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A Comparison of the Effects of Acetophenone, 1-Hexanol, and Hexane on *S. cerevisiae* and *Z. mobilis* in Batch and Continuous Immobilized-Cell Culture

Scientific Note

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INTRODUCTION

The yeast *Saccharomyces cerevisiae* has traditionally been employed in industrial ethanol production by fermentation because of its rapid specific growth and production rates in the presence of moderately high concentrations of alcohol. More recently, however, the bacterium *Zymomonas mobilis* has been used with sucess in laboratory studies (*1–3*). Although product formation by both organisms can be described by the same overall stoichiometric equation, there is an important metabolic difference between the two organisms. *S. cerevisiae* utilizes the Embden-Meyerhoff-Parnas pathway (*4*), which yields 2 mol of ATP/mol of glucose consumed. *Z. mobilis,* on the other hand, utilizes the Entner-Doudoroff pathway (*5*), which yields 1 mol of ATP/mol of glucose consumed. It has been postulated that, at the same specific growth rate, *Z. mobilis* would consume glucose at a rate at least twice as fast as that of *S. cerevisiae,* since only half as much energy is produced during the fermentations (*6*).

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The advantageous characteristics of *Z. mobilis* in comparison to *S. cerevisiae* have been summarized by Rogers et al. (*3*). They found that *Z. mobilis* had

1. Specific rates of sugar uptake and ethanol production three to four times faster than those of yeasts;
2. Higher ethanol yields and lower biomass yields; and
3. An ethanol tolerance comparable to, if not better than, that of yeasts.

Waldron et al. (*7*) compared the performance of *Z. mobilis* and *S. cerevisiae* in converting glucose to ethanol in a continuous, crosslinked immobilized-cell reactor. They found that reactor start-up was much more rapid with crosslinked *Z. mobilis*, and observed volumetric ethanol productivities (based on liquid holdup) that were three times those obtained with crosslinked yeast.

The substrate employed in each of the above studies was a highly purified sugar solution. However, it is generally recognized that much of the fuel alcohol produced industrially in the future will be formed from sugars produced from native sources, such as agricultural byproducts and municipal solid waste (MSW). If an acid-hydrolysis process is utilized in producing these sugars, trace quantities of hydrolysate byproducts or acid-recovery solvents may be present in the resulting sugar streams, which could reduce substrate conversion and cell growth. A study is thus needed to compare the results of glucose conversion with *Z. mobilis* and *S. cerevisiae* in the presence of small quantities of these potential inhibitors.

The purpose of this paper is to compare the performance of *Z. mobilis* and *S. cerevisiae* in converting glucose to ethanol in the presence of various concentrations of impurities in both batch culture and the crosslinked immobilized-cell reactor. Cell, substrate, and product profiles, as well as ethanol productivities, are compared in batch culture; overall ethanol productivity and column longevity are compared in continuous culture. The acid-recovery solvents acetophenone, 1-hexanol, and hexane were chosen as the impurities for study. Hydrolysate byproducts, such as furfural, have not been shown to cause significant decreases in ethanol production by *Z. mobilis* and *S. cerevisiae* at concentrations up to 2.0 g/L (*8*).

MATERIALS AND METHODS

Organisms and Media

Saccharomyces cerevisiae, ATCC 24860, was stored as slant cultures on a 21-g/L YM broth medium (Difco) containing 16 g/L glucose. Inocula were prepared by transferring cultures to a medium containing 10 g/L glucose and 3 g/L yeast extract (Difco) and incubating at 30°C for 20–30 h. *Zymomonas mobilis*, ATCC 31821, was stored in a tube containing 100 g/L glucose

Table 1
Media Compositions

Medium Component	S. cerevisiae	Z. mobilis
Yeast extract (g/L)	3	10
KH_2PO_4 (g/L)	--	1
$(NH_4)_2SO_4$ (g/L)	--	1
$MgSO_4 \cdot 7\,H_2O$ (g/L)	--	0.5
Glucose (g/L)	118	118
Initial pH	4.0	5.0

-- none present

and 10 g/L yeast extract (Difco) at 4°C. The stock culture was transferred to fresh medium monthly. Media utilized in batch and continuous studies involving the two organisms are shown in Table 1.

Solvents

Acetophenone, 1-hexanol, and hexane were the acid-recovery solvents selected for study. Acetophenone and 1-hexanol (99%) were obtained from Sigma Chemical Company (St. Louis, MO), and hexane (HPLC grade) was obtained from Fisher Scientific Company (Pittsburgh, PA).

The solubility of each of the solvents in a medium containing 3 g/L yeast extract and 100 g/L glucose at pH 4 and room temperature (26°C) was first determined, to serve as a guide for selecting the solvent concentrations in the inhibition studies. Acetophenone was found to have a solubility of 6.0 g/L; 1-hexanol and hexane had solubilities of 7.0 and 4.6 g/L, respectively.

Equipment and Procedures

The batch reactors were constructed from 500-mL Pyrex™ side-arm flasks, essentially sealed from the environment. During the fermentation, a tube was connected from the flasks to a water seal that allowed CO_2 produced during the fermentation to leave the system without air introduction. Agitation was provided by variable-speed magnetic stirrers at a level that ensured homogeneity. The temperature was controlled at 30°C in a constant-temperature room, and an inoculum of 10% by volume was employed. Varying quantities of acid-recovery solvents (acetophenone, 1-hexanol, or hexane) were added to the media prior to fermentation. Both organisms were incubated without pH control.

The immobilized-cell reactors consisted of plug-flow tubular columns constructed of Plexiglas™ tubing with an inside diameter of 3.16 cm and a wall thickness of 0.32 cm (47.0 cm long). The reactors were randomly filled

with spherical solid-glass beads (4 cm nominal size). The glass beads were dip-coated in a 25% gelatin solution and crosslinked to the cells using a 3% glutaraldehyde solution. The reactors were sterilized by passing 8% propylene oxide in carbon dioxide through the column. Various concentrations of the solvents were added to the column once a brief start-up without solvent had occurred. Again both organisms were incubated at 30°C without pH control. More detailed descriptions of the equipment and general operating procedures are given by Vega et al. (*9*) and Waldron et al. (*7*).

Analytical Methods

The following analytical methods were used as a routine basis for substrate, cell, and product concentration measurements:

Glucose Assay

Glucose was determined with a Yellow Springs Instrument Company (Yellow Springs, OH) Model 27 industrial analyzer.

Cell Concentration

Cell concentrations were determined by turbidity measurement at 520 nm for *S. cerevisiae* and at 580 nm for *Z. mobilis*, using a Spectronic 21 spectrophotometer (Milton Roy Company, Rochester, NY). Standard curves correlating dry cell weight to absorbance were prepared. It is realized that turbidity measurements can be a bit misleading as a result of changes in *Z. mobilis* cell morphology under stressed conditions. Nevertheless, turbidity measurements were employed in estimating cell concentration for both organisms because of the simplicity of the measurements.

Ethanol Analysis

Ethanol concentrations were determined by gas chromatography using a 3.2-mm × 1.2-m Chromosorb 102 column (60/80 mesh) and a thermal-conductivity detector. The oven temperature was maintained at 120°C, and the detector and injector temperatures were both 205°C. The carrier gas was helium at a flow rate of 40 mL/min.

RESULTS AND DISCUSSION

Batch Studies

The results of batch fermentations using *S. cerevisiae* in the presence of acetophenone with a 0.1-g/L inoculum are shown in Figs. 1–3. Similar results were obtained for 1-hexanol and hexane (data not shown). As is noted in Fig. 1, the concentration of acetophenone significantly affected growth, reaching only 30% of the maximum cell concentration, 3.5 g/L (without solvent), at a 2-g/L acetophenone concentration. Similarly, all the substrate could not be consumed at acetophenone concentrations above

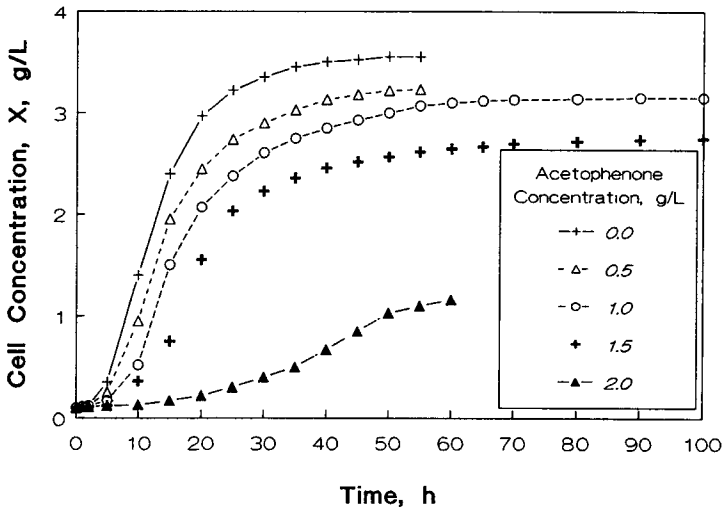

Fig. 1. Cell concentration as a function of time for *S. cerevisiae* in batch culture using various concentrations of acetophenone; 118 g/L glucose.

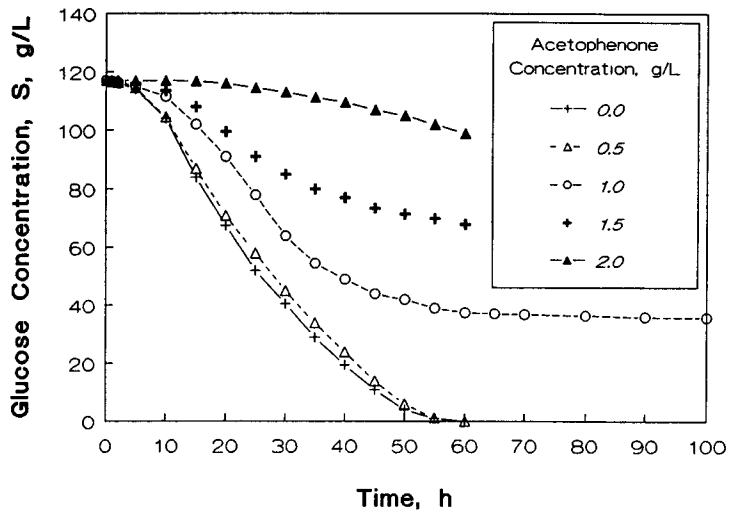

Fig. 2. Glucose concentration as a function for *S. cerevisiae* in batch culture using various concentrations of acetophenone.

0.5 g/L (*see* Fig. 2). However, the yield of cells from glucose, $Y_{x/s}$, and the yield of ethanol from glucose, $Y_{p/s}$, remained constant at 0.029 and 0.46 g/g, respectively, regardless of acetophenone concentration. Atkinson and Mavituna (*10*) reported a cell yield of 0.033 g/g and a product yield of 0.44 g/g for *Saccharomyces uvarum*.

The effects of acetophenone concentration on the fermentation using Z. *mobilis* are shown in Figs. 4–6. Again, similar results were noted for 1-hexanol and hexane. As was noted with *S. cerevisiae*, acetophenone limited cell growth, as evidenced by the significant decrease in cell concentration at an acetophenone concentration of 1 g/L (*see* Fig. 4). The time

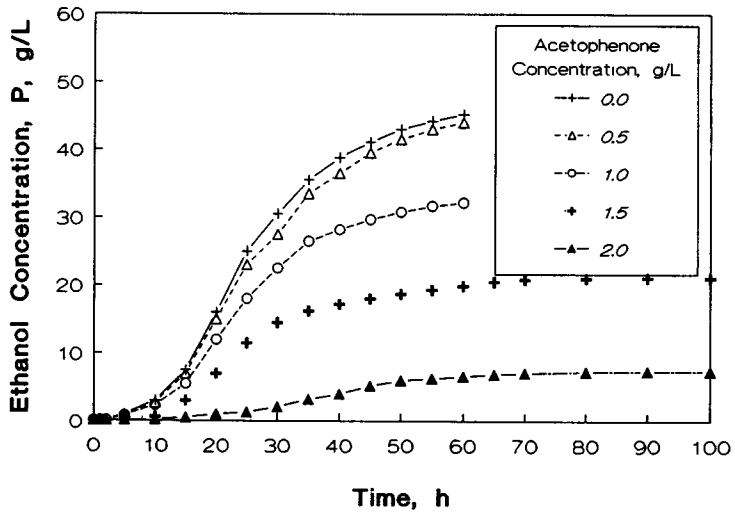

Fig. 3. Ethanol concentration as a function of time for *S. cerevisiae* in batch culture using various concentrations of acetophenone; 118 g/L glucose.

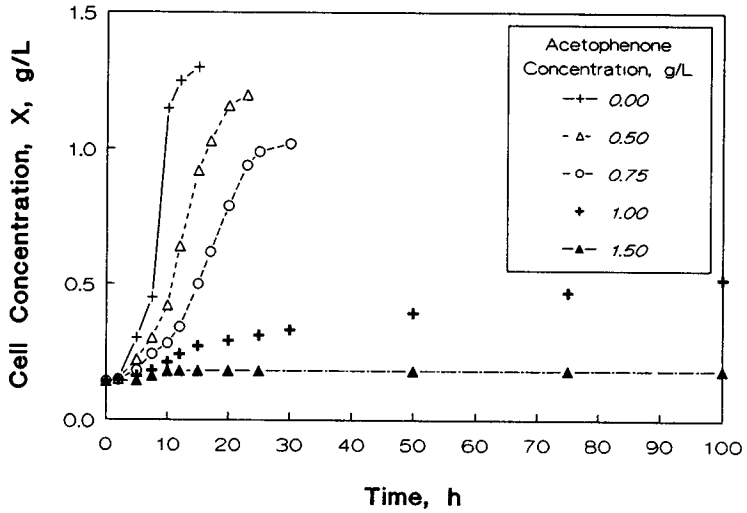

Fig. 4. Cell concentration as a function of time for Z. *mobilis* in batch culture using various concentrations of acetophenone; 118 g/L glucose.

for complete utilization was increased nearly 10-fold with a 1-g/L acetophenone concentration, and little substrate utilization was noted with a 1.5-g/L concentration (*see* Fig. 5). Again, $Y_{x/s}$ remained constant at 0.012 g/g and $Y_{p/s}$ remained constant at 0.47 g/g, regardless of the acetophenone concentration. Atkinson and Mavituna (*10*) obtained a product yield of 0.47 g/g and a cell yield of 0.019 g/g for Z. *mobilis*.

A comparison of overall batch productivities (based on medium volumes) for *S. cerevisiae* and Z. *mobilis* in the presence of various concentrations of solvents is shown in Table 2. Several interesting observations

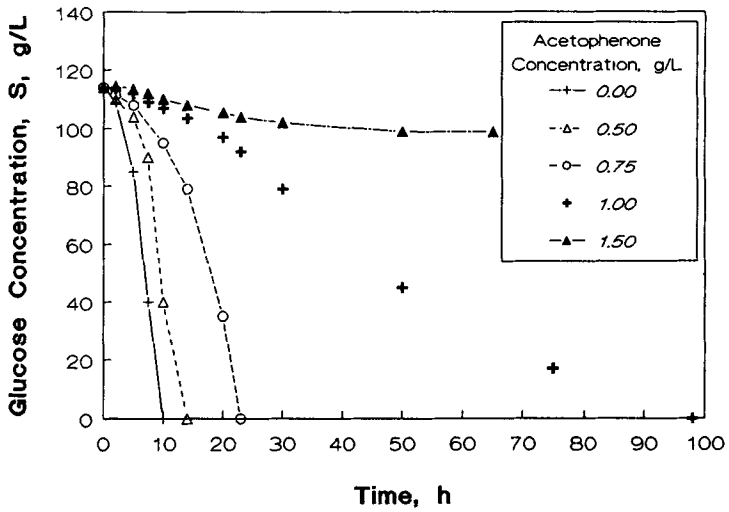

Fig. 5. Glucose concentration as a function of time for *Z. mobilis* in batch culture using various concentrations of acetophenone.

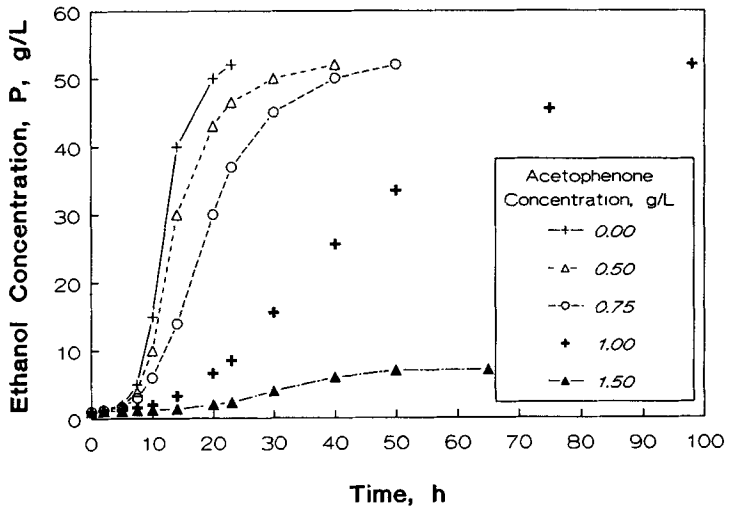

Fig. 6. Ethanol concentration as a function of time for *Z. mobilis* in batch culture using various concentrations of acetophenone; 118 g/L glucose.

may be noted from the results in this table: First, the productivities from *Z. mobilis* are generally higher than from *S. cerevisiae*. Second, each of the solvents inhibits productivity, but at different concentrations. Based mainly on the results with *Z. mobilis*, the solvent 1-hexanol appears to be a more potent inhibitor than acetophenone, which appears to be a more potent inhibitor than hexane. It is significant to note that this degree of inhibition is related to the degree of solubility in medium. These results are similar to the work of Gill and Ratledge (*11*), who found that the toxicity of several inhibitors to *Candida tropicalis* and *Saccharomyces carlsbergensis*

Table 2
Batch Ethanol Productivities for *S. cerevisiae* and *Z. mobilis* in the Presence of Various Acid Recovery Solvents

Solvent	Solvent Concentration (g/L)	Productivity, g/L·h	
		S. cerevisiae	*Z. mobilis*
No solvent present	--	1.0	4.6
Acetophenone	0.50	0.9	3.5
	0.75	0.8	2.0
	1.00	0.6	0.5
	1.50	0.4	0.2
1-Hexanol	0.50	0.9	3.5
	1.00	0.8	0.2
	1.50	0.5	--
Hexane	0.75	0.9	3.5
	1.50	0.7	0.9
	3.00	0.3	0.5
Acetophenone, 1-Hexanol and Hexane in equal concentrations	0.60 (total solvent concentration)	0.9	3.8
	1.20	0.8	0.7
	1.80	0.5	--

Inoculum Size: 0.1 g/L; 118 g/L glucose

-- not performed

was related to their solubilities in aqueous media. A third observation from Table 2 is that the productivity of Z. *mobilis* was more significantly inhibited by the presence of the solvents than that of *S. cerevisiae*. Finally, it is significant to note that the combined solvents have essentially the same inhibitory characteristics as the most potent of the inhibitors.

Vega et al. (*12*) showed that increasing the inoculum level decreased the severity of ethanol inhibition. It was thought that the same result might be found with solvent concentration. The effects of inoculum size on ethanol productivity for *S. cerevisiae* and *Z. mobilis* at a constant acetophenone concentration of 0.75 g/L are shown in Table 3. As is noted in the table, as the inoculum size increased, the productivity increased. Thus, the use of larger inoculum sizes might overcome the effects of higher solvent concentrations on ethanol productivity.

Continuous Studies

Fermentations were carried out in immobilized-cell reactors (ICRs) using *S. cerevisiae* and *Z. mobilis* with and without the presence of acetophenone. Figure 7 shows the overall volumetric column productivity in

Table 3
Batch Ethanol Productivities for S. cerevisiae and Z. mobilis on 118 g/L Glucose in the Presence of 0.75 g/L Acetophenone at Various Inoculum Sizes

Inoculum Size (g/L)	Ethanol Productivity (g/L·h)	
	S. cerevisiae	Z. mobilis
0.2	0.8	3.0
1.0	1.1	3.4
2.0	1.3	3.8
4.0	1.8	4.4
7.0	2.3	4.7
11.0	3.2	--

-- not performed

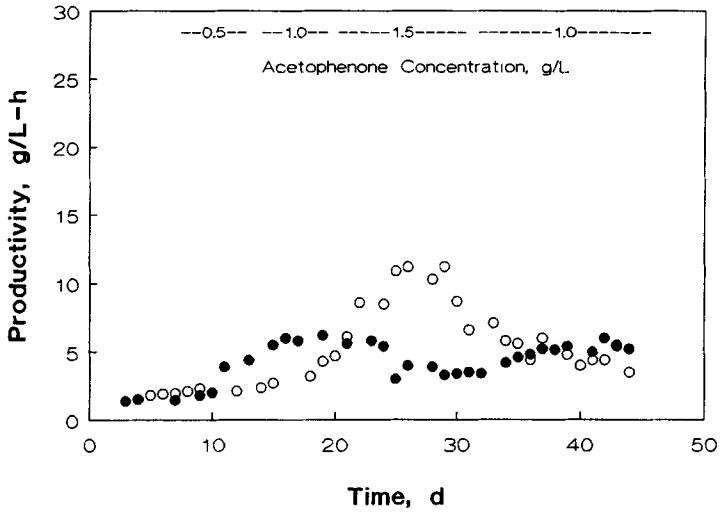

Fig. 7. Productivity in the immobilized-cell reactor for *S. cerevisiae* with (●) and without (○) acetophenone.

the ICR using *S. cerevisiae* with and without acetophenone present. Volumetric productivities were used, since the estimation of cell concentrations in biofilm reactors is not possible. However, measurements of the liquid holdup in each column showed nearly identical results, indicating that the columns had similar biomass loadings. Without acetophenone, the maximum productivity reached 12 g/L·h for an operating period of 3–5 d. After this time, cell overgrowth caused the productivity to drop, eventually decreasing to near zero productivity in 45–50 d. In the column in which acetophenone had been added, an acetophenone concentration of 1.5 g/L caused the productivity to drop, and an acetophenone

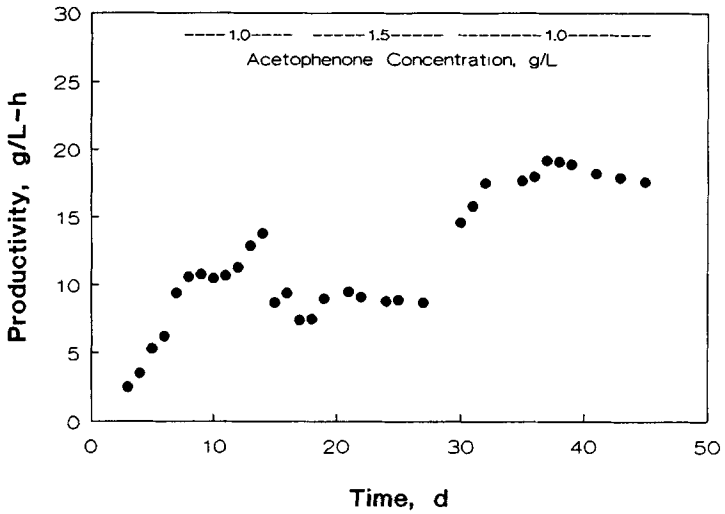

Fig. 8. Productivity in the immobilized-cell reactor for Z. *mobilis* using acetophenone.

concentration of 1.0 g/L controlled cell overgrowth. The drop in productivity as a result of cell overgrowth did not occur as it had in the column without acetophenone. Thus, an acetophenone concentration of 1.0 g/L is not only tolerated in the ICR, but also may improve column longevity. However, the maximum productivity will not be as high as without acetophenone. It should be noted that an acetophenone concentration of 1.0 g/L significantly inhibited growth and glucose uptake by *S. cerevisiae* in batch culture. The large cell density in the ICR may be responsible for the higher tolerance to acetophenone, although mass-transfer effects and detoxification reactions are possible.

Figure 8 shows overall volumetric column productivity in the ICR using Z. *mobilis* in the presence of acetophenone. As was noted for *S. cerevisiae*, column longevity was increased from 26 d without acetophenone (*13*) to at least 45 d in the presence of acetophenone. Furthermore, operation in the ICR in the presence of 1.0 g/L acetophenone was certainly possible, whereas a 1.0-g/L acetophenone concentration decreased growth and glucose uptake in batch culture. As was seen in batch culture, the productivities obtained with Z. *mobilis* were significantly higher (seven times larger) than those obtained with *S. cerevisiae*, even with an acetophenone concentration of 1.0 g/L.

It is expected that the addition of 1-hexanol or hexane to the medium of the ICR will show effects similar to those with acetophenone. However, it was found that, when using *S. cerevisiae*, furfural (a hydrolysate constituent), had quite different effects on ICR performance than did acetophenone (*14*). Lewis found that inhibition by furfural in the ICR was either absent or masked by the high concentration of cells within the reactor. Thus, different hydrolysate constituents or solvents might, quite

Table 4
Limiting Concentrations of Hydrolyzate Constituents

	Limiting Concentration (g/L)	
	S. cerevisiae	Z. mobilis
Batch Culture		
Acetophenone	0.6	0.6
1-Hexanol	0.5	0.5
Hexane	1.5	1.5
Acetophenone, 1-Hexanol and Hexane present jointly	0.5 (each solvent)	0.3
ICR		
Acetophenone	1.0-1.5	1.0-1.5

possibly, have different inhibitory effects on the ICR, especially when different microorganisms are used.

CONCLUSIONS

Both *S. cerevisiae* and *Z. mobilis* were found to be capable of producing ethanol from glucose in both batch and continuous cultures in the presence of low concentrations of acid-recovery solvents in the medium. However, *Z. mobilis* seemed to be more sensitive to the solvents when the concentrations were high or when two or more solvents were present in the medium. Among the three solvents tested, 1-hexanol had the most inhibitory effects, followed by acetophenone and then hexane. This order agrees will with the order of solubility in aqueous medium.

In comparing the continuous ICR with the batch reactor, it was found that the ICR yielded significantly higher volumetric productivities and tolerated higher concentrations of solvents. Low concentrations of solvents in the feed medium may, in fact, help to alleviate cell overgrowth, thereby increasing column longevity.

The limiting concentrations of solvents in the medium for both batch and immobilized-cell reactors are summarized in Table 4. The limiting concentration in batch culture was estimated as the maximum concentration permitting complete glucose uptake in a period not exceeding twice the time for complete conversion without solvent. The limiting concentration in the continuous ICR was estimated as the maximum allowable concentration not resulting in a decrease in volumetric productivity. Each of these values was estimated from the available data.

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