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Continuous Xylose Fermentation by *Candida shehatae* in a Two-Stage Reactor

M. A. ALEXANDER,[1] T. W. CHAPMAN,[1] AND T. W. JEFFRIES[2,*]

[1]Department of Chemical Engineering, University of Wisconsin, Madison, WI 53706; [2]Institute for Microbial and Biochemical Technology, USDA Forest Service; Forest Products Laboratory,† 1 Gifford Pinchot Drive, Madison, WI 53705

ABSTRACT

Recent work has identified ethanol toxicity as a major factor preventing continuous production of ethanol at the concentrations obtainable in batch culture. In this paper we investigate the use of a continuous two-stage bioreactor design to circumvent toxic effects of ethanol. Biomass is produced via continuous culture in the first stage reactor in which ethanol concentrations are either absent or maintained at low levels. The freshly grown cells are fed into the second bioreactor in which high ethanol concentrations are produced. The steady influx of fresh cells and continuous removal of spent cells helps minimize the loss of fermentative activity that results from anaerobiosis and exposure to high ethanol concentrations. A final ethanol concentration of 37 g L^{-1} and overall yield of .32 g g^{-1} were obtained with the two-stage reactor as compared to corresponding values of 38 g L^{-1} and .32 g g^{-1} obtained in batch. The volumetric rate in the two-state process was .96 g L^{-1} as compared to .46 g $L^{-1}h^{-1}$ in batch.

*Author to whom all correspondence and reprint requests should be addressed.

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Index Entries: Xylose fermentation, continuous fermentation; ethanol toxicity; *Candida shehatae*; anaerobic.

INTRODUCTION

Previous work with batch xylose fermentation of *Candida shehatae* showed that young inocula (24 h grown) in batch culture gave higher fermentation rates than older inocula (72 h grown) (*1*). The difference between metabolic performance of the inocula was caused by viability differences. Apparently, prolonged exposure to fermentative conditions leads to gradual cell death. Other work with continuous fermentation using immobilized cells gave concentrations and specific fermentation rates far lower than those obtainable in batch culture (*2*). The low metabolic activity of immobilized biomass was quite possibly a result of viability losses caused by long term exposure to fermentative conditions, as was observed in batch culture.

Further work (unpublished) was undertaken to study xylose metabolism using continuous culture. This work indicated that low concentrations of ethanol caused a gradual decline in growth and fermentation rates. Ethanol toxicity served to limit the concentration of ethanol formed at steady-state to maximum values of 13 g/L at 30°C and 24 g/L at 25°C. The low ethanol concentrations formed in the experiments with immobilized cells was probably a result of a gradual decline in cell viability caused by ethanol toxicity.

Continuous fermentation with nongrowing cells failed to produce high ethanol concentrations because of poisoning of the biocatalyst caused by lengthy exposure to ethanol. Continuous culture failed as well. If growth is more susceptible to ethanol toxicity than fermentation, as is the case for *Saccharomyces* species (*3–8*), then the growth requirement of continuous culture would prevent the continuous production of high concentrations of ethanol.

From the above considerations, we devised a continuous process to circumvent the performance-limiting effects of ethanol toxicity. Continuous cell production is necessary to avoid viability losses. Growth must occur in the absence of toxic ethanol concentrations to obtain active biomass. A continuous two-stage reactor design in which growth occurs in the first reactor and fermentation in the second meets these criteria. Growth is undesirable in the second stage and can be largely eliminated by using an unaerated reactor, because *C. shehatae* requires oxygen for growth (*9,10*). Ethanol can be absent or maintained at a low level in the first reactor by suitable adjustment of the aeration.

We tested these ideas in four experiments involving the two stage process. The objective was to circumvent the toxic effects of ethanol so as to allow continuous production of ethanol at concentrations typical of those obtained with batch, but at the high volumetric rates possible with continuous fermentation.

METHODS AND MATERIALS

Strain

The strain of yeast used was *Candida shehatae* ATCC 22984. It was maintained and grown on yeast malt peptone glucose agar (YMPG, Difco™) at 32°C.

Medium

The defined medium used was compounded according to the formula of du Preez and van der Walt (*11*), except that 5.0 g L^{-1} urea was substituted for casamino acids and NH_4Cl. Other details were as previously described (*2*).

Cultivation

Inocula were prepared on plates as previously described (*2*). Cells were initially cultivated under fully aerobic conditions for several days until they were fully adapted to respirative growth, as evidenced by attainment of a stable steady-state. Oxygen-limited conditions were imposed on aerobic cultures by reducing the agitation rate, and hence, the oxygen transfer rate.

Batch Fermentation

Batch fermentation was performed as previously described (*12*), except the temperature was 25°C.

Experimental Design

Reactor One Description

The first reactor was a New Brunswick Bioflo continuous fermentor of working volume 350 mL. It was operated as a chemostat at 25°C and pH 4.5. The culture was sparged with pure oxygen at a flow rate of 40 mL/min. Aeration was controlled by varying the agitation rate. The agitation rate used depended on the dilution rate: 550, 650, and 750 rpm were used with dilution rates of 0.046 h^{-1}, 0.096 h^{-1}, and 0.14 h^{-1}, respectively. These agitation rates were sufficient to maintain a cell density of about 10 g/L for each dilution rate.

Fully Aerobic Configuration

Two modes of operation were used, as shown in Figure 1. One experiment used the mode shown in Figure 1a, which employed a 2% xylose feed. The aeration used (see above paragraph) was enough to convert 20 g/L xylose completely into 10 g/L biomass—no ethanol or residual

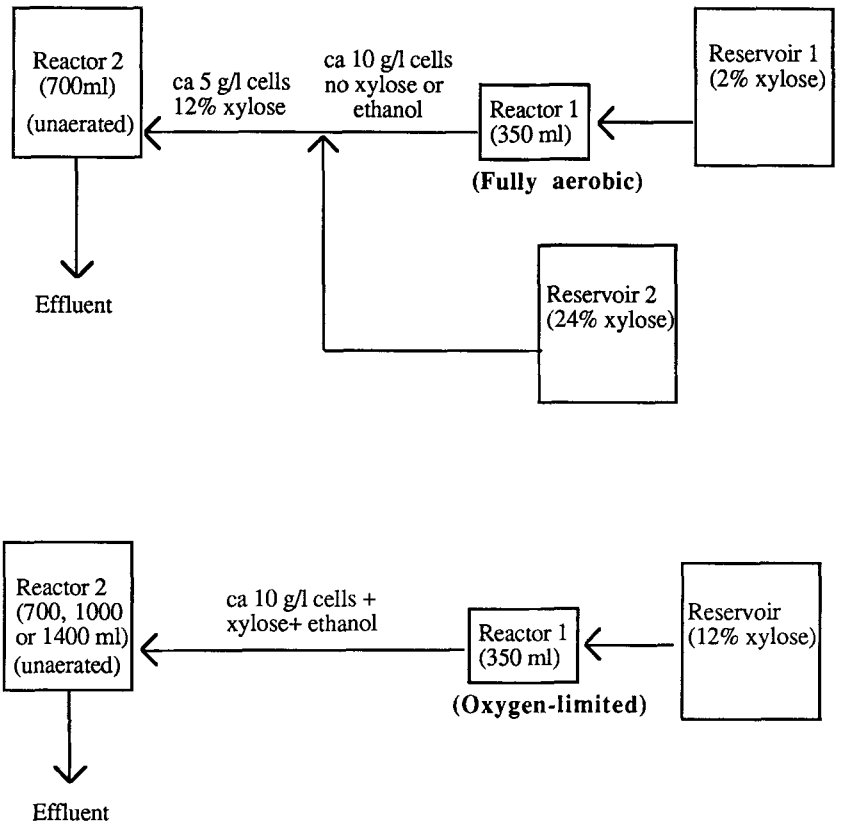

Fig. 1. Two-stage reactor configurations. Flow patterns for two stage xylose processing are shown for the two modes of operation used: fully aerobic first stage and oxygen-limited first stage.

xylose was present in the reactor effluent. Metabolism was completely respirative—hence the label "fully aerobic." An additional xylose feed was necessary to provide substrate for fermentation in reactor two because no xylose was left in the effluent stream of reactor one.

Oxygen-Limited Configuration

The configuration shown in Figure 1b was used for the remaining three experiments. As in the fully aerobic condition, aeration sufficient to produce 10 g/L biomass in the first reactor was used. However, the 12% xylose fed into the first reactor in this configuration was much larger than the 2% used in the fully aerobic configuration. Hence, large amounts of residual xylose (60–80 g/L) were present in the effluent of reactor one. This residual xylose was sufficient for fermentation substrate in reactor two. We use the term "oxygen-limited" to describe this configuration because growth in the first reactor was limited by the amount of oxygen provided.

Reactor Two Description

The second reactor was simply an unaerated vessel equipped with temperature control through which the effluent from reactor one (and a secondary feed, if necessary) was pumped. Operating temperature was 20°C. Dilution rates were changed by changing the volume of the reactor. A 40 mm × 1 m glass tube equipped with an internal coiled heat-exchanger was used to provide a reactor of volume 0.7 L. Larger volumes employed a 2 L New Brunswick Multigen batch fermenter equipped with a float switch (Micrometrics) that controlled culture withdrawal as necessary to maintain a constant volume. The height of the fluid surface, and hence the reactor volume, could be changed by raising or lowering the float switch.

Analytical Methods

Analytical methods for ethanol, xylose, and biomass were as previously described (2).

RESULTS

Table 1 shows the growth and fermentation performance of each stage. The aeration condition, dilution rate, volumetric fermentation productivity, and ethanol yield are reported for stage one. The dilution rate, specific growth rate μ, ethanol concentration, fermentation productivity, and ethanol yield are reported for stage two. The growth rate μ is the same as the dilution rate for stage one because stage one was operated as a chemostat. Little growth occurred in the unaerated stage two reactor as compared to the aerated stage one reactor.

The primary difference between fully aerobic (AR) and oxygen-limited (OL) operation lay in the fermentation performance of both reactors. No ethanol was produced in the first reactor under fully aerobic operation, as was intended. However, these respiratively-growth cells did not produce much ethanol in stage two either. The cells produced under oxygen-limited operation produced ethanol in both stages. The yields were better in stage two, reflecting the lack of yield-reducing cell production in that reactor.

Figure 2 shows that the steady-state obtained in reactor one for experiments 3 and 4 did not persist indefinitely. After a transient peak, ethanol levels settled into an apparent steady-state for several days. After the fifth day, a slow decline in fermentative activity began, which accelerated sharply after the eighth day. No change was made in the operating conditions for the first reactor throughout this period. On the other hand, fermentation performance in the second reactor was remark-

Table 1
Performance of Each Stage

	Stage One (continuous culture) (Aerated)				Stage Two (continuous fermentation) (Unaerated)				
Ex. No.	Aer.† cond.	Dilution rate (h^{-1})	Productivity ($g\ g^{-1}h^{-1}$)	Yield (g/g)	Dilution rate (h^{-1})	Growth μ (h^{-1})	EtOH (g/L)	Productivity ($g\ g^{-1}h^{-1}$)	Yield (g/g)
1	AR	0.046	0	0	0.049	0.003	1.5	0.07	—
2	OL	0.14	1.47	0.23	0.072	0.011	26	1.12	0.36
3	OL	0.096	1.52	0.26	0.035	0.004	37	0.74	0.41
4	OL	0.096	1.21	0.22	0.025	0.004	36	0.59	0.43

†AR refers to fully aerobic conditions, OL refers to oxygen-limited conditions.

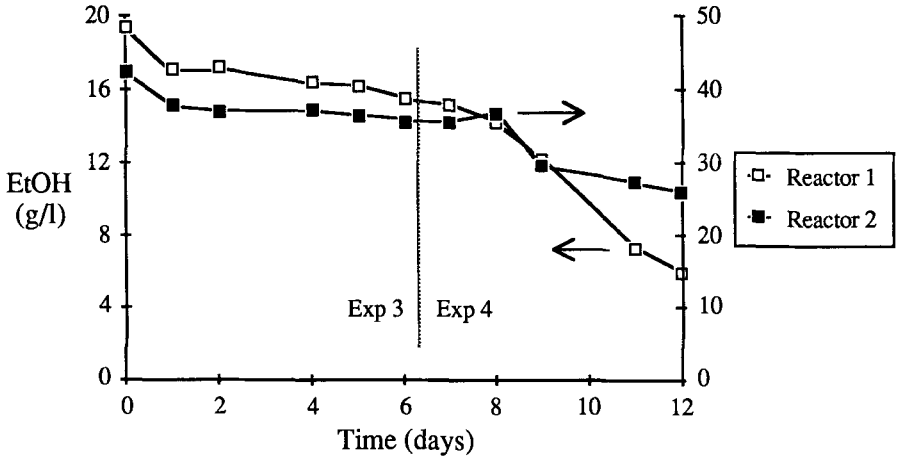

Fig. 2. Comparison of reactor stability of both reactors. Time profiles of ethanol concentration in both reactors are shown for experiments 3 and 4. The dilution rate of reactor 2 was changed from 0.035 h^{-1} to 0.025 h^{-1} between experiment 3 and 4. No change was made in operating conditions for reactor one; the dilution rate remained constant at 0.096 h^{-1}.

ably stable. The ethanol concentration in it was unaffected by the initial decline in reactor one activity. In fact, the increase in residence time at the start of experiment four resulted in no immediate change in ethanol concentration either. Only after the dramatic drop in fermentative activity in reactor one did ethanol concentration in reactor two change.

Figure 3 compares the performance of the two-stage process (experiment 3) with batch culture under our standard optimal conditions (*12*) at

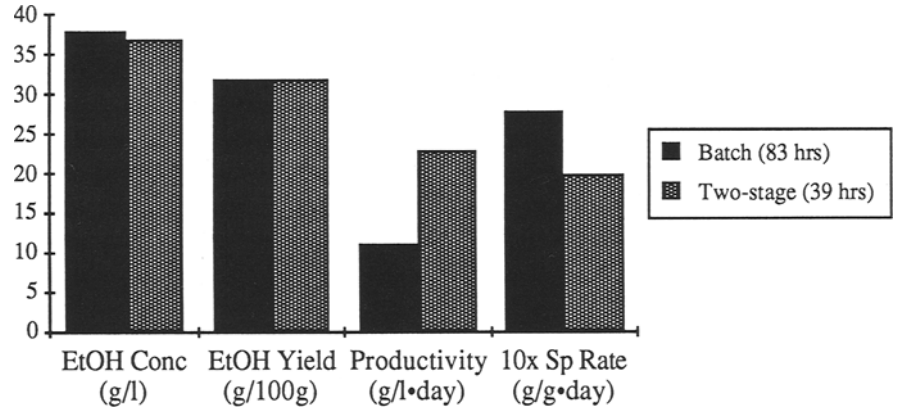

Fig. 3. Comparison of batch culture with the two-stage process. Batch culture is compared with the two-stage process. The batch example represented the maximum ethanol obtained from 12% xylose at 25°C. The fermentation time for the two stage reactor was the sum of the residence times of both reactors. The yield and productivity entries for the two stage reactor are overall values for both reactors combined.

peak ethanol concentration. Essentially identical concentrations and yields were obtained. The overall productivity of the two-state process was twice that of batch, but the specific productivity of batch remained higher.

DISCUSSION

The lack of substantial growth in the unaerated reactor is consistent with the observation that *C. shehatae* requires oxygen for growth (*9,10*). The absence of substantial ethanol production by respiratively-grown cells in the unaerated reactor is consistent with a similar finding using *Pachysolen tannophilus* in batch culture on xylose; shifting from fully aerobic to anoxic conditions results in little ethanol production (*13*).

Continuous cultivation of fermentative cells could not be sustained indefinitely. The initial gradual and later rapid decline in ethanol production by cells in reactor one was probably attributable to a shift in the predominant physiological state of the cells. This strain of *Candida shehatae* is known to undergo a step transition between respirative and fermentative states (*14*). Both cell types are present under any given condition, but proliferation of one can be favored over that of another. It is possible that continuous culture under oxygen-limited conditions selects for the nonfermentative state.

Fermentation in the second reactor was more robust. Apparently, it proceeded at whatever rate was necessary to produce a maximum ethanol concentration of about 36–37 g/L. Thus, increasing the residence time from experiment 3 to 4 (Fig. 2) only served to decrease the fermentation rate without increasing the concentration produced. Similarly, small increases in fermentation rate in the second stage compensated for small decreases in input ethanol concentration in such a way as to maintain a relatively constant final ethanol concentration (*see* Fig. 2). Eventually, when the input ethanol concentration fell too far, the second stage reactor could no longer compensate. Similarly, the fermentation capacity of stage two was insufficient to produce the maximum ethanol concentration for the short residence time used in experiment 2 (*see* Table 1).

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