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Kinetics of Insoluble Cellulose Fermentation by Continuous Cultures of *Ruminococcus albus*

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Data from analyses of continuous culture fermentation of insoluble cellulose by *Ruminococcus albus* 7 were used to derive constants for the rate of cellulose hydrolysis and fermentation, growth yield, and maintenance. Cellulose concentration was 1% in the nutrient reservoir, and hydraulic retention times of 0.5, 1.0, 1.5, 1.75, and 2.0 days were used. Concentrations of reducing sugars in the cultures were negligible (less than 1%) compared with the amount of hydrolyzed cellulose, indicating that cellulose hydrolysis was the rate-limiting step of the fermentation. The rate of utilization of cellulose depended on the steady-state concentration of cellulose and was first order with a rate constant (k) of 1.18 day^{-1}. The true microbial growth yield (Y) was 0.11 g g^{-1}, the maintenance coefficient (m) was 0.10 g g^{-1} h^{-1}, and the maximum Y_{ATP} was 7.7 g of biomass (dry weight) mol of ATP^{-1}.

Cellulose fermentation is an important process for ruminants and other herbivores since it provides them with carbon and energy. In addition, fermentation of cellulosic wastes such as urban refuse and paper mill, agricultural, and food wastes is useful for minimizing pollution and is potentially important for the production of a variety of fermentation products and single-cell protein. Despite the significance of the microbial fermentation of cellulose, there have been few studies of the kinetics of cellulose fermentation by bacteria.

Continuous culture methods are particularly useful for studying growth and fermentation kinetics. An anaerobic continuous culture apparatus was designed and used for studying the fermentation of cellulose by *Ruminococcus albus* as previously reported (6). This report deals with the development of a kinetic model for cellulose fermentation and the estimation of key kinetic constants.

MATERIALS AND METHODS

R. albus 7 was used as the cellulolytic bacterium. The nutrient suspension contained cellulose (Avicel, type PH-105; FMC Corp., Philadelphia, Pa.), Pfennig mineral and metal solutions (5), B vitamins, sodium bicarbonate, cysteine-sulfide, resazurin, and clarified rumen fluid. Five fermentors were constructed, and their operation was controlled by a personal computer. Details on medium constituents and preparation, analytical procedures, and continuous culture apparatus design and operation were previously reported (6).

The fermentors were operated at 0.5-, 0.75-, 1.0-, 1.5-, and 2.0-day hydraulic retention times. Nutrients were fed and fermentor contents were removed once per hour. Solids in the withdrawal lines settled back into the fermentors between the hourly withdrawals, resulting in increased retention times of both particulate cellulose and biomass. The effluent volume that settled back into the fermenters was 1 ml each hour (i.e., Z = 0.024 liter day^{-1}), and all the solids present in this volume appeared to return to the fermentors (completely clear liquid). This led to the following solids retention times (respective hydraulic retention times shown in parentheses): 0.52 (0.50), 0.78 (0.75), 1.06 (1.00), 1.65 (1.50), and 2.27 (2.00) days.

To express all parameters on a uniform basis, fermentation products and the biomass were converted to equivalent glucose units by use of the theoretical oxygen demand, as is demonstrated in the following examples: for glucose, $C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$ or 6 mol of oxygen mol (180 g) of glucose^{-1}; for ethanol, $CH_3CH_2OH + 3O_2 = 2CO_2 + 3H_2O$ or 3 mol of oxygen mol of ethanol^{-1}. Therefore, 1 mol of ethanol is equivalent to 180/(6/3) = 90 g of glucose. The conversion factors for the other products were calculated in the same fashion and are 60, 15, 15, and 150 g of glucose per mol of product for acetate, formate, H_2, and biomass, respectively. To convert biomass dry weight units to molar units, we assumed that the empirical formula of the organic fraction of the biomass was $C_5H_7O_2N$ (commonly used for bacterial cells [4]) and that the organic fraction was 90% of the cell dry weight.

RESULTS

Kinetics equations. The disappearance of cellulose in continuous cultures of *R. albus* (6) followed first-order kinetics with respect to the concentration of particulate cellulose:

$$dC/dt = -kC \quad (1)$$

where C is the particulate cellulose concentration and k is the rate constant for cellulose disappearance.

The relationship between bacterial growth rate and the concentration of the growth-limiting substrate is usually expressed by the Monod equation (7):

$$\mu = \mu_M S/(K_S + S) \quad (2)$$

where $\mu = (1/X)(dX/dt)$ is the specific growth rate, μ_M is the maximum specific growth rate, S is the substrate concentration, K_S is the half-saturation constant, and X is the biomass concentration. In the present analysis, S represents the soluble sugar concentration since this is the actual substrate taken up by the cells.

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The substrate utilization rate is expressed as:

$$dS/dt = -(1/Y)\mu X = -\mu_M SX/Y(K_S + S) \quad (3)$$

where Y is the microbial growth yield (bacterial mass produced per mass of substrate used).

The net microbial growth rate is expressed as:

$$dX/dt = (\mu - a)X \quad (4)$$

and substituting μ from equation 2 we obtain:

$$dX/dt = [\mu_M SX/(K_S + S)] - aX \quad (5)$$

where a is the specific maintenance rate which accounts for endogenous metabolism and maintenance requirements.

For completely mixed fermentors at steady state, the following mass balances can be derived: for particulate cellulose,

$$VdC/dt = QC_0 - (Q - Z)C - VkC = 0 \quad (6)$$

for soluble sugars,

$$VdS/dt = QS_0 - QS + VkC - V\mu_M SX/[Y(K_S + S)] = 0 \quad (7)$$

for biomass,

$$VdX/dt = QX_0 - (Q - Z)X + [V\mu_M SX/(K_S + S)] - VaX = 0 \quad (8)$$

for products,

$$VdP/dt = QP_0 - QP + V\mu_M SX/[Y(K_S + S)] - V\beta X\{[\mu_M S/(K_S + S)] - a\} = 0 \quad (9)$$

where Q is the hydraulic flow rate; V is the fermentor liquid volume; Z is the solids flow rate settled back into the fermentor (see Materials and Methods); C_0, S_0, and X_0 are the influent particulate cellulose, soluble sugar, and biomass concentrations, respectively; P_0 and P are the influent and effluent product concentrations, respectively; and β is a factor used to convert biomass to equivalent substrate units (i.e., β = 1.2 g of glucose equivalent g of biomass (dry weight)$^{-1}$; see Materials and Methods).

Mass conservation reasoning was used to derive the last mass balance (equation 9), the substrate utilized is either incorporated into cellular material or converted to products. Therefore, the amount of products should always be equal to the difference between the substrate utilized and the net cellular biomass produced.

The above equations were used to algebraically derive explicit solutions for each variable. The model equations are:

$$C = (R_C/R)C_0/(1 + kR_C) \quad (10)$$

$$S = K_S[-X_0/R + X(1/R_C + a)]/[X_0/R + X(\mu_M - a - 1/R_C)] \quad (11)$$

$$X = \{X_0 + Y[C_0 - (R/R_C)C + S_0 - S]\}/[R(1/R_C + a)] \quad (12)$$

$$P = P_0 + C_0 + S_0 - S + \beta X_0 - (R/R_c)(C + \beta X) \quad (13)$$

where R is the hydraulic retention time (= V/Q) and R_C is the solids retention time [= $V/(Q - Z)$]. (Note: Retention time is the inverse of dilution rate.)

Evaluation of kinetic constants. The three kinetic constants (k, Y, and a) were estimated. Equation 10 in a linear form becomes:

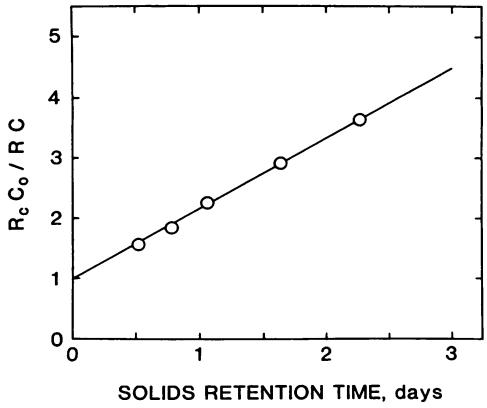

FIG. 1. Estimation of the rate constant for cellulose disappearance according to equation 14. R_C and R are the solids and hydraulic retention times, respectively. C_0 and C are the influent and fermentor cellulose concentrations, respectively.

$$R_C C_0/RC = 1 + kR_C \quad (14)$$

The quantity $R_C C_0/RC$ was plotted versus R_C (Fig. 1) and resulted in a regression equation: $y = 0.98 + 1.18x$ (r^2 = 0.99). Based on the slope of the regression line, the value of k was estimated as equal to 1.18 day^{-1}.

Equation 12 for X_0 and S_0 equal to zero becomes:

$$X = Y[(R_C/R)(C_0 - S) - C]/(1 + aR_C) \quad (15)$$

By linearization and substitution of $(1/X)[(1/R)(C_0 - S) - (C/R_C)] = U$ (= specific substrate utilization rate; mass substrate utilized per mass biomass present per day), the following expression results:

$$1/R_C = YU - a \quad (16)$$

When the experimental data were plotted according to equation 16 (Fig. 2), the regression equation was: $y = -0.30 + 0.11x$ (r^2 = 0.99). From the values of the slope and y intercept, the values of the growth yield and specific maintenance were determined, respectively. Therefore, Y = 0.11 g g^{-1} and a = 0.30 day^{-1}.

Pirt (7) showed that $m = a/Y_{EG}$, where Y_{EG} is the true growth yield (i.e., substrate energy entirely utilized for growth without any utilization of energy for maintenance). Y_{EG} can be estimated as:

$$Y_{EG} = cY/(c - dY) \quad (17)$$

where Y is the true yield based on total use of substrate for growth with zero maintenance requirement, c is the carbon fraction of the substrate, and d is the carbon fraction of biomass (7). For the present study, c = 0.4 (since substrate was expressed as glucose) and d = 0.48 (assuming that the organic fraction is 90% of the cell dry weight and is represented by the formula $C_5H_7O_2N$). Thus, for Y = 0.11 g g^{-1}, Y_{EG} = 0.124 g g^{-1}, and for a = 0.30 day^{-1}, the maintenance coefficient (m) is equal to 0.10 g g^{-1} h^{-1}.

The maximum microbial ATP yield can be estimated by the following equation (7):

$$Y_{ATP} = MY_{EG}/n \quad (18)$$

where n is the moles of ATP available to the microorganism per mole of energy source utilized and M is the gram-molecular weight of the energy source. For the case at hand, it was assumed that cellobiose was hydrolyzed to glucose, 2

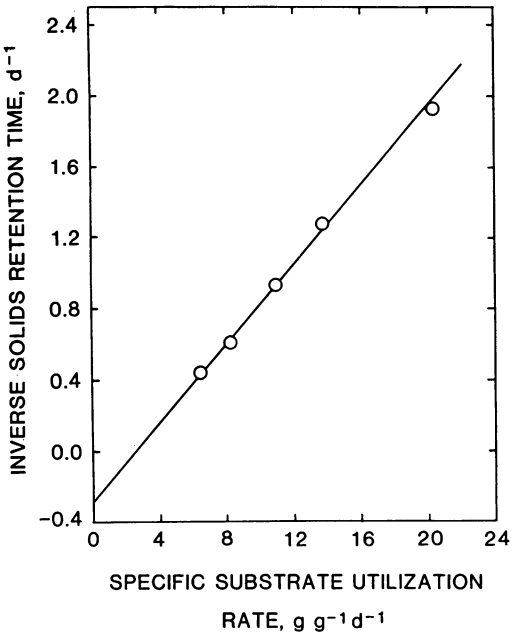

FIG. 2. Estimation of growth yield and specific maintenance rate according to equation 16. d, Day.

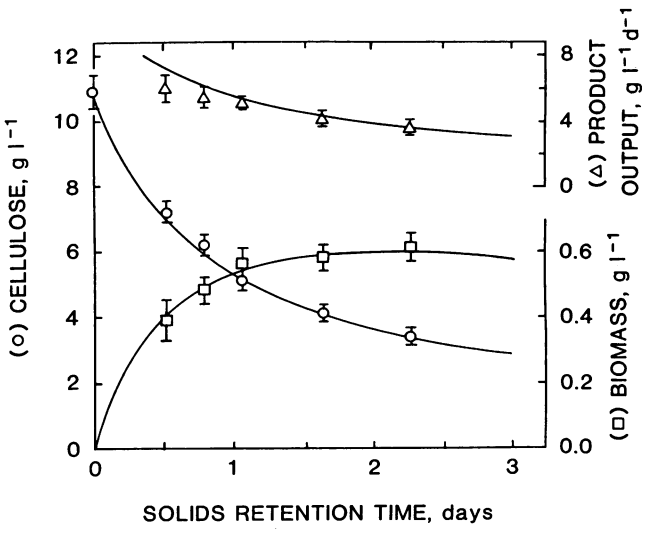

FIG. 3. Effect of solids retention time on cellulose, biomass, and specific product output rate; ——, model predictions (bars indicate 95% confidence intervals). d, Day.

mol of ATP were obtained by the Embden-Meyerhof-Parnas pathway, and 1 mol of ATP was produced from the formation of acetate from pyruvate (3, 10). Based on the average acetate production (90 mol of acetate 100 mol of glucose equivalents used^{-1}), n = 2.9. For M = 180 g mol^{-1}, the maximum yield is then estimated as 7.7 g of cell (dry weight) mol ATP^{-1}. If cellobiose is converted to glucose by a mechanism that leads to the formation of phosphorylated glucose, the maximum yield would be lower, i.e., 6.6 g of cell (dry weight) mol of ATP^{-1}.

The constants μ_M and K_S theoretically can be estimated by linearization of equation 11. However, such a plot of data resulted in a negative correlation because the observed soluble sugar concentrations showed a slight increase (instead of decrease) with increasing retention time (6). However, the soluble sugar concentrations were very low (32 to 52 mg liter^{-1}) when compared with the concentration of the hydrolyzed cellulose (3,900 to 7,900 mg liter^{-1}). Therefore, for all practical purposes, soluble sugar concentrations were negligible.

The specific product output rate (R_P; mass of products generated per fermentor volume per day) can be derived from equation 13. For P_0, S_0, and X_0 equal to zero, the following expression results:

$$R_P = QP/V = (1/R)(C_0 - S) - (1/R_C)(C + \beta X) \quad (19)$$

By use of equations 10, 15, and 19 and the kinetic constants estimated based on the data of this study, the following parameters were calculated as a function of solids retention time: cellulose concentration, biomass concentration, and specific product output rate. Figure 3 depicts the results of the model predictions as well as the observed data.

The above equations can be applied beyond the range of solids retention times used in the present study. The equations and the estimated kinetic constants can be used to predict culture values (e.g., fermentor cellulose and biomass concentrations) for different influent (reservoir) cellulose concentrations and a broader range of solids retention times. For a case in which no settling of solids in the withdrawal line occurs or no solids recycle is applied (i.e., $R = R_C$) and ignoring S (since it is negligible compared with the mass of hydrolyzed cellulose) the following equations result:

$$(C_0 - C)/C_0 = 1 - 1/(1 + kR_C) \quad (20)$$

$$X/(C_0 - C) = Y/(1 + aR_C) \quad (21)$$

Both equations are dimensionless and, in contrast to equations 10 and 15, can be used without specifying influent cellulose concentration (C_0). Therefore, these equations have a universal applicability within certain physical constraints. Equation 20 essentially represents the fraction of influent cellulose that is hydrolyzed at a specified retention time. Equation 21 is the ratio of biomass to hydrolyzed cellulose, or the observed microbial growth yield (Y_{OBS}). Figure 4 depicts equations 20 and 21 for an extended range of solids retention times. The values of k, a, and Y estimated in the present study were used for these predictions. For any given solids retention time and a specified influent cellulose concentration, the effluent cellulose concentration (C) can be estimated from the lower curve of Fig. 4. The upper curve in the same figure can be used to estimate $X/(C_0 - C)$, which in turn yields the value of X.

DISCUSSION

The amount of carbon recovered in products and biomass was virtually equivalent to the amount of particulate cellulose carbon that disappeared, indicating that significant pools of soluble hydrolysis intermediates did not accumulate. This was confirmed by the fact that soluble reducing sugar concentrations were negligible (less than 1%) when compared with the amount of particulate cellulose used. The results show that the conversion of particulate cellulose to soluble products governed the rate of the fermentation. The soluble sugar concentrations showed a slight increase with increasing retention time (6). Undegraded extracellular carbohydrates or intracellular storage carbohydrates released upon death and lysis of nonviable cells may have contributed to the observed soluble carbohydrate. Both sources would

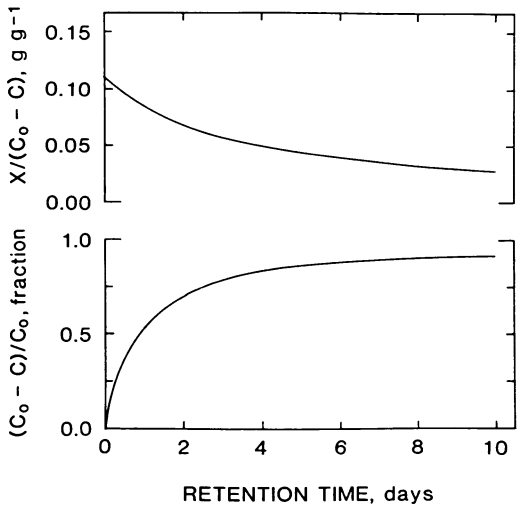

FIG. 4. Model predictions for an extended range of solids retention times. C_0 and C are the influent and fermentor cellulose concentrations, respectively. X is the fermentor biomass concentration.

be expected to increase as the retention time increases. The increase of soluble sugars with increasing retention time precluded any estimation of μ_M and K_S for the soluble substrate taken up by the cells and fermented.

It is difficult to interpret the observed first-order kinetics of cellulose disappearance found in the present study in terms of models of enzyme kinetics developed from studies of the decomposition of particulate cellulose by cell-free enzymes (2). For example, a recent study showed that the rate of production of cellobiose and glucose from Avicel by cellulase from *Penicillium funiculosum* increased with increasing substrate concentration even when substrate was supplied at high percentage concentrations (e.g., 10 to 20% Avicel), but the kinetics of hydrolysis were not simple first-order kinetics (1). A model was developed that took into account the heterogeneity of the substrate, the operation of multienzyme reactions, enzyme adsorption and inactivation, and product inhibition to explain the observed kinetics of cellulose hydrolysis (1). However, Stack and Cotta (8) showed that cellulose disappearance in batch cultures of *R. albus* 7 was first order with a rate constant of 0.12 h^{-1} for cultures grown on ball-milled Whatman no. 1 filter paper in a medium supplemented with 3-phenylpropanoic acid. The rate constant found in the present study was 1.18 day^{-1} (ca. 0.05 h^{-1}). The different rate constants are probably due to the differences in the type of cellulose used in the two experiments.

The finding in the present study that the rate of cellulose disappearance appeared to be independent of the biomass concentration in the fermentors was unexpected. Whatever the mechanism of enzymatic hydrolysis of cellulose, it was expected that the amount of hydrolytic enzyme would vary with biomass concentration and influence the rate of disappearance of cellulose. If both substrate and biomass concentration limited the rate, disappearance of cellulose should have followed second-order kinetics in which both biomass and substrate concentrations are rate limiting. For example, second-order kinetics apply when very low, rate-limiting concentrations of xenobiotics are decomposed by microorganisms (9). A possible reason for first-order kinetics for cellulose disappearance in the present study is that the molar concentration of cellulase in the continuous cultures was always in excess of the molar concentration of cellulose regardless of biomass concentration and that only the concentration of cellulose limited the rate of fermentation. An alternative explanation is that the molar concentration of cellulase was the same at all retention times but much lower than the molar concentration of cellulose. This would imply regulation of cellulase production by cells as a function of growth rate. Additional experiments, especially experiments that include direct measurements of enzymes responsible for the conversion of particulate cellulose to cellobiose, are necessary to investigate the basis of the first-order kinetics of cellulose fermentation observed in the present study.

First-order kinetics may govern the rate of microbial hydrolysis of other insoluble substrates. In addition to substrates that are insoluble under the usual conditions used to grow microorganisms, polymeric substrates which are artificially rendered soluble by heat, e.g., starch, may undergo first-order hydrolysis when untreated. The method we developed for supplying relatively homogeneous suspensions of insoluble cellulose to continuous cultures can be used for studying the kinetics of microbial growth on a variety of insoluble substrates.

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