Continuous-Culture Responses of *Candida shehatae* to Shifts in Temperature and Aeration: Implications for Ethanol Inhibition

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Temperature and aeration shifts were used to perturb steady-state continuous cultures to determine the effects of ethanol on xylose metabolism by *Candida shehatae*. The accumulation of ethanol exerted a delayed inhibitory effect on the specific rate of substrate utilization. A second effect was also observed in which the specific rate of xylitol production increased at the expense of the specific rate of ethanol production. Both effects were enhanced at higher temperature. Inhibitory effects also occurred in glucose metabolism.

Ethanol inhibition of yeasts and other microorganisms has received much attention (4). Ethanol inhibition has been studied less in xylose than in glucose fermentations. Here, we report on inhibitory effects of ethanol in oxygen-limited continuous cultures of the xylose-fermenting yeast *Candida shehatae*.

Understanding the factors affecting the maximum ethanol concentration obtainable is of practical importance because high concentrations can reduce recovery costs. Continuous culture is of interest because it can result in high volumetric rates of ethanol production. Oxygen-limited yeast xylose fermentations are of fundamental interest because they enable us to study simultaneous respiration and fermentation.

Xylose-fermenting yeasts do not grow under anoxic conditions and do not ferment when fully aerobic. Fermentation and growth occur simultaneously only under oxygen limitation. Achieving steady-state oxygen-limited growth in continuous culture presents a number of technical problems and has been accomplished only recently (3).

Lucas and van Uden (14) investigated the effects of temperature on ethanol tolerance and thermal death of *C. shehatae* and determined that it was more tolerant of ethanol at lower temperatures. du Preez and co-workers (6) quantitatively evaluated the effects of ethanol on the growth of the xylose-fermenting yeasts *C. shehatae* and *Pichia stipitis* using Luong kinetics (15).

Effects of ethanol on metabolic rates have often been examined with ethanol added exogenously. Both Lucas and van Uden (14) and du Preez et al. (6) placed cells into media containing different concentrations of ethanol and measured the specific growth rate which ensued. Unfortunately, less inhibition is observed with exogeneous ethanol than with the same concentration of ethanol produced endogenously (8, 16, 17). Some have claimed that the apparently greater inhibition by endogenously produced ethanol reflects the tendency of actively fermenting cells to accumulate ethanol intracellularly (4). However, recent demonstrations that the yeast plasma membrane is very permeable to ethanol cast doubt on this hypothesis (7, 13). Whatever the reason for the different effects of externally added and internally generated ethanol, realistic assessments of ethanol inhibition ought to involve ethanol generated in situ.

Chung and Lee (5) examined the effect of in situ ethanol removal on the batch fermentation of xylose by the yeast *Pachysolen tannophilus*. They found that removing ethanol increased growth but had no effect on the specific rate of ethanol production for ethanol concentrations up to 18 g liter⁻¹. In a previous continuous fermentation with *C. shehatae* employing cell recycle (1), we examined the effect of ethanol on the fermentation productivity without the complicating effects of growth inhibition. No correlation between productivity and ethanol concentration was evident over the range of 10 to 18 g liter⁻¹, indicating no inhibition by concentrations of less than 18 g liter⁻¹ at 30°C. Concentrations higher than 18 g liter⁻¹ could not be obtained, however, even when very long residence times were used.

Under fermentative conditions, the growth of *C. shehatae* is oxygen limited (2, 3). The specific substrate (xylose) utilization rate (*Q*<sub>S</sub>) is independent of xylose concentration or dilution rate and is equal to a constant *Q*<sub>S max</sub>. Values for *Q*<sub>S max</sub> of 0.50 ± 0.03 g g<sup>-1</sup> h<sup>-1</sup> at 25°C and 0.55 ± 0.03 g g<sup>-1</sup> h<sup>-1</sup> at 30°C have been previously reported (3). Unlike *Q*<sub>S</sub>, the specific rate of ethanol production (*Q*<sub>E</sub>) is inversely related to dilution rate. This relation reflects the increasing fraction of *Q* that is utilized for respirative growth as the dilution rate increases. The fraction available for ethanol production is thereby reduced. Thus, an effect of ethanol inhibition is best demonstrated in terms of *Q*<sub>E</sub> rather than *Q*<sub>S</sub>.

Here, we report the results of a number of continuous-culture experiments involving shifts from nonfermentative to fermentative conditions. In these experiments, fermentation was induced at will in a steady-state culture and the effects of the rising ethanol concentration on *Q*<sub>E</sub> were observed. Effects of temperature shifts were examined also. Ethanol exerted effects on both *Q*<sub>S</sub> and the ethanol-polyol product mix. Both these effects were enhanced at higher temperatures.

**MATERIALS AND METHODS**

**Nomenclature.** *D*, dilution rate (hour⁻¹). *OTR*, oxygen transfer rate in millimoles of *O*₂ per liter per hour. *Q*<sub>S</sub>, grams of substrate (xylose) utilized per gram of biomass (dry weight) per hour. *Q*<sub>E</sub>, grams of ethanol produced per gram of biomass (dry weight) per hour. *Q*<sub>x</sub>, grams of xylitol pro-
duced per gram of biomass (dry weight) per hour. $Q_{S_{\text{max}}}$, constant value of $Q_S$ observed under oxygen limitation in the absence of ethanol inhibition.

**Methods.** The continuous-culture techniques used here have been described previously (3). Specific rates were calculated by using an unsteady-state mass balance as previously described (1). $Q_E$ was corrected for ethanol evaporation by using evaporation rates measured in water at the same temperature and aeration rate.

**RESULTS**

$Q_S$ is equal to $Q_{S_{\text{max}}}$ at 30°C only for ethanol concentrations less than 13 g liter$^{-1}$. Attempts to produce steady-state ethanol concentrations higher than 13 g liter$^{-1}$ in continuous culture at 30°C were unsuccessful. At a biomass concentration of 3.5 g liter$^{-1}$ with $D = 0.05$ h$^{-1}$, 11 to 12 g of ethanol liter$^{-1}$ was obtained at steady state and $Q_S$ was close to $Q_{S_{\text{max}}}$ as expected. Employing a higher biomass concentration of 9 g liter$^{-1}$ with $D = 0.05$ h$^{-1}$, 11 to 12 g of ethanol liter$^{-1}$ was obtained at steady state and $Q_S$ was close to $Q_{S_{\text{max}}}$ as expected. Employing a higher biomass concentration of 9 g liter$^{-1}$ with the same dilution rate gave essentially the same steady-state ethanol concentration (12 to 13 g liter$^{-1}$). At the higher cell density, $Q_S$ at steady state (0.30 g g$^{-1}$ h$^{-1}$) was much less than $Q_{S_{\text{max}}}$. Following a shift from a fully aerobic to oxygen-limited condition, $Q_S$ rapidly increased to a value near $Q_{S_{\text{max}}}$, leading to the accumulation of nearly 20 g of ethanol liter$^{-1}$ (Fig. 1). At this point (20 h), $Q_S$ began to decrease, resulting in a declining ethanol concentration. Between 60 and 80 h, the ethanol concentration was only 10 g liter$^{-1}$ well below the 13 g liter$^{-1}$ limit obtained earlier. Likewise, $Q_S$ was equal to 0.35 g g$^{-1}$ h$^{-1}$, well below $Q_{S_{\text{max}}}$. Shifting the temperature from 30 to 25°C increased the ethanol concentration to levels exceeding 20 g liter$^{-1}$ (Fig. 1).

A similar experiment was performed at 25°C instead of 30°C with a substantially higher OTR. This generated a higher cell density at a higher dilution rate ($D = 0.086$ h$^{-1}$). We expected over 20 g of ethanol liter$^{-1}$. Instead, oscillating ethanol and biomass concentrations and shifting metabolic rates were observed (Fig. 2). Reducing the OTR produced a steady state. The effect of shifting the temperature from 25 to 30°C was then investigated. Ethanol concentrations fell below 13 g liter$^{-1}$ following a shift in temperature from 25 to 30°C. As the ethanol concentration fell, there was a sharp increase in the specific rate of xylitol production (Fig. 2).

Metabolic oscillations even more exaggerated than those described above also occurred with glucose culture of C. shehatae (Fig. 3) and during the continuous fermentation of xylose by Pichia stipitis (data not shown).

**DISCUSSION**

One can conclude from the simultaneous decline in metabolic rates and the accumulation of ethanol that ethanol inhibited metabolic activity. Furthermore, the effect of temperature is consistent with such an interpretation. Since similar effects were observed with glucose, such inhibition is not specific to xylose metabolism and since it was also observed with Pichia stipitis, it is not peculiar to C. shehatae. By reducing the growth temperature, we were able to increase ethanol concentrations in continuous processes with C. shehatae. Steady-state ethanol concentrations of about 25 g liter$^{-1}$ have been obtained in continuous fermentation of xylose at 25°C (3). and steady-state concentrations as high as 37 g liter$^{-1}$ have been obtained by a two-stage process at 20°C (2).

Oscillating ethanol concentrations in continuous culture and temperature effects similar to those described in this report have been observed by Lee and co-workers (11, 12) with Zymomonas mobilis. They hypothesized that such
oscillations were caused by ethanol (11, 12). Jöbjes et al. (10) also studied oscillating metabolism with Z. mobilis and formulated a model to describe it. These authors pointed out that a delayed effect of ethanol on metabolism was required to result in oscillating ethanol concentrations. By analogy, the oscillations observed with C. shehatae may also reflect a delayed effect of ethanol.

Direct evidence for such a delay is given here. The downshift in temperature led to little immediate change in \( Q_e \). Only after a delay of about 20 h did \( Q_e \) begin to climb. The response of \( Q_e \) was biphasic: an abrupt increase immediately after the shift, mostly at the expense of \( Q_{so} \) and a delayed rise which paralleled that of \( Q_s \). Here we see two effects temporally separated: a delayed effect involving \( Q_s \) and an effect on the xylitol-ethanol product mix which shows no delay. Increasing the process temperature from 25 to 30°C did not affect \( Q_s \) but did exert a marked effect on xylitol production (Fig. 2). That is, only one of the two effects was operative. Ethanol concentrations declined after the temperature shift, but this was the result of a gradually declining biomass concentration, rather than a decrease in \( Q_e \) or \( Q_s \).

The effect of the OTR on the oscillations (Fig. 2) suggests an interplay between the respirative and fermentative modes of metabolism and ethanol inhibition. Changes in the relative amounts of respirative and fermentative metabolism should affect the redox balance, which might lead to the observed changes in the relative amounts of xylitol and ethanol formed. Such changes could also affect the equilibrium state that is hypothesized to exist between respirative and fermentative metabolism. Since, however, OTR also affects growth, the higher ethanol concentrations attained at higher OTR might reflect higher cell viability.

Considerable evidence exists for involvement of cell membranes in ethanol inhibition (9). Since the volubility of ethanol in lipid is directly related to temperature (9), a higher temperature will increase the concentration of ethanol inside a cell membrane. Since both effects observed in this work show a temperature dependency, it is possible that membranes are involved in both.

LITERATURE CITED