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Experimental and theoretical discrepancies in growth yields of *Acinetobacter calcoaceticus*: a correction of published data

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Abstract. *Acinetobacter calcoaceticus* can incompletely oxidize aldose sugars to the corresponding aldonic acids. This reaction can serve as an auxiliary energy source for the organism. An increase in biomass yields is observed in acetate-limited chemostat cultures grown in the presence of, for example, xylose. However, experimental and theoretical discrepancies exist with respect to the magnitude of the yield enhancement as a result of xylose addition. We previously observed increases in cell yields that were unexpectedly high. In contrast, other data were in agreement with the theoretical predictions. In this paper, evidence is presented indicating that this discrepancy is likely to be due to errors in the methodology used for our previous investigation, in particular with respect to the determination of biomass concentrations.

Introduction

Acinetobacter calcoaceticus is a versatile Gram-negative bacterium capable of aerobic growth on a wide variety of organic compounds (Juni 1978). Aldose sugars, however, generally do not support growth, although they are incompletely oxidized to the corresponding aldonic acids, with aldonolactones as intermediates. This reaction is catalysed by a periplasmic pyrroloquinoline quinone (PQQ)-dependent aldose dehydrogenase (GDH, EC 1.1.99.17; Duine et al. 1986).

A. calcoaceticus has been used as a model organism to study the energetics of the GDH reaction. Oxidation of aldose sugars by cytoplasmic membrane vesicles prepared from this organism generated a proton-motive force, which could energize the accumulation of solutes (van Schie et al. 1987a). Furthermore, addition of aldose sugars to energy-starved cells resulted in the quantitative conversion of the sugars into aldonic acids and the accumulation of intracellular ATP (van Schie et al. 1987a).

For an in-vivo analysis of the role of GDH in energy metabolism, chemostat experiments have been performed (Müller and Babel 1986; van Schie et al. 1987b). In these experiments an increase in cell yield was observed upon the addition of aldose sugars to the reservoir medium of acetate-limited chemostat cultures. Xylose rather than glucose was used as a model substrate in our studies (van Schie et al. 1987b), because glucose-assimilating variants of *A. calcoaceticus* accumulate after prolonged chemostat cultivation in the presence of glucose (van Schie et al. 1989). The xylose added to the chemostat cultures was quantitatively recovered as xylonic acid and less than 1% of the xylose carbon appeared as biomass. The increase in cell yield upon xylose addition was nearly threefold higher than calculated on the basis of expected P/O ratios (van Schie et al. 1987b).

Subsequent work of Noorman (1991) in our laboratory confirmed the role of xylose as an auxiliary energy source for acetate-limited chemostat cultures of *A. calcoaceticus*. However, the increase in cell yield was much lower than observed in our studies (van Schie et al. 1987b). In view of the differences in experimental conditions and methodology, we have reinvestigated growth of *A. calcoaceticus* in chemostat cultures grown under dual substrate limitation.

Materials and methods

Organism and growth conditions. Lyophilized cultures of *A. calcoaceticus* LMD 79.41 were obtained from the Netherlands Culture Collection. The organism was grown on a mineral medium in acetate-limited chemostat cultures (1 l working volume) in the presence or absence of 30 mM xylose, as described by van Schie et al. (1987b). In order to make comparison with the earlier experiments of van Schie et al. (1987b) possible, the cultivation conditions differed from those used by Noorman (1991) in that:

1. No antifoam was used.

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2. In order to prevent foaming, air was not sparged through, but passed over the culture.
3. Effluent removal occurred from the surface rather than from the centre of the culture.
The purity of chemostat cultures was routinely checked by phase-contrast microscopy and by plating on complex media.

Substrate determinations. Xylose and acetate concentrations in reservoir media were measured by HPLC using a Aminex HPX-87H column (300×7.8 mm, BioRad) at room temperature. The column was eluted with 0.5 $g \cdot l^{-1}$ of H_2SO_4 at a flow rate of 0.6 $ml \cdot min^{-1}$. A dual detection system was used, consisting of a Waters 441 UV detector and an ERC-7515A RI detector (Erma, Tokyo, Japan).

Culture dry weights. Instead of the total-organic-carbon method used by van Schie et al. (1987b), biomass dry weights were determined by filtering culture samples over preweighed membrane filters (0.45 μm pore diameter, Schleicher and Schüll, Dassel, Germany). The filters were washed three times with demineralized water and dried to constant weight at 70° C. Biomass concentrations were determined both in samples taken directly from the culture and in samples taken from the culture effluent.

Table 1. Biomass yields in aerobic, acetate-limited chemostat cultures of *Acinetobacter calcoaceticus* LMD 79.41 (dilution rate = 0.15 h^{-1}, T = 30° C, 30 mM acetate) grown at pH 7.0 and at pH 8.2, and in the presence and absence of 30 mM xylose in the reservoir medium

Xylose (mM)	pH	Sampling mode	Biomass yield (g dry wt·mol^{-1} acetate)	Yield increase (%)
0	7.0	Culture	12.8±0.1 (12.0)	
0	7.0	Effluent	14.7±0.5	
30	7.0	Culture	14.2±0.1 (12.0)	11 (0)
30	7.0	Effluent	16.0±0.6	10
0	8.2	Culture	14.2±0.8 (12.0)	
0	8.2	Effluent	15.5±0.7	
30	8.2	Culture	16.2±0.2 (18.5)	14 (54)
30	8.2	Effluent	17.0±0.2	10

Culture were sampled either directly from the fermentor or from the effluent line. Biomass yields are average data from two independent steady states. Data of van Schie et al. (1987b) are given in brackets

Results

It was previously observed (van Schie et al. 1987b) that the addition of xylose to the reservoir medium of acetate-limited chemostat cultures resulted in a drastic enhancement of the biomass yields. At a fixed concentration of 30 mM acetate, increases in biomass yields were linearly proportional to the reservoir xylose concentration up to 25 mM. Peculiarly, this increase in cell yield was observed at pH 8.2, but not at pH 7.0. Since quantitative conversion of xylose was observed irrespective of the culture pH, it was speculated (van Schie et al. 1987b) that the effect of pH on cell yields might be related to the hydrolysis kinetics of xylonolactone to xylonic acid. This chemical reaction proceeds much more slowly at pH 7.0 than at pH 8.2. Noorman (1991) has pointed out that at pH 7.0 hydrolysis of xylonolactone, although slower than at pH 8.2, is still substantial. Therefore, if lactone hydrolysis contributed to yield enhancement, an increase in cell yields would also be expected at pH 7.0. This was indeed observed by Noorman (1991).

To investigate the above theoretical and experimental discrepancies, we have studied the biomass yields in chemostat cultures of *A. calcoaceticus* grown in the presence and absence of xylose, at pH 7.0 and at pH 8.2. Particular attention was paid to the sampling of biomass from the cultures. It has been reported (Noorman et al. 1992) that, depending on the way in which effluent is removed, substantial differences may occur between the biomass concentrations in the effluent and in the culture. The degree to which selective biomass removal occurs may be also be dependent on growth conditions, for example due to a change of the surface properties of the organism under study. This in turn could lead to an unjustified comparison of different steady-state situations. Noorman (1991) used a special device (Noorman et al. 1992) to prevent this problem, which was not recognized by van Schie et al. (1987b). In the present study, we therefore measured biomass concentrations both in samples taken from the fermentor and in samples from the effluent line.

The results of our present investigation are summarized in Table 1. For comparison, also the data of van Schie et al. (1987b) are included in this table. From Table 1, a number of conclusions can be drawn:
1. With the effluent removal system used by van Schie et al. (1987b), differences in biomass concentration between culture and effluent did indeed occur.
2. In contrast to our previously published data (van Schie et al. 1987b) and in line with the results of Noorman (1991), an enhancement of the biomass yields in the presence of xylose also occurred at pH 7.0.
3. The magnitude of the yield enhancement observed here is lower than we reported previously (van Schie et al. 1987b), as was also found by Noorman (1991).

Discussion

The results of our present work, as summarized in Table 1, show that the large increases in cell yields reported previously (van Schie et al. 1987b) cannot be maintained. The reported increment in yield was only approximately 10%, in contrast to the 54% increase reported by van Schie et al. (1987b).

The explanation for the different results found in the present study as compared to our previous work (van Schie et al. 1987b) cannot solely be attributed to the mode of effluent removal. Although selective removal of biomass from the culture did indeed occur, the high increase in biomass concentrations observed by van Schie et al. (1987b) was not found, neither in samples taken directly from the fermentor nor in samples from the effluent line.

Accurate determination of culture weights is essential for quantitative studies in microbial physiology. With *A. calcoaceticus* this is not a simple enterprise,

since the organism tends to clog up the filters routinely used for dry weight measurements. Therefore, Noorman (1991) chose to collect cells by centrifugation, followed by freeze-drying. We found that direct filtration of *A. calcoaceticus* culture samples is possible, provided that small samples are treated. When carried out in triplicate, the accuracy of the filter method was within 3%. A similar accuracy can be obtained with the freeze-drying method used by Noorman (1991). Van Schie et al. (1987b) used total-organic-carbon (TOC) analysis: the carbon content of culture supernatants obtained by centrifugation was subtracted from that of whole culture samples.

The TOC method used by van Schie et al. (1987b) is not reliable in all situations. In acetate-limited chemostat cultures of *A. calcoaceticus*, the supernatants contain very low concentrations (<25 mg·l^{-1}) of organic carbon. However, during cultivation on mixtures of acetate and xylose, high amounts of organic carbon in the form of xylonic acid are present in the culture supernatant (30 mM xylonic acid corresponds to 1800 mg C·l^{-1}, in the presence of approximately 240 mg C·l^{-1} biomass, giving a TOC concentration of 2040 mg·l^{-1}). Although the accuracy of individual TOC measurements is within 2%, this method may give rise to large errors in the calculated biomass concentration, which is obtained from the subtraction of the two large figures. A rigorous statistical analysis of the experimental data and procedures would have prevented this error.

The re-evaluation presented herein shows that the inconsistencies between theory and experimental results reported in our previous paper (van Schie et al. 1987b) in fact do not exist and are likely to be explained by methodological errors.

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