Effect of Growth Rate and Nutrient Limitation on the Composition and Biomass Yield of *Acinetobacter calcoaceticus*

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*Acinetobacter calcoaceticus* was grown on ethanol in a chemostat as a model system for single-cell protein production. The substrate yield coefficient (*Yσ*, grams of biomass/gram of ethanol), protein yield coefficient (*Yπ*, grams of protein/gram of ethanol), and biomass composition were measured as a function of the specific growth rate. Nucleic acid, protein, *Yπ* and *Yσ* all increased at higher growth rates. Although protein content increased only 14% (from 53 to 67%), *Yσ* almost doubled over the same range of growth rates. The increase in *Yσ* was due to the higher protein content of the biomass and to higher values of *Yσ*. The higher values of *Yσ* were attributed to maintenance metabolism, and the value of the maintenance coefficient was found to be 0.11 g of ethanol per g of cell per h. When *A. calcoaceticus* was cultivated under a phosphorus limitation protein content, *Yπ* and *Yσ* were lower than in carbon-limited cultures. It was concluded that a single-cell protein fermentation using *A. calcoaceticus* should be operated at a high growth rate under ethanol-limiting conditions in order to maximize both the protein content of the biomass and the amount of biomass and/or protein made from the substrate.

The composition of a microorganism is not invariant but is strongly dependent on its growth rate and environment. The chemostat is an ideal tool for studying changes in cell composition because both environment and growth rate can be controlled readily and accurately.

By using chemostat cultures, it was found that there is a close correlation between specific growth rates (μ) and ribonucleic acid content of microorganisms (5, 12, 16, 19, 22). This variation is due primarily to the changes in ribosome content (6, 10). Protein content also varies with growth rate, but unlike ribonucleic acid, the extent of variation usually is not great (10, 21). The carbohydrate content usually does not change greatly when cells are grown at different rates under carbon-limiting conditions; however, chemostat cultures of nitrogen- or sulfur-limited cells exhibit wide variations in carbohydrate content (10, 21).

The growth rate of a microorganism influences not only its composition but also the substrate yield coefficient (*Yσ*, grams of biomass produced per gram of substrate consumed). Usually one of two types of relationships between μ and *Yσ* are reported: (i) *Yσ* increases as μ increases, attaining a maximum value as the growth rate approaches *μ*max (13); or (ii) *Yσ* attains a maximum value at some intermediate growth rate (11). The first type of behavior has been attributed to the maintenance metabolism of the microorganism (13, 14), which is a measure of the consumption of substrate by microorganisms for functions other than the production of new biomass. These functions include maintaining concentration gradients, providing energy for motility, resynthesizing unstable macromolecules, etc. The amount of substrate consumed for these purposes is a progressively smaller proportion of the total substrate consumption as the growth rate increases. Thus, more substrate goes to biomass production, causing *Yσ* values to increase. This relationship can be expressed as:

\[
\frac{\mu}{Y_\sigma} = \left(\frac{1}{x}\right) \left(\frac{ds}{dt}\right) = \frac{\mu}{Y_\sigma} + m
\]

where \((1/x)(ds/dt)\) is the specific rate of substrate consumption (grams of substrate consumed per gram of cell per hour), *m* is the maintenance coefficient (grams of substrate consumed for maintenance per gram of cell per hour), and *Yσ* is the yield coefficient when *m* = 0 or when *μ* approaches infinity (14). By plotting \((1/x) (ds/dt)\) versus *μ*, *m* and *Yσ* can be found as the intercept and the reciprocal of the slope, respectively.

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The chemostat, in addition to its usefulness for studying changes in biomass yield and composition, is the preferred method of cultivation for the production of single-cell protein. In developing this process, it is important to establish how biomass composition can be varied, since the composition is a determinant of the nutritional and toxicological properties of the product. The protein content of single-cell protein also influences its market value, and the cost of production is strongly dependent on the protein yield coefficient, \( Y_p \) (grams of protein produced/gram of substrate consumed). Thus, for single-cell protein production, it is desirable to increase \( Y_p \), \( Y_a \), and protein content, three factors that may vary with the growth rate of the microorganism or the nutrient limitation under which it is grown.

**MATERIALS AND METHODS**

**Organism.** The organism used was *Acinetobacter calcoaceticus* strain 4736. Taxonomic characteristics and procedures for maintaining stock cultures were described previously (1, 2).

**Fermentation.** *A. calcoaceticus* was cultivated in a mineral salt medium containing ethanol as a sole carbon source. The medium, temperature, and pH for chemostat cultivation were described previously (1). For ethanol-limited cultures, the entering medium contained 6 g of ethanol per liter. Similar medium, temperature, and pH were used for phosphorus-limited cultures, except that \( H_2 PO_4 \) was deleted from the medium and added as a dilute solution via a second stream. The rate of \( PO_4 \) addition was gradually reduced until about 0.05% ethanol accumulated in the culture broth.

At low growth rates, the chemostat was operated for longer periods of time before biomass yields and composition were determined. At least four changes of medium were made at all growth rates to insure steady-state conditions. The dissolved oxygen tension in the fermentor was continuously monitored by a galvanic electrode and was maintained at greater than 20% of air saturation by varying agitation and aeration rate. At each steady state the concentrations of ethanol, acetate, and acetaldehyde in the culture broth were measured (1, 2). In ethanol-limited cultures, the steady-state concentration of these metabolites was less than 0.05 g/liter. In phosphate-limited cultures, the ethanol concentration in the broth was measured by quantitative gas chromatography (1, 2) and subtracted from the amount added to obtain the amount of ethanol consumed.

**Analytical methods.** (i) **Cell mass.** Cell mass was determined by dry weight measurements. A portion (5.0 ml) of the cell suspension from the steady-state chemostat was transferred into a tared centrifuge tube. The biomass was sedimented by centrifugation at 12,000 \( \times \) g for 15 min, and the supernatant fluid was discarded. The pellet was resuspended in 5.0 ml 0.1 N HCl, sedimented, and washed with distilled water. The washed pellet was dried at 105 C, cooled in a desiccator over CaCl\(_2\), and weighed.

The amount of precipitated salts in carbon-limited cultures was about 0.3 mg/ml, but there was very little precipitation in phosphorus-limited cultures. The HCl wash removed precipitated salts that sedimented with the biomass, compensating for the differences in the two types of medium. Control experiments indicated that the HCl and distilled water washes did not cause appreciable cell lysis. The dry weight measurements were performed in quadruplicate, and the averages are reported.

(ii) **Carbohydrate.** For total carbohydrate measurements, 0.5 ml of the biomass suspension was added to 5.0 ml of anthrone reagent (7). The mixture was placed in a boiling-water bath for 10 min and cooled, and the absorption was read at 620 nm in a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). A standard curve was prepared by using glucose, and the determinations were performed in triplicate.

(iii) **Lipid.** A cell suspension (1.0 liter) was removed from the chemostat, centrifuged, and resuspended in 200 ml of cold 1.0 N HCl. This suspension was incubated for 1 h to remove the low-molecular-weight (cold acid-soluble) components of the biomass. The supernatant fluid was discarded, and the pellet, containing high-molecular-weight components, was resuspended in 200 ml of 10\(^{-4}\) N HCl. A portion of this suspension was retained for protein and nucleic acid analyses, and the remainder (100 ml) was used to determine lipid content.

For lipid analysis, the biomass was extracted first with 30 ml of acetone at room temperature. The material remaining after the acetone extraction was extracted with a mixture of 15 ml of ethanol and 5 ml of ether at 100 C for 5 min. The solvents were recovered by centrifugation, and the remaining biomass was refluxed for 1 h with a mixture of 5 ml of methanol and 15 ml of chloroform. The solvent extracts were combined, filtered, dried, and weighed.

(iv) **Nucleic acids.** The biomass pellet remaining after removal of the cold acid-soluble (low-molecularweight) fraction was used for protein and nucleic acid analyses. The pellet from 10.0 ml of suspension was resuspended in 0.5 N perchloric acid and incubated at 90 C for 45 min. The suspension was cooled and the supernatant fluid was recovered after centrifugation. The supernatant was adjusted to pH 7.0 with KOH and the absorbance at 260 nm was determined in a Gilford spectrophotometer. An extinction coefficient of 0.0315 cm\(^2\) / mg was used to convert absorbance to nucleic acid concentration (8).

(v) **Protein.** The cell pellet remaining after the perchloric acid-catalyzed hydrolysis of nucleic acid was used for the protein analysis. The pellet was resuspended in 1.0 N KOH and incubated at 90 C for 45 min. The reaction mixture was cooled and the supernatant fluid was used for a biuret protein determination (18). A standard curve was prepared with bovine serum albumin.

Protein content was also expressed as (N% \( \times \) 6.25) - % nucleic acid. Total nitrogen was determined by a carbon, hydrogen, nitrogen analysis (Perkin-Elmer Corp., Norwalk,
of the biomass fraction that remained after removal of the low-molecular-weight fraction. \( Y_p \) was calculated from the relationship \( Y_p = (Y_d) \% \text{ protein in biomass} \).

**RESULTS**

A. calcoaceticus was cultivated in a chemostat under ethanol-limiting conditions. A steady state was obtained at various growth rates, and the substrate yield coefficient, carbohydrate, lipid, nucleic acid, and protein content were measured.

**Substrate yield coefficient.** \( Y_s \) of A. calcoaceticus was strongly dependent on the growth rate, particularly at low specific growth rates. The specific rate of substrate consumption,

\[
\left( \frac{1}{X} \right) \left( \frac{ds}{dt} \right) = \frac{\mu}{Y_s},
\]

was calculated at each growth rate and plotted as a function of \( \mu \) (Fig. 1). A straight line was obtained that intersected the ordinate at a positive value, indicating that the variation of \( Y_s \) with \( \mu \) was due to maintenance metabolism. The values of \( m \) and \( Y_d \) calculated from Fig. 1 were 0.11 g of ethanol per g of biomass per h and 0.775 g of biomass/g of ethanol, respectively.

**Cell composition.** The carbohydrate content of the biomass remained essentially constant when the growth rate was varied (Fig. 2), whereas nucleic acid varied from about 7.5 to 12% (Fig. 3).

There were no large differences in protein content when protein was measured by the biuret assay; however, the results obtained were highly variable and these variations would obfuscate all but large changes. In addition, when protein was determined by an amino acid analysis, it was found that the protein content was

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**Fig. 1.** Effect of specific growth rate on the specific rate of ethanol consumption by A. calcoaceticus in ethanol-limited chemostat culture.

**Fig. 2.** Effect of specific growth rate on the protein, lipid, and carbohydrate content of A. calcoaceticus growing under an ethanol limitation in chemostat culture.

**Fig. 3.** Effect of specific growth rate on the nucleic acid content of A. calcoaceticus growing under an ethanol limitation in chemostat culture.
much higher than that indicated by biuret assay. Closer agreement with the amino acid analysis and more consistent results were obtained by determining protein content from the nitrogen and nucleic acid content of the biomass (see Materials and Methods). The resultant data suggested that the protein content of *A. calcoaceticus* increased from about 53 to 67% of the biomass as the growth rate was increased from 0.1/h to 0.53/h (Fig. 2).

Although the protein content of the microorganism varied by only 14%, the protein yield coefficient ($Y_p$) almost doubled, increasing from 0.24 to 0.43 over the same range of growth rates (Fig. 4). This large variation was due to the additive effects of the higher values of $Y_\varepsilon$ and the higher protein content of the microorganism.

**Phosphorus limitation.** *A. calcoaceticus* was cultivated in a chemostat on ethanol under phosphorus-limiting conditions. A steady state was achieved at a specific growth rate of 0.5/h, and analyses were made of the biomass composition and the ethanol and phosphate yield coefficients. The steady state was maintained for 3 days, and daily measurements were made. $Y_\varepsilon$ was significantly lower under phosphorus-limiting conditions than under an ethanol limitation (Table 1). These yield coefficients were corrected for unused ethanol in the culture broths. The protein content appeared to be somewhat lower, whereas the total nucleic acid content was slightly higher (11.8 versus 14.2%) than in ethanol-limited cultures growing at a similar rate (Table 1). As a result of the declines in protein content and $Y_\varepsilon$, $Y_p$ was about one-third less in phosphorus-limited cultures.

**DISCUSSION**

Our data suggest that the protein content of *A. calcoaceticus* in an ethanol-limited chemostat increases when the growth rate increases. In contrast, others have reported that the protein content of microorganisms remains relatively constant or decreases when the growth rate increases.

**Table 1. Yield coefficients and protein content of *A. calcoaceticus* in phosphorus-limited and ethanol-limited chemostat cultures**

| Trial          | Nucleic acid (%) | Protein (%) | $Y_\varepsilon$ (g of biomass/g of ethanol) | Protein content (g of protein/g of biomass) | $Y_p$ (g of protein/g of ethanol) |
|----------------|------------------|-------------|---------------------------------------------|--------------------------------------------|----------------------------------|
| **Phosphorus limited** |                  |             |                                             |                                            |                                  |
| 1              | 13.8             | 56.2        | 0.479                                       | 13.7                                       | 0.30                             |
| 2              | 14.2             | 63.0        | 0.441                                       | 12.9                                       | 0.29                             |
| 3              | 14.6             | 66.8        | 0.460                                       | 13.7                                       | 0.29                             |
| Avg            | 14.2             | 62.0        |                                             |                                            |                                  |
| **Ethanol limited** |                  |             |                                             |                                            |                                  |
| 1              | 11.0             | 68.0        | 0.628                                       |                                            | 0.450                            |
| 2              | 11.1             | 68.0        | 0.662                                       |                                            | 0.416                            |
| 3              | 12.4             | 66.5        | 0.627                                       |                                            | 0.433                            |
| Avg            | 11.5             | 67.3        |                                             |                                            |                                  |

*a Biomass was harvested from a chemostat operated at steady state under phosphorus-limiting conditions at a specific growth rate of 0.5/h.

*b Biomass was harvested from a chemostat operated at steady state under ethanol-limiting conditions at specific growth rates between 0.50 and 0.53/h.
rate increases (3, 4, 9, 21). The protein content, as we measured it, was based on the total nitrogen content of the biomass. As a result, these protein values may be higher than the true protein content because of the presence of non-protein nitrogen other than nucleic acids (e.g., N-acetylglycosamine and muramic acid in cell walls). Nitrogen-based protein analyses also are dependent on the somewhat arbitrary extinction coefficient used for the total nucleic acid estimation. These sources of error influence primarily the magnitude of the protein content, but they should not hinder the determination of relative differences in protein values unless large changes in non-protein or non-nucleic acid nitrogen content occur.

The identity of proteins responsible for the apparent increase in protein content of *A. calcoaceticus* at higher growth rates is not known. At least part of the increase may reflect the increase in enzyme level that presumably must occur to sustain high specific growth rates. Also, part of the increase may be due to ribosomal protein. Schaechter et al. (17) and Ecker and Schaechter (6) reported that the rate of protein synthesis per ribosome is independent of growth rate. Thus, larger numbers of ribosomes are needed to support higher growth rates. Although Sykes and Young (20) have shown that the rate of protein synthesis per ribosome increases with increases in growth rate, the rate of synthesis per ribosome was constant at growth rates above \( \mu = 0.5/h \).

Of particular significance to single-cell protein production is the variation of \( Y_p \) with growth rate. This variation was due primarily to maintenance metabolism. At higher growth rates, the amount of substrate diverted to maintenance becomes a progressively smaller proportion of the total amount of substrate consumed. As a result, \( Y_p \) increases at a greater rate than \( Y_x \) because both protein content and \( Y_x \) are higher at higher growth rates.

Earlier reports indicated that the protein content of a microorganism may be higher under a phosphorus limitation than under a carbon limitation (15, 21). A similar increase in a single-cell protein fermentation would have a beneficial impact on process economics if concomitant decreases in \( Y_x \) and \( Y_p \) did not occur. The present study indicates that *A. calcoaceticus* contained slightly less protein in phosphorus-limited cultures, and this decrease was accompanied by substantial declines in \( Y_x \) and \( Y_p \).

In conclusion, a single-cell protein fermentation using *A. calcoaceticus* should be operated under ethanol-limiting conditions at high growth rates in order to maximize the protein content of the product and the amount of protein and/or biomass produced from ethanol.

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