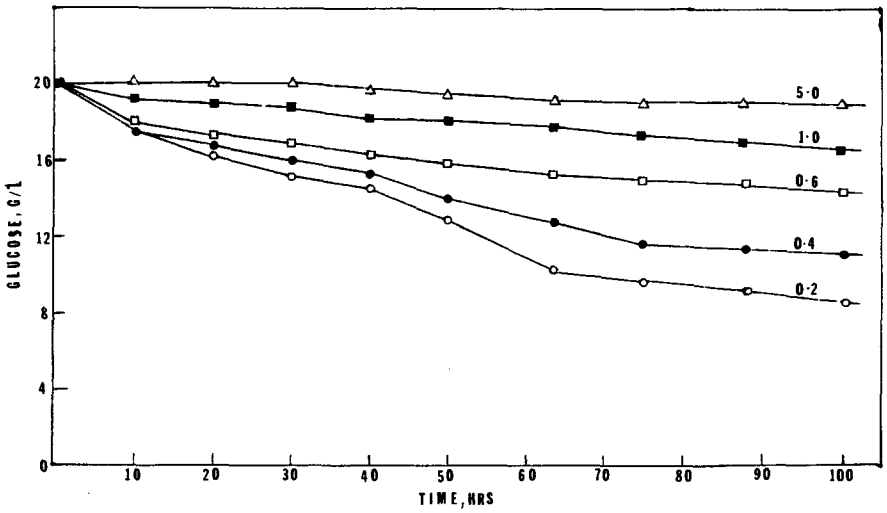

Fig. 3. Effects of 1-octanol in medium on glucose consumption. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

The formation data of acetic acid and butyric acid are shown in Figs. 6 and 7 for both solvents, and the trend is similar. The biomass formation data are plotted in Figs. 8 and 9 and these results are consistent with glucose consumption observations. The pH drops from 6.4 and later stabilizes. There is no dramatic change in pH because of the presence of solvent in the medium. The effects of extracting solvent concentration levels

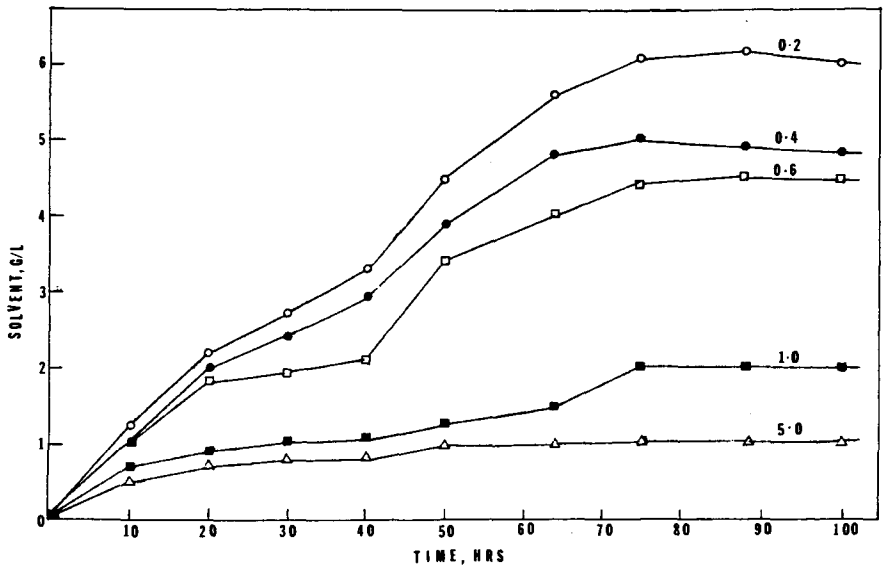

Fig. 4. Effects of 2-ethyl-1-hexanol in medium on solvents production. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

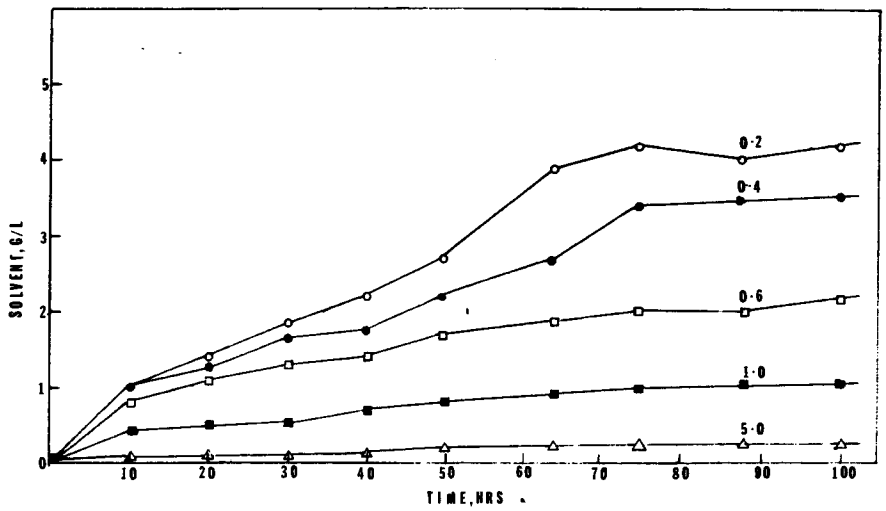

Fig. 5. Effects of 1-octanol in medium on solvent production. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

on glucose consumption, neutral solvents formation, acids formation, and biomass formation are shown in Fig. 10 at 100 h. The inhibition constants may also be determined by plotting the production rates with the solvent concentrations (*10*).

It is evident from Figs. 2–9 that 1-octanol is more toxic than 2-ethyl-1-hexanol, though both extracting solvents are isomers and have the same molecular weight. The major difference is in their structure, i.e., the former is a straight chain compound, whereas an ethyl group is present

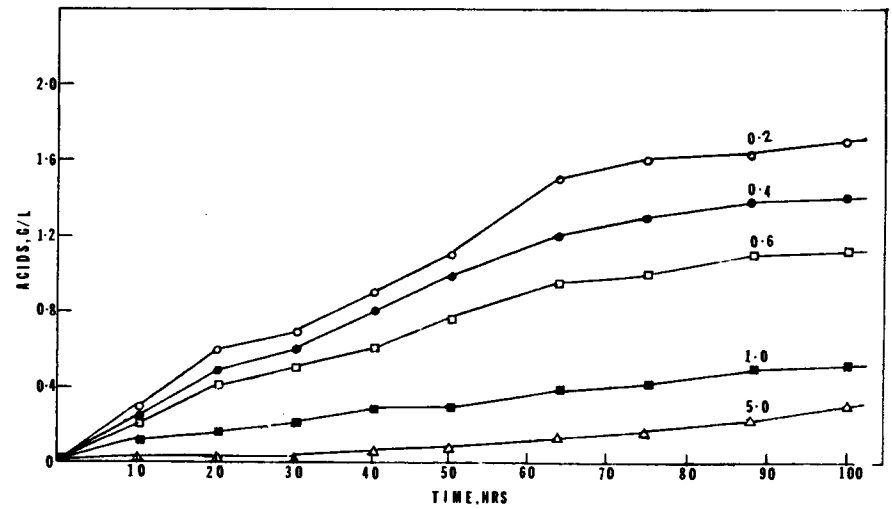

Fig. 6. Effects of 2-ethyl-1-hexanol in medium on acids production. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

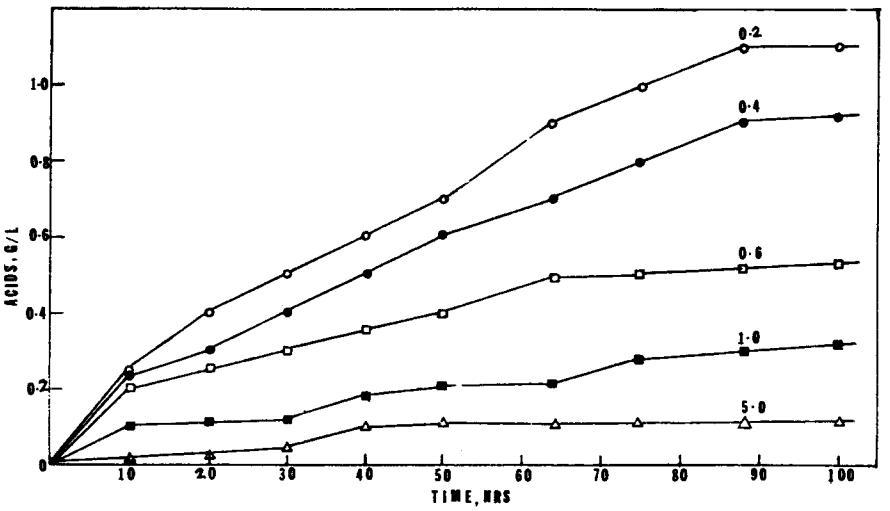

Fig. 7. Effects of 1-octanol in medium on acids production. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

at the second carbon atom in the case of the latter. Although both molecules can diffuse through the cell membrane, 2-ethyl-1-hexanol may not diffuse so freely because of branching. Hence, it is probably less toxic than 1-octanol. The partitioning of the solvent in the cell membranes can also influence the concentration of solvent molecules in the cells and in cell membrane.

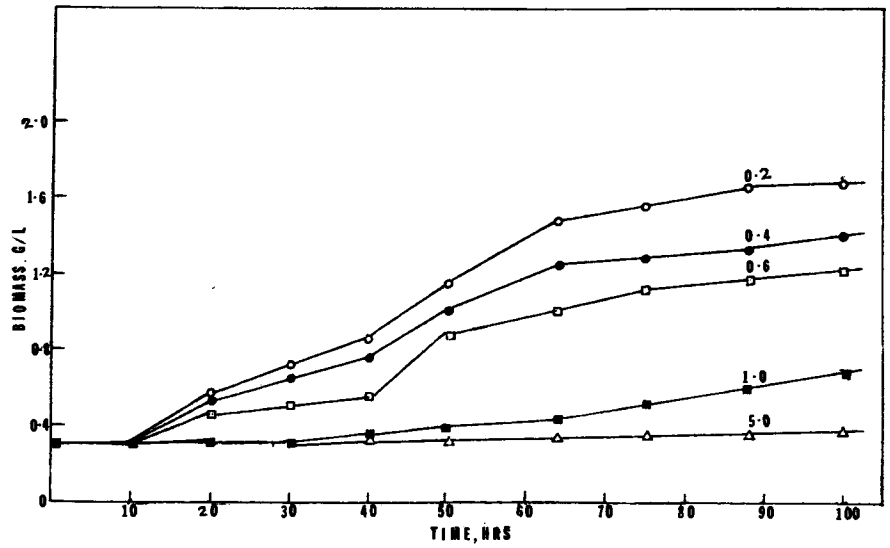

Fig. 8. Effects of 2-ethyl-1-hexanol in medium on cell density. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

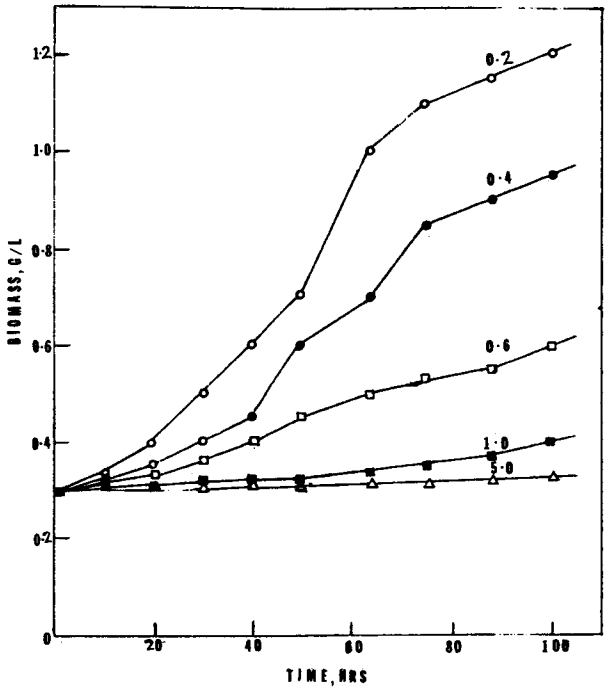

Fig. 9. Effects of 1-octanol in medium on cell density. Solvent concentration is shown in volume percent. ○ - .2; ● - .4; □ - .6; ■ - 1.0; △ - 5.0% (v/v).

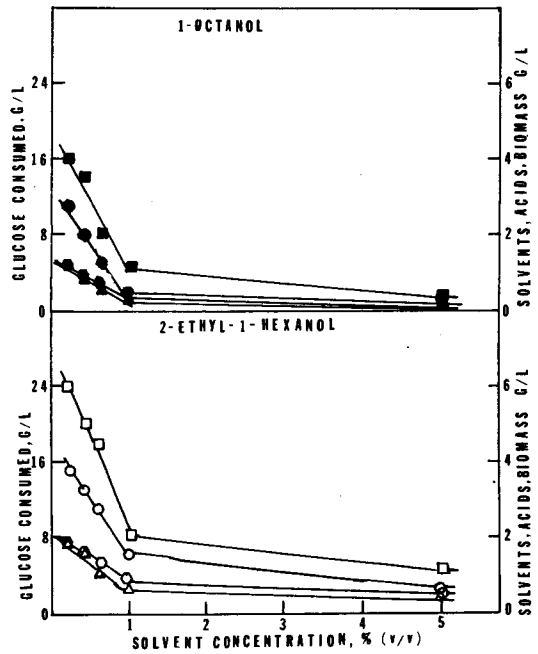

Fig. 10. Effects of solvent concentrations on glucose consumption, neutral solvents, acids and biomass formation at one hundred hours. ○ ● glucose consumption; □ ■ neutral solvent; △ ▲ acids; ⬡ biomass.

Bar and Gainer (7) have pointed out that toxicity can be present at molecular level as well as phase level. According to them, molecular toxicity is caused by dissolved molecules, whereas phase toxicity appears when two phases are quite distinct. The latter could lead to solvent coatings over the cell membrane, the immobilization of cells on the micelle interface, and the associated ill effects. We believe that in our case at .2, .4, and .6% (v/v) solvent levels, the toxicity is at the molecular level, whereas at 1 and 5% (v/v) it is at both molecular and phase levels. The phase level toxicity and molecular level toxicity, when combined, reduce the activity of cells drastically. This explains the much lower activity of cells at 5% solvent concentration levels. It may be pointed out that we have tried to maintain the same inoculum level for each set of experiments. The toxicity problem may be mitigated somewhat by incorporating more and more cells.

In a microporous hollow fiber membrane based fermentor-extractor being studied in our laboratory, the volume inside the fibers is much less than the shell side volume. On the other hand, the cells are immobilized in the shell side and they are not in direct contact with the solvent. Thus, the extent of extracting solvent buildup in the broth is likely to be very low, and its effect on the cells still lower. We will report elsewhere the role of the extracting solvents in our extractive fermentor.

ACKNOWLEDGMENTS

The authors thank New Jersey Commission on Science and Technology for funding this study under the Biotechnology Initiative of the Innovation Partnership Program.

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