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A MODEL OF ACETIC ACID AND 2,3-BUTANEDIOL INHIBITION OF THE GROWTH AND METABOLISM OF KLEBSIELLA OXYTOCA

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SUMMARY

The main product of fermentation by Klebsiella oxytoca is 2,3-butanediol. This organism also produces acetic acid, ethanol, and acetoin. In this report, product inhibition due to 2,3-butanediol and acetic acid is considered. Although the acetate ion has little effect on growth, acetic acid is a strong inhibitor. Acetic acid inhibits growth more strongly than it inhibits respiration. The neutral product 2,3-butanediol is not a strong inhibitor; its effect on growth is no more than is expected by the decrease in water activity it causes. The effect of 2,3-butanediol on respiration can also be explained by a decreased water activity. It appears that it is possible to accumulate as much as 130 g/L butanediol while as little as 0.45 g/L acetic acid completely inhibits growth.

INTRODUCTION

In producing useful chemicals from biomass, the utilization of both cellulose and hemicellulose is important. The 2,3-butanediol fermentation by Klebsiella oxytoca is of interest because this organism rapidly ferments both five- and six-carbon sugars. For the fed-batch fermentation of xylose or glucose, it is possible to obtain a final butanediol concentration of 88-100 g/L (Fond et al., 1985). For these fermentations, product inhibition becomes an important consideration. We have identified 2,3-butanediol and acetic acid as the most inhibitory metabolites based on their final concentrations and respective inhibitory power. According to Thauer (1977), it is the undissociated form of the acids and not the corresponding salts (i.e., acetic acid and not acetate) which is responsible for the inhibition. High xylose concentration is also known to be inhibitory to butanediol fermentation, presumably due to the effect of decreased water activity on the organism (Esener et al, 1980: Jansen et al., 1984a).

When oxygen is available in excess (dissolved oxygen above 3%), K. oxytoca grows at an exponential rate while when oxygen becomes limiting the growth rate is dependent upon the oxygen supply rate. We focus our study on the oxygen-limited metabolism, which is when the production of acetate, acetoin, 2,3-butanediol, and ethanol occurs.

Our objective is to study the inhibitory effects of acetic acid and 2,3-butanediol on the growth of K. oxytoca and on 2,3-butanediol production. The goal is to model end-product inhibition such that it can be included in a bioenergetic model of the type proposed by Jansen et al. (1984b).

MATERIALS AND METHODS

Organism: We used a strain of Klebsiella oxytoca which was adapted from strain B199 (ATCC 8724), obtained from the United States Department of Agriculture (Peoria, IL).

Medium: The composition of "PA medium" was given by Jansen et al. (1984a). This medium consists of phosphate and ammonium salts, and a number of trace elements. The initial xylose concentration was 40 g/L unless specified.

Culture Conditions: Inocula were prepared in 250 ml shake flasks containing 50 ml PA medium. Flasks were incubated 18 h at 37°C. To minimize lag time in experiments with high solute concentration, the organism was "acclimatized" by serial transfers to flasks containing increasing concentrations. A 5% inoculum was used for precultures. The experiments in which cell growth and butanediol production were measured were also performed in 250 ml flasks containing 50 ml medium. In flasks, the pH varied from an initial value of 6.0 to 5.5 at the end. The experiments in which oxygen utilization was measured were carried out in a 2 L fermentor (New Brunswick Scientific Co., New Brunswick, NJ) containing 1 L medium. Fermentations were controlled at pH 5.5 by the automatic addition of 2N KOH. Other procedures and a description of the microcomputer-controlled gas analysis (CO_2 and O_2) were previously reported (Jansen et al., 1984a). In flasks and fermentors, we used a large inoculum (initial cell concentration = 2 g/L) to be sure oxygen was limiting.

Calculations: Substrates and products were analyzed as reported previously (Jansen et al., 1984a). Acetic acid was determined by injecting acidified samples into a Varian 3700 gas chromatograph with a 6′ x 2 mm ID glass column packed with chromosorb 101 (80/100 mesh). The initial concentration of un-ionized acetic acid was calculated from the amount of acetate added and the pH by the Henderson-Hasselbalch equation. Metabolic rates were estimated from the slopes of the product versus time curves and from measurements of the concentrations of oxygen and carbon dioxide in the exhaust gas. The rates are reported as a normalized rate which is defined as 100 times the measured specific rate in the presence of the inhibitor divided by the specific rate in the absence of the inhibitor.

Catabolic activity (respiration and fermentation) was estimated from the specific ATP production rate, which was calculated from the assumed stoichiometry of the energy-producing reactions and the measured extent to which they occurred (Jansen et al., 1984b).

To estimate the viability of the cells, small samples were used to inoculate aerobic shaken flasks such that the initial cell density was ~0.1 g/L. After determination of the exponential growth curves, we extrapolated back to zero time to estimate the initial concentration of

viable cells, assuming negligible lag time. The fraction of viable cells was calculated as the concentration of viable cells at time zero divided by the total concentration of cells (as measured by optical density).

RESULTS

We chose to consider the specific growth rate and the specific oxygen uptake rate because they give an indication of overall metabolic activity, rather than the activity of a particular pathway. Butanediol production is considered because it accounts for the majority of substrate fermented, and it is a neutral product. Figure 1 shows the normalized oxygen limited growth rate as a function of the acetic acid concentration. Although it is reported that acetate stimulates butanediol production (Stormer, 1977; Yu and Saddler, 1982), acetic acid (undissociated) is a very powerful inhibitor of growth. In Figure 2, we depict the normalized specific butanediol production and oxygen uptake rates as functions of the protonated acetic acid concentration. Although cell growth ceased at acetic acid concentrations above 0.45 g/L, other metabolic activities continued until the acetic acid concentration reached 1.4 g/L.

Inhibition due to 2,3-butanediol is described by Figures 3 and 4, which show the normalized oxygen-limited growth rate, and the specific oxygen uptake and butanediol production rates as functions of the butanediol concentration. K. oxytoca can sustain growth at butanediol concentrations up to 105 g/L while other metabolic activities appear to continue at up to 130 g/L butanediol. Butanediol does not appear to be a strong inhibitor. Indeed, the decreased growth rate at higher butanediol concentrations can be completely explained by a decreased water activity, which is known to impede growth. Figure 3 indicates that the oxygen-limited growth rate at a butanediol concentration of 80 g/L is about 17% maximal. According to Esener et al. (1980), the specific growth rate of Klebsiella pneumoniae is 22% of the maximum at a water activity of 0.9805. This water activity corresponds to a solution of 80 g/L 2,3-butanediol in water; other solutes present in the fermentation broth will cause a slightly lower water activity.

Inhibition Model

The growth inhibition shown in Figures 1 and 3 was modeled with a linear inhibition function of the type used by Dagley and Hinshelwood (1938).

$$\mu = \mu^{o} \left(1 - \frac{[BDL]}{105}\right) \left(1 - \frac{[HAc]}{0.45}\right) \qquad (1)$$

Equation (1) simulates this inhibition where [BDL] is the butanediol concentration and [HAc] is the acetic acid concentration (g/L), μ is the specific growth rate (hr^{-1}) and μ^{o} is the specific growth in absence of inhibitors at the same specific oxygen supply rate.

The difference between Figures 1 and 2 suggests that there are important differences between the inhibition of cell growth and the inhibition of respiration and fermentation. The decreased growth depicted in Figure 1 is not sufficient to account for the decreased catabolism

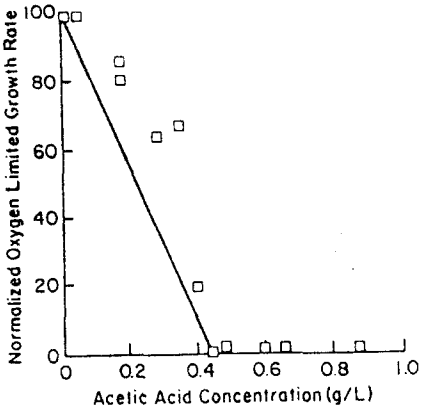

Fig. 1. Normalized oxygen-limited growth rate (□) as a function of the protonated acetic acid concentrated. Solid line, calculated from Eq. (1).

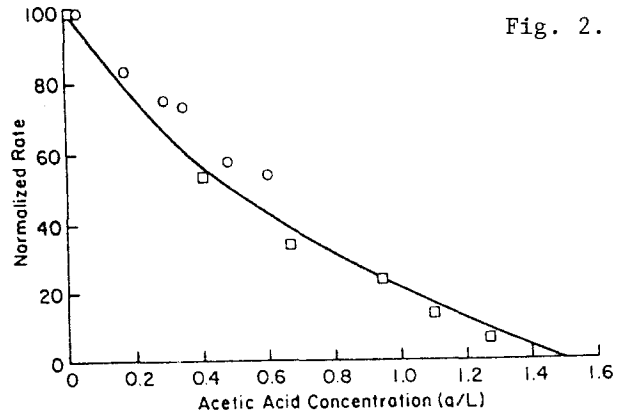

Fig. 2. Normalized butanediol production rate (○) and oxygen uptake rate (□) as functions of the ions of the protonated acetic acid concentration. Solid line, calculated from Eqs. (1) and (4) after Jansen et al. (1984b).

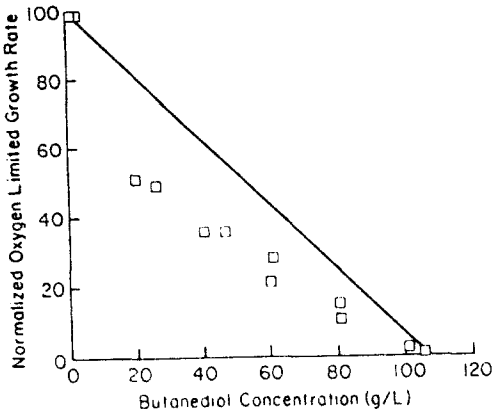

Fig. 3. Normalized oxygen-limited growth rate (□) as a function of the 2,3-butanediol concentration. Solid line, calculated from Eq. (1).

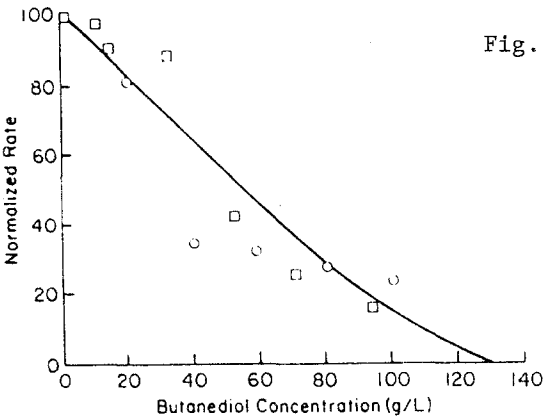

Fig. 4. Normalized butanediol production rate (○) and oxygen uptake rate (□) as functions of the 2,3-butanediol concentration. Solid line, calculated from Eqs. (1) and (4) after Jansen et al. (1984b).

shown in Figure 2. Even in the absence of growth, considerable maintenance metabolism is expected. We postulate that the inhibition of respiration and fermentation depicted by Figures 2 and 4 can be modeled by assuming that this inhibition is related to a reduction in the energy (ATP) consumption of the culture, which is the product of the cell density and the specific ATP requirement ($X\ q_{ATP}$). The specific ATP requirement consists of a growth-associated term and a maintenance term (Pirt, 1965).

$$q_{ATP} = \frac{\mu}{Y^{max}_{ATP}} + m_e \qquad (2)$$

Here Y^{max}_{ATP} is the maximum cell yield from ATP (g cells/mole ATP) and m_e is the maintenance energy requirement (mole ATP/g cells/hr). If growth is inhibited according to Eq. (1), it seems reasonable to expect that maintenance metabolism is also inhibited. Since there is little growth, maintenance accounts for the majority of ATP needed. If we assume that the total ATP requirement of the culture is inhibited according to either Eq. (3) or Eq. (4), we are able to use the bioenergetic model of Jansen et al. (1984b), to simulate the data in Figures 2 and 4 as shown by the solid lines.

$$m_e = m^o_e \left(1 - \frac{[BDL]}{130}\right)\left(1 - \frac{[HAc]}{1.5}\right) \qquad (3)$$

$$X_v = X \left(1 - \frac{[BDL]}{130}\right)\left(1 - \frac{[HAc]}{1.5}\right) \qquad (4)$$

Here m^o_e is the maintenance energy requirement in the absence of inhibition, X is the total cell concentration, and X_v is the "viable" cell concentration. Butanediol and acetic acid concentrations are expressed in g/L. To check the possibility that this "inhibition" is due to a decreased viability, we determined the cell viability of samples withdrawn from a fermentor at different times with different acetic acid and butanediol concentrations. Table 1 compares measured cell viabilities to those calculated from Eq. (4).

Table 1: Comparison of the experimental cell viability to the calculated values for samples withdrawn from fermentor.

Acetic acid (g/L)	0.03	0.3	0.2	0.67
Butanediol (g/L)	10.6	65	95	83
Calculated Cell Viability (%)	90	39	22	20
Measured Cell Viability (%)	100	35	11	20

Table 1 suggests that the accumulation of acetic acid and butanediol decreases the viability of the cells; however, our experiments do not conclusively prove whether the inhibition observed in Figures 2 and 4 is due to an actual loss of viability (Eq. (4)) or simply

decreased metabolic activity (Eq. (3)). Acetic acid has been shown to affect Salmonella typhimurium survival and indeed, accelerate cell death (Meyer et al., 1981). In considering this, it becomes important to make distinctions between living, non-growing, dormant, and dead cells. The maintenance requirements of cells in each state needs to be quantified. It is also important to distinguish between cell activity and cell viability. While we are unable to do this at the present time, we do show that acetic acid inhibits growth more strongly than it inhibits respiration and fermentation.

Since acetic acid but not acetate inhibits K. oxytoca, pH control is very important. To minimize acetic acid concentration, it is desirable to keep the pH as high as possible. However, above pH 6.0, the activity of the butanediol-producing enzymes decreases sharply (Stormer, 1968), and acetate production increases (Neish and Ledingham, 1949). We have found that at pH 6.0, acetic acid inhibition is minimized and it is possible to produce 88 g/L 2,3-butanediol from xylose in a 52 hour fed-batch fermentation (Fond et al., 1985). Since butanediol itself does not appear to be inhibitory except in as much as it decreases the water activity, it becomes important to develop osmotolerant strains of K. oxytoca.

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