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Maintenance energy demand affects biomass synthesis but not cellulase production by a mesophilic *Clostridium*

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SUMMARY

Secretion of cellulolytic activity by the mesophil *Clostridium* strain C7 was studied while the bacterium underwent progressive carbon/energy starvation and the ensuing continuous decline in growth rate. In the slowest range of growth rates studied the organism was in full response to the global regulation imposed by guanosine 5′,3′-bispyrophosphate (ppGpp). The exoenzymes of the cellulase complex were produced at the same volumetric rate whether or not the response was active. However, the volumetric rate of biomass synthesis was reduced 45% or more by the response. Energy necessary to maintain the ppGpp-regulated state (i.e., maintenance energy) was, therefore, diverted from energy going to synthesis of biomass but not from that going to exoenzyme synthesis, making the yield of cellulase activity per mole of carbon-energy substrate independent of growth rate and the exoenzyme complex produced from the substrate with equal efficiency at all growth rates. The primary consideration in improving exoenzyme productivity by bacteria with this type of energy distribution between secretion, growth, and maintenance is simply increasing yield per mole of carbon-energy substrate, with growth rate effects on yield a secondary and minimum concern.

INTRODUCTION

The definition of maintenance energy as energy demanded for preservation of cell vitality was introduced in the 19th century, while more recently the cost of maintenance energy demand to the cell has been defined in terms of mass transfer equations (reviewed in Refs. 20 and 21). This permits the cost to be estimated by applying these equations to cultures whose growth rate is limited by the rate at which energy, or energy substrate, is provided. The estimates are made by extrapolation from specific rates of substrate utilization, and corresponding specific mass growth rates, to the value of the specific rate of utilization at zero specific growth rate. This value is taken as the specific rate of energy supply, or energy substrate supply, needed for maintenance. Calculating maintenance energy demand by mass transfer analysis in this way defines it as energy consumed without a corresponding synthesis of biomass. This definition overlaps the original definition, but differs from it significantly by omitting the further requirement that the maintenance energy consumption be dedicated to sustaining vitality [6].

For *Escherichia coli* [7,26], *Bacillus licheniformis* [4,10], *Bacillus polymyxa* [3], *Clostridium beijerinckii* [1], and *Paracoccus denitrificans* [26] the apparent cost of biomass synthesis increases more than 4-fold over the mass doubling time (t_d) range between 10–100 h, with a precipitous doubling in the apparent cost occurring at 50–60 h when ribosome synthesis is checked by the regulation imposed by guanosine 5′,3′-bispyrophosphate (ppGpp). The mass transfer equations just cited, as well as those of Monod and at least three others [6], describing the relationship between utilization of energy substrate and synthesis of biomass in energy-limited cultures fail to predict this increase in the apparent cost of biomass synthesis at mass doubling times (t_d) longer than 10–12 h, t_d that are low, but well above zero. This predictive failure is due primarily to lack of terms for the onset of the metabolic regulation necessary for survival at these longer t_d, particularly the onset of regulation due to elevated cell levels of cAMP and ppGpp [1,2,5,23,25] and the associated energy cost to the cell. The energy required to synthesize regulatory nucleotides and sustain their activities meets the definition of maintenance energy by any criterion so far suggested [23].

Other influences that also are not accounted for by

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these equations [4] appear with falling specific growth rate, contributing further to their inadequacy in relating specific rates of substrate utilization to corresponding specific mass growth rates across t_d ranges between 10–100 h. This growth rate range corresponds to the post-exponential idiophase in which antibiotics, toxins, and exoenzymes are often produced by bacteria both in natural environments and when employed industrially.

Clostridium strain C7 is a dinitrogen-fixing [15], cellulolytic mesophil [13] that is easily co-cultured [14], all of which make it an attractive candidate for industrial development. We have studied the secretion of cellulolytic activity by this bacterium during carbon-energy limited growth across the t_d range from 5 to longer than 100 h. A relationship between biomass synthesis, exoenzyme synthesis and secretion, and maintenance energy has appeared which is likely to represent a new found regulatory arrangement in the cell between competing energy needs for maintenance and for biosynthesis: when energy is required for maintenance demands, it is deducted from the synthesis of biomass with little or none taken from the synthesis of cellulase activity.

MATERIAL AND METHODS

Bacteria and culture methods

Clostridium strain C7 was obtained as a gift of S. Leschine and E. Canale-Parola, University of Massachusetts. Spore stocks were prepared by first growing the organism in a basal medium containing per l (w/v) glucose, 2.0; yeast extract, 2.5; tryptone, 1.0; dibasic potassium phosphate, 3.5; ammonium sulphate, 1.0; biotin, 0.01; para-aminobenzoic acid, 0.01; cysteine-HCl, 0.05; and 1 ml of the mineral stock solution described by George et al. [11]. One ml of an 18-h culture in this medium was added to a screw-capped, glass culture vial half filled with a 1:1:1 mixture of sand/soil/$CaCO_3$, total volume about 4 ml. One week after making this preparation, it was ready for use as a spore source.

Fresh cultures were prepared for each experiment by heat-shocking a loopful of the spore preparation in 10 ml of the basal medium at 80 °C for 10 min. After this spore-seeded culture had incubated anaerobically at 30 °C for 24 h, it was used to seed other media. The seed volume was generally 3% to 10% of the final volume. Cultures were kept in an anaerobic chamber (Coy Lab Products, Ann Arbor, MI) with an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 and maintained at 30 °C.

The isogenic *Escherichia coli* strains 859 (*arg met*) and 859X (*relA1 arg met*) [1] were obtained from the Department of Microbiology (UNH) culture collection. Frozen stocks were thawed for each experiment and used to seed Davis-Mingioli medium [8] supplemented with 20 $\mu g\,ml^{-1}$ of the appropriate amino acids.

Recycling fermenter

The recycling fermentor was a Marubishi Bench Top fermentor MD series (L.E. Marubishi Co., Tokyo, Japan) with a 2.6-l reaction vessel modified for biomass feedback. A magnetically driven bottom-drive impeller operating at 200 rpm provided agitation.

When the fermentor was operated as a chemostat, medium was transferred through stainless steel tubing from the medium reservoir to the reaction vessel by a pump head, and a peristaltic action pump was used to pump broth from the reactor vessel to waste at the same rate medium was added.

The recycling loop consisted of a variable speed DC motor (Dayton Electric Manufacturing Co., Chicago, IL) with a rotary vane pump head (Procon Products, Murfreesbro, TN) which continuously drew broth from the reaction vessel at a rate of 500 ml min^{-1}. The broth was passed into a thin channel filtration apparatus (Amicon Corp., Danvers, MA; model No. TC-1R) containing a polycarbonate filter with 0.2 μm pores (Nuclepore, Pleasanton, CA). The retentate, which contained the cells, was passed back into the reaction vessel and the cell-free filtrate sent to waste. The rate of filtrate flow was regulated by a micrometering valve (Whitey Co., Highland Heights, OH) to equal the medium inflow to the reactor, maintaining the fluid volume in the reactor at 1 l.

Anaerobiosis was maintained by a flow of O_2-free N_2 through the entire system. The gas used was 99.998% pure and was scrubbed through a heated copper coil to remove remaining oxidant, then filtered through sterile microfiber filter tubes (Balston Filter Products, Lexington, MA). Gas from the reaction vessel and reservoir was vented through further sterile filters.

A temperature probe was used to control the reactor temperature through a solenoid-driven combination heating-cooling circuit. A gel electrode connected to a pH meter was used to monitor pH. The pH of the reaction vessel was maintained at 7.0 by slow continuous addition of sterile 0.5 M NaOH. Neoprene tubing and a peristaltic pump were used to supply base to the vessel.

Sterilization was accomplished by filling the reaction vessel with 5% formaldehyde. The temperature was raised to 60 °C and the system kept this way for 48 h. The recycling loop and chemostat line were washed with 5% formaldehyde pumped from the reaction vessel. The entire system was then rinsed with 15 l of sterile RO water.

Samples were taken by three methods. Chemostat samples for total cell counts and biomass estimation were collected in a sterile centrifuge tube as culture was pumped from the reactor vessel, and immediately made 5% in formaldehyde. Samples for extracellular product analysis were obtained from the same position in the chemostat and the culture was immediately centrifuged at 10000 × g

for 10 min in a Sorval RC-2 superspeed centrifuge (Dupont Instrument Co., Newton, CO). The supernate was saved and frozen at −20 °C. During recycling operation, biomass and cell count samples were taken from the reaction vessel by syringe through a two-way ball valve which interrupted the recycling loop directly over the reaction vessel, and treated with formaldehyde. Recycling loop filtrate samples for extracellular product analysis were taken from the exit port of the filter apparatus.

The medium used for the recycling fermentor experiments was the basal medium with 0.05 g sodium thioglycollate per l substituted for cysteine-HCl. Sugars were filter sterilized with the mineral salts solution and added aseptically after the broth had been autoclaved.

Analyses

Biomass samples were filtered through preweighed 25 mm, 0.2-μm polycarbonate filters (Nucleopore, Pleasanton, CA). Two volumes of 5% formaldehyde were used to wash cells trapped on the filters and the filters placed in a desiccator and allowed to dry for 48 h at 55 °C. The dry weights of the filters were determined on an electrobalance (Cahn Balance, Ventron Instrument Corp., Paramount, CA).

Cell numbers were determined by a Petroff-Hauser cell counting chamber (C.A. Hauser and Son, Philadelphia, PA). Culture turbidity was determined at a wavelength of 600 nm with a Spectronic 20 (Bausch and Lomb Inc., Rochester, NY).

Cell protein was determined by a modified Lowry method [7]. Culture samples were centrifuged at $10000 \times g$ for 10 min, the supernate discarded and the cell pellet resuspended in a volume of 1 M NaOH equal to the discarded supernatant fluid. The samples were then frozen at −20 °C until protein analysis was performed.

Protein for cellulase analysis was collected from the samples taken for analysis of extracellular products. Ammonium sulphate was added to bring the solutions to 80% saturation at 4 °C and the samples shaken at maximum speed on a wrist action shaker. After 2 h they were centrifuged at $20000 \times g$ for 20 min. The supernate was discarded, the pellet resuspended in 0.1 M succinate (pH 5.6), to a volume equal to that of the original sample, and the protein concentration determined.

Carboxymethylcellulose hydrolysis (CMCase) as a measure of endoglucanase activity [22] in the resuspended protein solutions was determined by the method of Miller et al. [18]. One ml of enzyme preparation was added to 3 ml of a 1% carboxymethylcellulose solution made in 0.1 M succinate buffer (pH 5.6). The mixture was incubated for 15 min at 45 °C and the reaction stopped by addition of 3 ml of dinitrosalicylic acid (DNS) reagent and boiling for 10 min [19]. After the mixture was cooled, 1 ml of 40% potassium sodium tartrate was added to stabilize the color of the reaction. Sample absorbance at 600 nm was measured in a Spectronic 20. Glucose was used as a standard and activity units were expressed as μg of glucose equivalents released min^{-1}.

Solubilizing activity as a measure of 'cellulase' activity [22] was determined by a cellulose-azure assay (azurase) based on the assay described by Fernley [9]. One ml of cellulase sample was incubated with 3 ml of a 0.4% cellulose azure suspension made in 0.1 M succinate buffer (pH 5.6), for 1 h. During this time the tubes were gently rotated in a cylindrical housing designed to rotate on a cell culture rotator. The reaction was stopped by the addition of 1 ml of 1 M Na_2HPO_4. Samples were centrifuged for 10 min at $10000 \times g$ and absorbance of the supernate measured at 570 nm in a Spectronic 20. Activity units were expressed as mg cellulose-azure released h^{-1}.

For determination of ppGpp, 20 μCi of $^{32}PO_4$ ml^{-1} was added to cultures and allowed to equilibrate with cellular phosphate. One ml of the equilibrated culture was withdrawn and centrifuged at $10000 \times g$ for 10 min in a Microfuge 11 (Beckman, Wakefield, MA). The supernate was discarded and 100 μl of 2 N formic acid added to the pellet. The pellet plus formic acid was immediately frozen at −20 °C for at least 1 h. Treated samples were thawed and centrifuged at $10000 \times g$. Portions of the supernates were used for the nucleotide analysis.

Separation of ppGpp was carried out by PEI-cellulose thin-layer chromatography. The glass plates were prewashed in deionized water and dried. Ten μl aliquots of the $^{32}PO_4$-labelled nucleotide extracts were spotted and chromatographed in 1.5 M potassium phosphate buffer (pH 3.4) [5]. Extracts of *Escherichia coli* strain 859 and 859X were used as standards. The procedure for their handling was identical except that valine was added to the cultures after the 20-min equilibration period to produce a concentration of 100 μg ml^{-1}.

Exoprotein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the procedure of Laemmli [12]. Samples were periodically withdrawn from the recycling fermentor. Exoprotein was concentrated from the samples by the ammonium precipitation described and then loaded onto the gel apparatus as 60-μl subsamples.

The analysis of solvents and organic acids by gas chromatography was by methods previously described [3].

Mass transfer calculations and statistical analyses

The value of Y_x, the apparent molar growth yield (g dry biomass mol^{-1} growth limiting substrate), in the chemostat mode was estimated with the usual relationship for steady-state chemostat growth,

$$Y_x = X S_r^{-1} \quad (1)$$

in which X is the biomass concentration (g dry biomass l^{-1}) and S_r is the carbon-energy concentration in the growth medium (mol l^{-1}). Similarly, the specific growth rate, μ (h^{-1}), and mass doubling time, t_d (h), were estimated from:

$$\mu = D = C t_d^{-1} \quad (2)$$

where D is the medium flow influent to the fermentor, F_r ($l\ h^{-1}$), divided by the fluid volume of the fermentor and C for exponential growth is 0.69.

The volumetric growth rate, $dX dt^{-1}$, is the product of the substrate provision rate, SPR (mol h^{-1} l^{-1}), itself the product of $S_r F_r$, and Y_x [7], i.e.,

$$dX dt^{-1} = (\mathrm{SPR})(Y_x) \quad (3)$$

whether the culture is growing in chemostat or recycle mode, and will be the same in either mode providing that neither SPR nor Y_x change. However, growth in the recycling fermentor with 100% biomass is not in steady state, and μ is not equal to D. Since:

$$\mu = dX dt^{-1} X^{-1} \quad (4)$$

and biomass increases continuously during recycling fermentation, unless SPR (under the experimenter's control) or Y_x (under endogenous control in the culture) change, $dX dt^{-1}$ is constant, and μ must fall continuously. That is, when SPR is constant, a constant input of carbon/energy substrate is being shared among more cells in successive time intervals. Consequently, the cells undergo progressive chronic starvation and μ decreases. The relationship:

$$\mu = C t_d^{-1} \quad (5)$$

where the value of C is 0.69 for exponential growth and 1.0 for linear growth, applies in any growth system since μ and t_d, are reciprocal functions [7,26]. The utility of t_d for describing growth kinetics at values of μ less than 0.05 h^{-1} has been pointed out [23].

Statistical analyses were made with the Statview + Graphics software package for the MacIntosh computer system (BrainPower, Seattle, WA).

RESULTS

Production of cellulolytic enzymes in batch culture

Clostridium C7 was grown with glucose, cellobiose, arabinose or xylose as the carbon-energy source and the activities of the cellulose-solubilizing enzyme complex (azurase) and carboxymethylcellulase (CMCase) were measured in the supernates.

The maximum growth rate of strain C7 differed for each of the four monosaccharides. Xylose-grown C7 cultures had the shortest generation time (T_d), 2.5 h (Fig. 1A), while the T_d of glucose-grown cultures was never less than 4.3 h. Relatively little secreted enzyme activity of either kind was found with xylose as the substrate, but both activities were produced in substantial amounts by the populations growing on the other monosaccharides (Fig. 1B–C). The correlation coefficient of secretion of the cellulolytic activities with cell number increase varied, the lowest being 0.83 for cellobiose and carboxylmethylcellulase and the highest 0.99 for cellobiose and solubilizing activity.

The relative yield of enzyme activity per g substrate carbon was determined for each energy source (Table 1). The highest yields of both enzyme activities were found when glucose was the carbon-energy source. Although arabinose- and glucose-grown cells produced carboxymethylcellulose activity in equal yield, the yield of solubilizing activity from arabinose-grown cells was only 75% the yield from glucose-grown cells. Cellobiose-grown cells yielded approx. 60% of the enzyme activities yielded from glucose-grown cells.

The relatively greater amount of enzyme produced by glucose-grown cells, with the production decreasing on the other sugars to the least amount being produced on xylose, was the reverse of the relative maximum growth rates and suggested that synthesis of exoenzyme by glucose-grown cells might be at the expense of biomass, resulting in the slower growth rate observed.

Growth rate regions and secretion at low growth rates in a recycling fermentor

To relate substrate uptake and growth at t_d longer than 14 h, strain C7 was cultured in a recycling fermentor with glucose as the carbon-energy limitation. The system was

TABLE 1

Relative enzyme activities produced per g substrate carbon by *Clostridium* strain C7 grown on four different sugars

Substrate	CMCase activity/g carbon[a]	Solubilizing activity/g carbon[a]
Glucose	100	100
Arabinose	100	76
Cellobiose	66	53
Xylose	40	40

[a] Units expressed as percent maximal enzyme activity produced during growth on glucose.

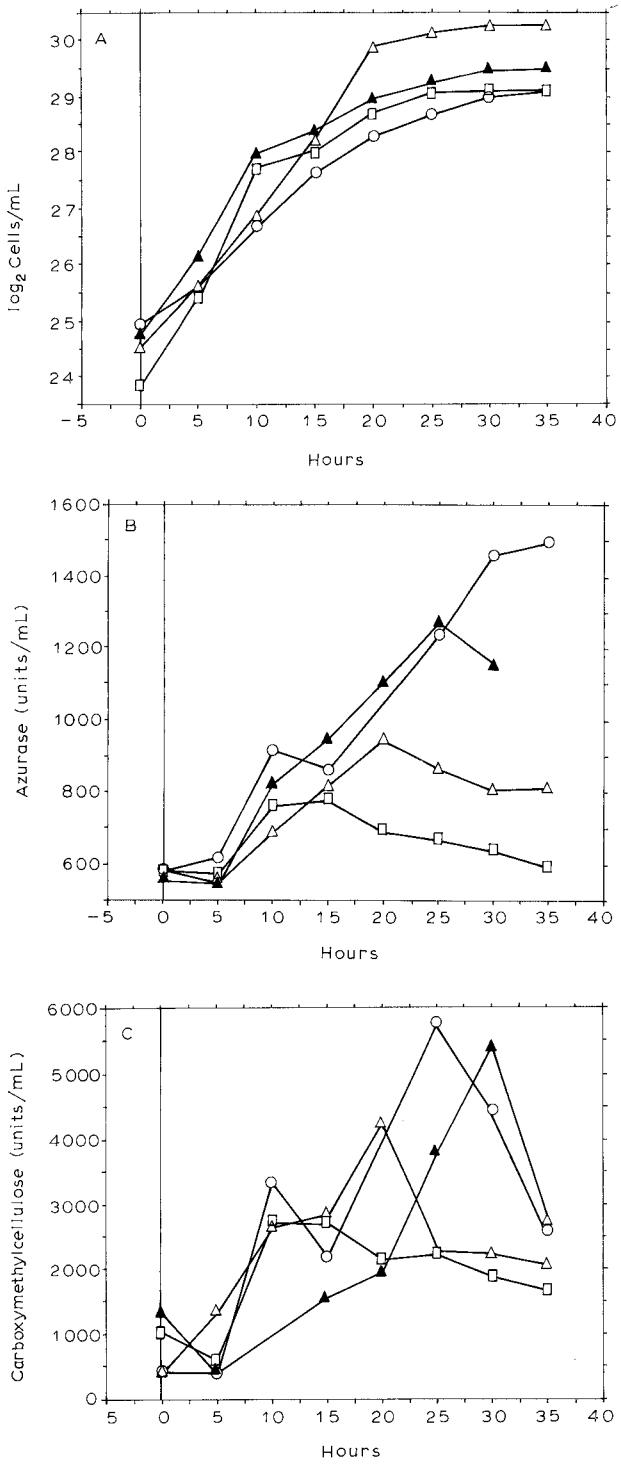

Fig. 1. Growth and secretion of cellulolytic activities by *Clostridium* strain C7 grown in batch mode on 4 sugars. A, biomass accumulation; B, production of exocellular azurase (solubilizing activity); C, production of exocellular carboxymethylcellulase. ○, glucose; □, xylose, ◆, arabinose, △, cellobiose.

operated first in chemostat mode to establish a constant growth rate and measure the apparent molar growth yield, Y_x, (Eqn. 1) then shifted to recycling mode. Time-dependent changes in biomass, X, under these conditions are shown in Fig. 2A, with time at the shift from chemostat to recycling operation taken as zero.

To establish the three sequential lines fitted to the points in the recycle portion of the experiment shown in Fig. 2A, advantage was taken of the observation that a linear function described the range of slowest growth of *B. licheniformis* [4,10], *B. polymyxa* [3], *C. beijerinckii* [1], *E. coli* [2,7], and *P. denitrificans* [26] in the recycling fermentor, and all exhibited stringent regulation of ribosome synthesis with maximum cellular concentrations of ppGpp in this growth rate range. Extracts of strain C7 also contained elevated concentrations of the nucleotide at slow growth rates, demonstrable by chromatography and radioautography of ^{32}P-labelled extracts, and a regression line with a coefficient of determination of 0.96 described the last segment of the growth curve shown in Fig. 2A. Consequently, this line was used to define the range of slowest growth of strain C7.

To establish the volumetric growth rate, $dX dt^{-1}$, in the first interval of recycling, the mass transfer parameters of the chemostat-recycle system immediately after the shift to recycling were used. Immediately after the shift to recycle shown in Fig. 2A the value of $dX dt^{-1}$ was the same as in the chemostat mode (Eqn. 3). The value of Y_x in chemostat mode was estimated as 16.8 g biomass mol^{-1} glucose using Eqn. 1. This value of Y_x used in Eqn. 3 predicts that $dX dt^{-1}$ will be 20.5 mg h^{-1} immediately after shifting to recycle. A regression line having a coefficient of determination of 0.93, and a slope corresponding to a volumetric growth rate of 20.5 mg h^{-1}, fitted the points for the first 10 h. To complete the growth curve, the points between 10–34 h were fitted by regression with a linear segment having a coefficient of determination of 0.96 and a slope of 14.0 mg h^{-1}.

The mass transfer description of growth from 0 to 34 h indicates that the fraction of energy substrate used by the culture for maintenance demand increased at 10 h after the shift to recycling, thereby decreasing the fraction available for biomass synthesis. As a result, at 10 h post-shift Y_x fell from 16.8 to 11.6 g biomass mol^{-1} glucose. Thereafter, the maintenance demand remained nearly constant until restriction of ribosome synthesis occurred at the start of the final segment, when the volumetric growth rate fell to 2.7 mg h^{-1}, and Y_x fell to 2.2 g biomass mol^{-1} glucose.

Each of the two intersections of the segments of the recycling growth curve in Fig. 2A includes an experimental point. This posed the problem, in calculating μ at these points, that the value of $dX dt^{-1}$ used in the calculation could be either the slope of the segment preceding the

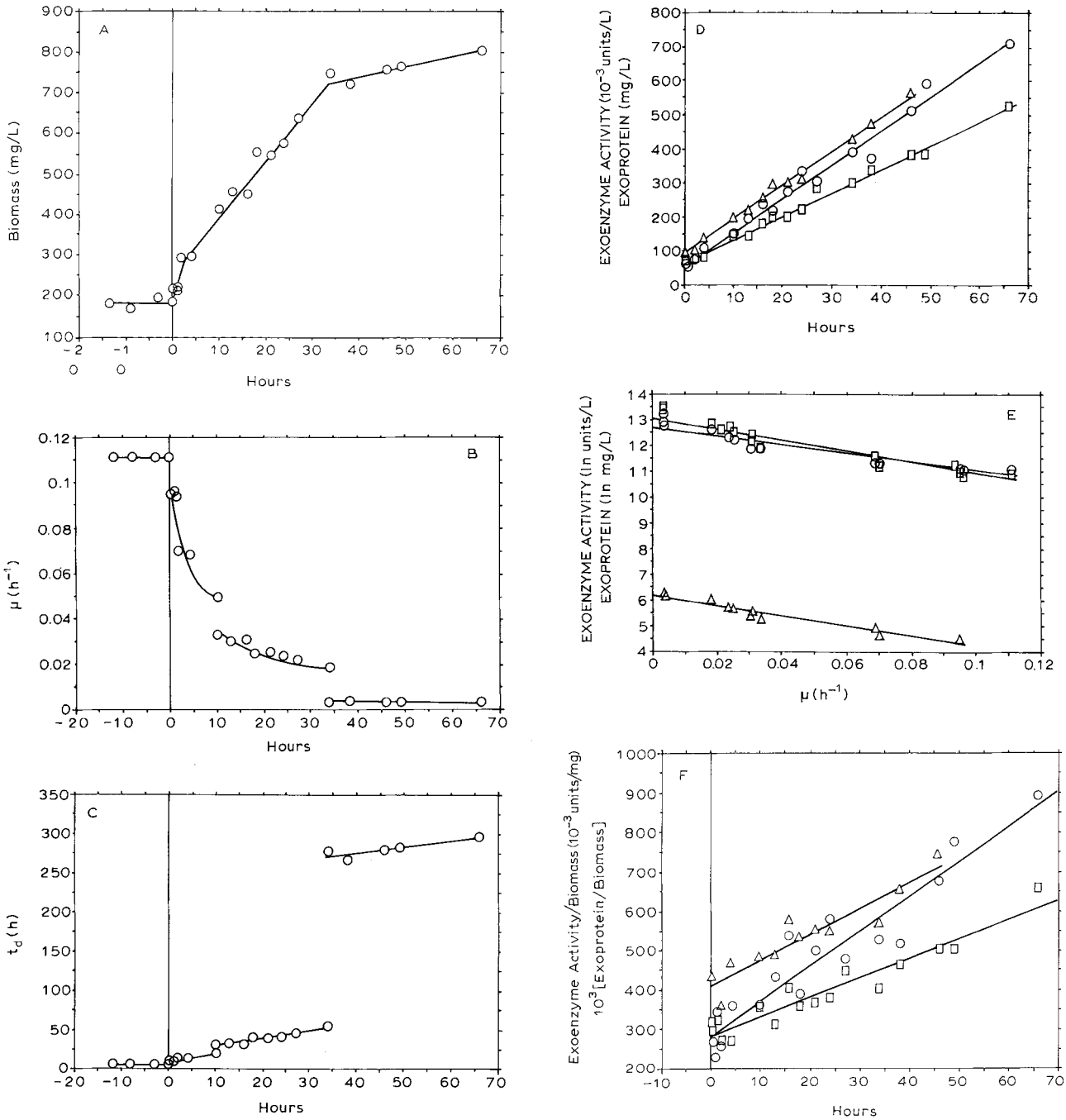

Fig. 2. Growth, secretion of cellulolytic activities, and secretion of protein by *Clostridium* strain C7 in a recycling fermentor. The fermentor was operated as a chemostat with glucose limiting at 10.9 mM and $D = 0.111\ h^{-1}$, then shifted to recycling at time 0, while keeping the same medium and flow rate. A, biomass accumulation; B, specific growth rate (μ) changes with time; C, changes in mass doubling timewith time; D, accumulation of exoenzyme activity and exoprotein mass with time; E, accumulation of ln exoenzyme activity and ln exoprotein mass with μ; F, accumulation of specific exoenzyme activity and specific exoprotein mass with time. ○, exocellular azurase (solubilizing activity); □, exocellular carboxymethylcellulase; △, exocellular protein.

point or that postceding it. To deal with this ambiguity, the points in the intersections were assigned both values of $dXdt^{-1}$ in calculating values of μ. The values of μ, calculated from X and $dXdt^{-1}$ for the experiment shown in Fig. 2A, are shown in Fig. 2B. They decrease in three successive stages rather than continuously, reflecting the

discontinuities in volumetric growth rate between the three linear growth segments.

The value of μ at the discontinuity occurring 34 h after the shift to recycling, 0.019 h^{-1}, corresponds to μ values occurring at the discontinuity in growth rate that occurs in all the bacterial genera mentioned previously, when ribosome synthesis becomes fully interdicted by elevated levels of ppGpp. Fig. 2C characterizes this point further. Thirty-four h after the shift to recycle, the conspicuous, ppGpp-associated break occurred when the t_d was 55 h. This is near the value at which it appears in the other bacterial genera. After the break, the t_d increased immediately to 270 h, then fell continuously until the experiment was terminated.

The cumulative amounts of exoprotein, azurase and CMCase secreted by *Clostridium* C7 during this experiment are shown in Fig. 2D. The data for all three products during recycle can be fitted with regression lines having coefficients of determination of greater than 0.98. Thus, the secretion of exoprotein and enzyme activities proceeded at constant volumetric rates throughout the chemostat and recycle periods.

The hyperbolic relation between μ and elapsed time (Fig. 2B) and the linear relation between the volumetric secretion rate and elapsed time (Fig. 2D), predict an exponential relation between secreted products and μ, and a plot of ln[secreted products] against μ (Fig. 2E) yields the linear relationship expected. When the specific secretion of enzyme activities and exoprotein are plotted with elapsed time (Fig. 2F), all three specific secretions increase with time, because the volumetric secretion rate is constant, while the rate of biomass synthesis falls in each domain of progressively slower growth.

The experiment was repeated with the SPR kept the same, but with F_r halved and S_r doubled by doubling the glucose concentration in the medium reservoir. Fig. 3A shows biomass and exoenzyme accumulation in the fermentor with time. In chemostat mode, the biomass concentration doubled in the steady-state compared to the first experiment, since biomass concentration is a simple multiple of S_r in the chemostat (Eqn. 1), but the volumetric growth rate in recycle mode is a function of SPR (Eqn. 3), and remained the same as in the first experiment. Biomass again increased in stages with progressively slower volumetric rates and the exoenzyme activities appeared at invariant rates. Fig. 3B compares the production of biomass with the production of exoprotein and with the sum of the two. Over the course of the experiment, strain C7 secreted an exoprotein mass equal to about 70% of the biomass formed. When the amount of protein secreted is added to the biomass produced, the changes in the rates of biomass synthesis become less distinct but the change at the onset of stringent regulation remains perceptible.

Samples of exoprotein from various time points during recycling fermentor operation were separated and visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic pattern of the proteins secreted by strain C7 (data not shown) remained constant across the three domains of growth indicating that exoprotein secretion was not likely to have been supplemented by cell-bound proteins released by lysis in the slowly growing cell populations.

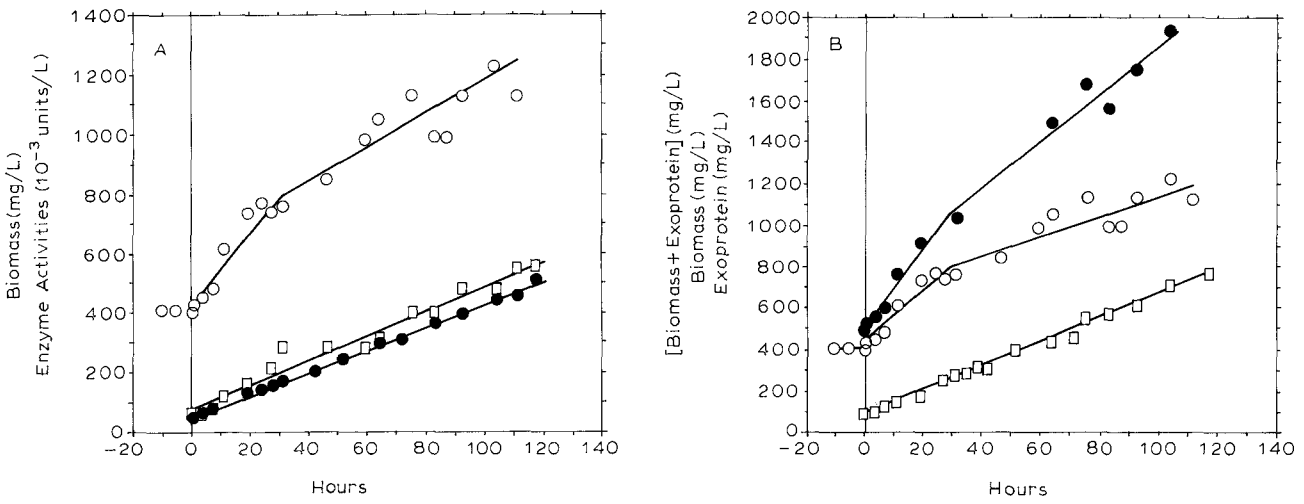

Fig. 3. Growth, secretion of cellulolytic activities, and secretion of protein by *Clostridium* strain C7 in a recycling fermentor. The fermentor was operated as a chemostat with glucose limiting at 18.6 mM and $D = 0.066$ h^{-1}, then shifted to recycling at time 0 while keeping the same medium and flow rate. A, accumulation of biomass (○), exocellular azurase (solubilizing activity) (□), and exocellular carboxymethylcellulase (●) activities with time; B, accumulation of biomass (○), exoprotein mass (□), and the sum of the two with time (●).

Growth rate ranges and growth yields in the recycling fermentor with xylose as the carbon-energy source

A recycling experiment was conducted at the same SPR with xylose as the carbon-energy substrate. The segments in the growth curve in recycling mode were calculated using Eqns. 1–3 as has been done for growth on glucose. The results are shown in Fig. 4A–C.

The culture was maintained at a μ of 0.066 h^{-1} in chemostat mode. After the switch to recycling at time 0, the stages of linear growth appeared in sequence. The final growth rate range with the culture in stringent regulation occurred when the second stage terminated at a μ of 0.014 h^{-1} and a t_d of 70 h, slightly lower and longer, respectively, than the comparable values for the glucose-grown culture.

Analyses of catabolite output by strain C7 during recycling growth demonstrated that glucose and xylose were nearly completely dissimilated. Carbon recovery calculations of runs with glucose as the energy source showed that 101% of the glucose carbon was recovered as acetate, ethanol, and CO_2. With xylose as the energy source 92% of the carbon could be accounted for as acetate, ethanol, and CO_2 (data not shown). The recoveries indicated that energy, rather than carbon, was growth limiting in these experiments.

Table 2 shows analyses of the yields in the three growth rate domains with glucose and xylose as the energy sources. For domain 1, Y_x for xylose was 90% that of glucose and in domain 2, 88% that of glucose (xylose contains about 83% as much carbon as glucose does on a molar basis). However, at domain 3 growth rates, Y_x for xylose was 150% that for glucose. Similarly, Y_X^C for xylose in domains 1 and 2 was 107 and 100% that for glucose, while in domain 3 it was 150%.

The yields for domains 1 and 2 supported the premise

TABLE 2

Cell biomass, exoprotein, and anabolic product yields in three growth rate domains by *Clostridium* strain C7 growing on glucose and xylose based on mmol substrate carbon or mmol substrate

Yields	Domain 1		Domain 2		Domain 3	
	glucose	xylose	glucose	xylose	glucose	xylose
[a] Y_x^c	2.8	3.0	1.5	1.6	0.4	0.6
[b] Y_p^c	1.4	1.3	1.4	1.3	1.4	1.0
[c] $Y_{(x+p)}^c$	4.3	4.4	2.9	2.9	1.7	1.6
[d] Y_x	16.8	15.2	9.1	8.0	2.2	3.2
[e] Y_p	8.3	6.4	8.3	6.4	8.3	4.3
[f] $Y_{(x+p)}$	25.7	21.6	17.3	14.4	9.9	8.0

[a] g biomass/mol substrate carbon.
[b] g exoprotein/mol substrate carbon.
[c] Sum of biomass and exoprotein/mol substrate carbon.
[d] g biomass/mol substrate.
[e] g exoprotein/mol substrate.
[f] Sum of biomass and exoprotein/mol substrate.

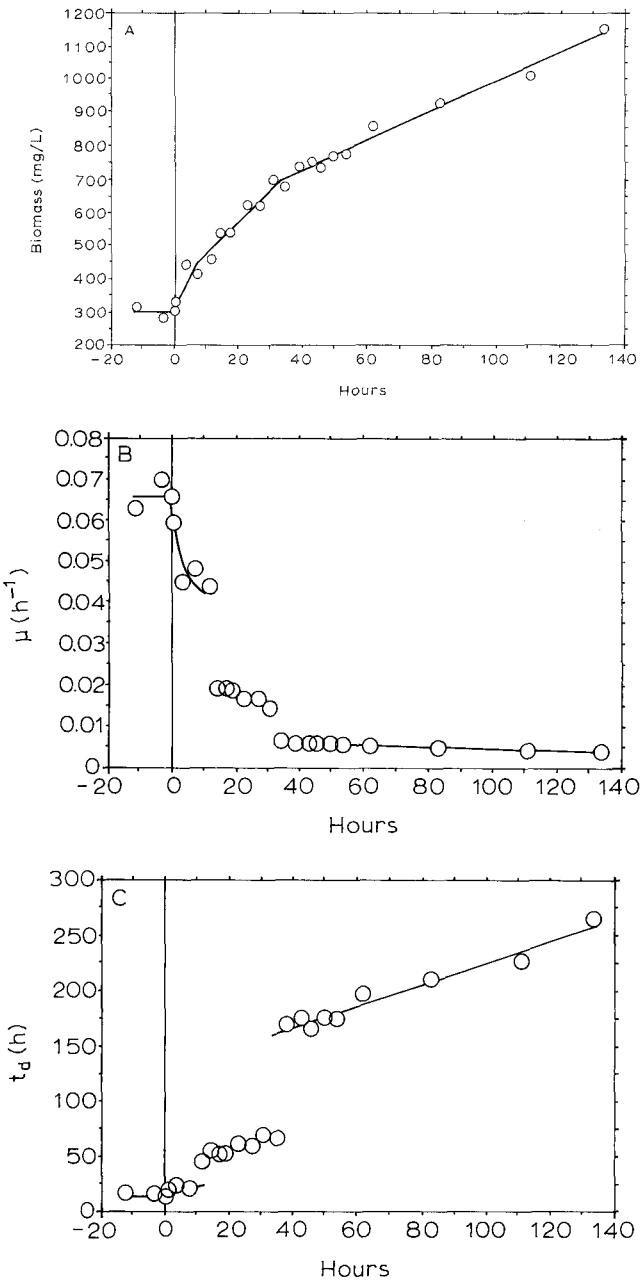

Fig. 4. Growth of *Clostridium* strain C7 in a recycling fermentor. The fermentor was operated as a chemostat with xylose limiting at 18.6 mM and $D = 0.066$ h^{-1}, then shifted to recycling at time 0, while keeping the same medium and flow rate. A, biomass accumulation; B, specific growth rate (μ) changes with time; C, changes in mass doubling time (t_d) with time.

that dissimilation of the two substrates was similar, producing ATP in proportion to their carbon content, but comparison of the yields in domain 3 suggested either that the substrates were not catabolized in the same way and so did not give proportionate ATP yields (although the fermentation products had not changed qualitatively nor quantitatively) or that energy from the substrates was distributed in different ways in domain 3.

Total anabolic product on the two substrates was calculated as the sum of cell biomass, determined from cell dry weights, plus exoprotein, determined by the Lowry method. Anabolic product yields were calculated for xylose- and glucose-grown cells in the three growth rate domains as g, anabolic product per mol substrate, $Y_{(X+P)}$, or g, anabolic product per mol substrate carbon, $Y^{C}_{(X+P)}$. The yields are shown in Table 2. When exoprotein production was accounted for in growth yield calculations, $Y_{(X+P)}$ for xylose-grown cultures was about 80% that of glucose-grown cultures in domains 1, 2, and 3, respectively, while $Y^{C}_{(X+P)}$ for xylose-grown cultures was close to 100% that of glucose-grown cultures. The near approach to the theoretical ratios for yields from the two substrates when exoprotein was summed with biomass in all three domains, including the third, indicated that the energy available to the cells in domain 3 was partitioned differently in glucose-grown cells than it was in xylose-grown cells, with synthesis and secretion of exoprotein being proportionately more of the total biosynthetic cost for glucose-grown cells.

DISCUSSION

Three growth-rate ranges, or domains, can be found in the growth of *Clostridium* C7 between the shortest t_d the culture attained in the recycling system, 6.3 h, to t_d longer than 150 h. These domains are similar to those found with other bacterial genera in this growth system [1–4,7,26]. Within each domain, Y_x was constant, but fell to lower values in successive domains of slower growth. The drop in Y_x occurred at values of t_d marking the boundaries of the two domains of slower growth. That is, the boundaries were characterized by relatively abrupt shifts of part of the culture's energy flow away from biosynthetic reactions and toward reactions that did not in themselves increase biomass. Energy demands of the latter type are maintenance energy demands by definition [20,21,23]. In the four bacterial genera and six species so far studied in this way, the domain of slowest growth results from the onset of full stringent regulation by ppGpp, which is at a maximum cellular concentration in this domain, and whose editing and biosynthesis-inhibiting activities account for the increased maintenance energy demand [5,23].

The relationship between biomass increase, energy substrate utilization, and the secretion of cellulolytic enzymes at the growth rates typical of domains 2 and 3 revealed that the rates of these enzymes' syntheses were controlled differently than the rate of general biomass synthesis: a constant fraction of the energy available to the cultures was used for synthesis of the secreted enzymes at all growth rates, while progressively smaller fractions of the available energy were directed to synthesis of biomass and progressively greater fractions directed to maintenance reactions.

A different relationship was found when the secretion of alkaline protease by parent and ppGpp-defective (*rel*$^-$) cultures of *B. licheniformis* were studied as a function of growth rate across a broad range of μ [4,10,23,24]. The parent secreted protease only in the growth rate domain of stringent regulation, but the *rel*$^-$ culture produced the exoenzyme at a specific rate of secretion that was a function of μ, with the maximum rate at a μ of 0.22 ($t_d = 3.1$ h). Secretion of the enzyme by the *rel*$^-$ culture ceased when μ was less than 0.04 h^{-1} ($t_d > 17$ h), and at lower values of μ the fraction of substrate used in production of the exoenzyme was redirected to the synthesis of biomass.

Two expressions that described the relation between secreted products, substrate utilization, and growth rate in the *rel*$^-$ strain were proposed that also characterized the usefulness of bacterial strains chosen for production of exocellular products [24]:

$$dP\,dt^{-1} = a\,X + b\,dX\,dt^{-1} \tag{6}$$

$dP\,dt^{-1}$ being the volumetric rate of product formation. The constants a and b are strain-specific parameters characterizing the degree in which product formation is linked with biomass and volumetric growth rate, respectively. Eqn. 6 is tautological with

$$q_p = a + b\mu \tag{7}$$

That is, the specific rate of product formation, q_p, is a linear function of μ over specific ranges of μ.

The conclusion drawn from these studies on protease secretion by *B. licheniformis* was that for production systems in which the bacterium is growing at low values of μ and long t_d for a significant fraction of the total fermentation time, e.g., fed-batch fermentations, it is desirable that the organism chosen show both a high maximum value for q_p (i.e., the maximum rate of extracellular enzyme produced per h per unit biomass is high) and high values for a and b in Eqn. 7 (i.e., the maximum value of q_p occurs at slower growth rates).

Growth and secretion of cellulase activity by *Clostridium* C7 represents a new, second case of regulation of the response of exoenzyme synthesis to changes in μ and

maintenance energy demand. The specific rate of cellulase production fell at domain boundaries in the recycling fermentor in the same way μ fell at these boundaries. But, while both specific and volumetric rates of biomass production fell as the maintenance energy demand increased in each domain of slower growth, the volumetric rate of cellulase production was constant in all domains. Production of cellulolytic activity therefore obeyed the simple definition:

$$P = Y_p S \tag{8}$$

in all domains, where P is product formed, S is substrate utilized, and Y_p a molar yield coefficient for the product that remains constant in all domains, i.e., at all growth rates.

When substrate-saturated cultures are growing exponentially in batch mode, product formation will obey Eqns. 6, 7 and 8, and exoprotein formation will show an apparent dependence on growth rate, as the correlation of exoenzyme activity with growth for strain C7 growing in batch mode on glucose, cellobiose or arabinose showed. That is, exoenzyme formation will require a constant fraction of the energy substrate used (Eqn. 8) in substrate-saturated cultures, and as biomass concentration increases, more substrate will be used per unit time with a constant fraction being used for exoenzyme production. The volumetric rate of exoenzyme synthesis will, therefore, increase as the culture grows, which yields the apparent dependence of exoprotein production on growth rate, but conceals the immunity of product formation to changes in maintenance energy demand. It is not until the culture becomes substrate limited, and Eqns. 6 and 7 no longer describe exoenzyme formation, that the independence of exoenzyme formation from biomass and growth rate can be seen.

For bacteria showing this type of protein secretion, strain selection would be concerned primarily with increasing the dP/dS ratio, i.e., increasing the value of Y_p in Eqn. 8, by simply selecting for strains that are higher producers at any growth rate, since exoenzyme production rate is not linked to growth rate and increases in maintenance energy costs are deducted solely from the energy used for biomass synthesis. Parameters such as a and b in Eqn. 7 would not apply since dP/dt is not linked to growth rate when the culture is undersaturated for carbon-energy.

In an energy-limited culture of the C7 type, the choice of growth rate can be determined by factors other than maintenance coefficients or growth rates. For example, in domain 1 the Y_x of *Clostridium* strain C7 was 22.9 mg mmol glucose^{-1} and the Y_p of exoenzyme production 8.3 mg mmol glucose^{-1}. In domain 3, Y_x had decreased to a minimum of 3 mg mmol glucose^{-1}, while the Y_p for secreted protein remained at 8.3 mg mmol glucose^{-1}. In this circumstance, domain 3 growth rates could be chosen for operations if accumulation of biomass presented either additional costs in disposal or increased difficulty in process operations.

The potential of *Clostridium* C7 for industrial application has been pointed out previously [14]. Our experience reinforces the conclusion that it is an attractive bacterium for industrial application. Maintenance costs apparently are higher in prokaryotes than in eukaryotes [23,24]. Because of this, eukaryotes, and the filamentous fungi in particular, could be expected to have a higher Y_p for anabolic products than do prokaryotes. This conclusion was reached from the analysis of the *B. licheniformis* case, where increase in maintenance energy costs affect both biomass and secreted protein syntheses. *Clostridium* C7 represents a second case in which increases in maintenance energy costs are at the expense of biomass only, which makes the strain more competitive with eucaryotic genera as in a choice for engineered synthesis of secreted proteins.

Since strain C7 is an obligate anaerobe, Y_p is necessarily only 0.4–0.1 that of aerobic organisms. Balanced against this in considering it for biotechnological employment are the inherently lower energy costs of anaerobic fermentation, the ability of C7 to employ inexpensive substrates, its ability to fix dinitrogen [15], and its synthesis of as much or more exoprotein as cell-bound protein during growth.

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