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The use of acetate as an additional co-substrate improves methylotrophic growth of the acetogenic anaerobe *Eubacterium limosum* when CO_2 fixation is rate-limiting

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Growth of the acetogenic anaerobe *Eubacterium limosum* on methanol/CO_2 mixtures is limited by the rate at which CO_2 can be assimilated. This limitation can be offset by the consumption of acetate as an additional co-substrate. Growth on methanol/CO_2/acetate mixtures improves growth rates but stimulates production of an unidentified polymer leading to cell aggregation and wall growth under chemostat conditions. Production of butyrate as major fermentation end-product leads to growth inhibition visualized by an increased maintenance requirement as demonstrated by Y_{ATP} estimations.

Introduction

Eubacterium limosum is an acetogenic anaerobe able to grow on a variety of substrates including methanol (Genthner *et al.*, 1981). Methylotrophic growth is possible only if a more oxidized co-substrate, i.e. CO_2 is presented. The fermentation end-products are butyrate and acetate, the relative proportions of which vary with the culture conditions. Fastest growth rates have been obtained with the addition of acetate (or rumen fluid), conditions which also prevent prolonged lag phases occurring (Genthner *et al.*, 1981; Lynd & Zeikus, 1983). To date, the role of acetate has remained largely obscure as regards its effect on growth rates, though the use of acetate supplements to drive the metabolism towards the biotechnologically interesting homobutyric fermentation has been reported (Pacaud *et al.*, 1986*a*). Under such conditions some acetate is consumed, and recent work has shown that the micro-organism produces only butyrate during the relatively short-lived exponential phase during growth on methanol (Loubière *et al.*, 1990). Mixed acid production takes place only after the onset of the extended period of decelerating growth. It might be imagined, therefore, that some consumption of acetate may occur during the initial period of rapid growth though this has never been clearly demonstrated.

In this study the growth of *E. limosum* has been studied, using both batch and continuous modes of cultivation, in order to ascertain the role of both acetate and CO_2 as co-substrates to methanol under various nutritional environments.

Methods

Micro-organism and culture conditions. Eubacterium limosum was isolated from pea processing wastes (Samain, 1983). The growth medium was the semi-defined medium of Pacaud *et al.* (1985). The major carbon source was methanol (100 mM), but CO_2 (60 mM) and/or acetate (50 mM) were presented as potential co-substrates as detailed in the text. The medium was prepared under strict anaerobic conditions and reduced after autoclaving by addition of cysteine.HCl (0·5 g l^{-1}). The initial pH of the medium was 7·3 and this value was maintained throughout growth by automatic addition of NH_4OH (4 M). An incubation temperature of 37 °C was used for all cultures. Inocula (5% of final culture volume) were prepared by three succesive 24 h transfers through identical medium. Glass fermenters (800 ml working volume) were used for both batch and continuous cultures. The feed-bottle gas-phase was maintained constant with a membrane-valve-operated 0·01 bar positive-pressure device using a defined gas-mixture (85% N_2 + 15% CO_2, v/v) passed over a hot copper catalyst. In addition to maintaining the liquid phase CO_2 concentration constant this system also proved to be a more reliable method of avoiding oxygen contamination than the sulphate/pyragallol traps often employed. Biomass concentrations were followed by spectrophotometric measurements at 660 nm and by gravimetric analysis of washed cell samples dried to constant weight.

Substrate and product analysis. Substrate and product concentrations were measured by gas chromatography techniques as described by Pacaud *et al.* (1985). The dissolved CO_2 concentration was calculated from the gas-phase composition and the culture pH assuming equilibrium conditions.

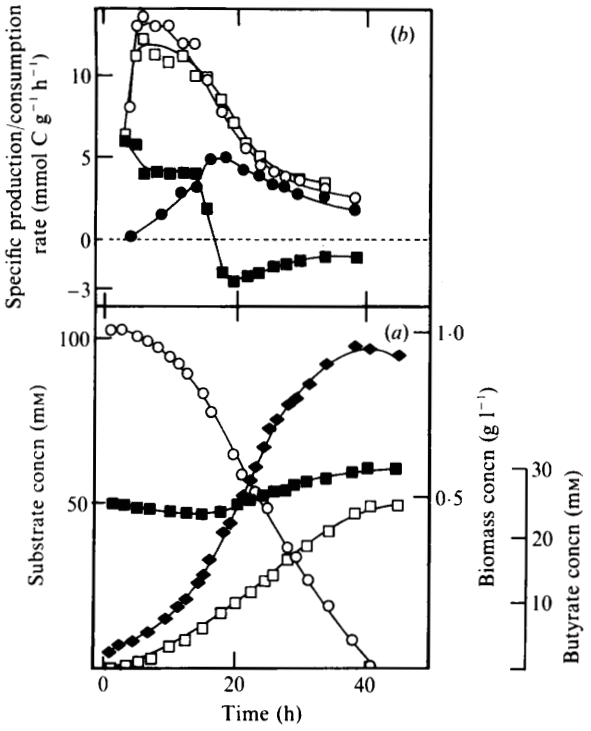

Fig. 1

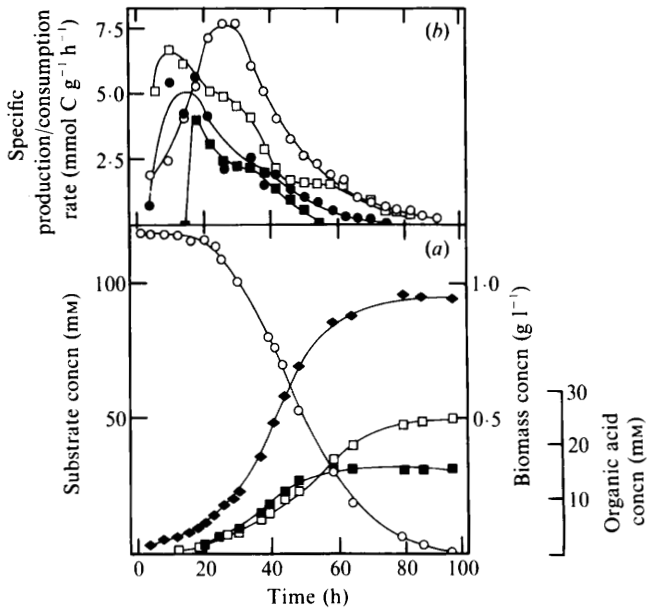

Fig. 2

Fig. 1. Batch fermentation profile for the growth of *E. limosum* on a methanol, CO_2 and acetate mixture, showing substrate and product concentrations (*a*) and specific rates of consumption and production (*b*). Negative values for acetate in (*b*) indicate a shift from consumption to production. ○, methanol; ●, CO_2; □, butyrate; ■, acetate; ◆, biomass.

Fig. 2. Batch fermentation profile for the growth of *E. limosum* on a methanol and CO_2 mixture, showing substrate and product concentrations (*a*) and specific rates of consumption and production (*b*). ○, Methanol; ●, CO_2; □, butyrate; ■, acetate; ◆, biomass.

Fermentation balances. All experiments were analysed to ensure that major elements (C, H and O) and reducing equivalents could be accounted for. Biomass was taken to have the formula of $C_4H_7O_2N_{0.6}$ with an overall degree of reduction of 4·3 per carbon atom (Erickson, 1980). Biomass yields were calculated relative to ATP, the production of ATP being estimated from the known biochemical pathways (Kerby *et al.*, 1983) and stoichiometric balance equations. Production of ATP is limited to three enzymic reactions: formyltetrahydrofolate synthetase and the acetate and butyrate kinases, all of which can be reliably estimated from fermentation balance equations.

Results and Discussion

Batch growth of *E. limosum* on methanol/CO_2 in medium supplemented with acetate was characterized by a short-lived exponential growth phase (μ = 0·12 h^{-1}) followed by a prolonged period of decelerating growth (Fig. 1). During the period of exponential growth the fermentation was homobutyric and acetate was consumed. Specific substrate consumption rates during this phase of the culture were as follows: methanol, 14 mmol g^{-1} h^{-1}; CO_2, 2 mmol g^{-1} h^{-1}; and acetate, 2 mmol g^{-1} h^{-1}. Acetate consumption diminished towards the end of the exponential growth phase to become negative (i.e. production of acetate) during the phase of decelerating growth. During this second phase, specific rates of methanol and CO_2 consumption also diminished.

In comparison with the above fermentation, batch growth on methanol/CO_2 in the absence of an acetate supplement progressed at a lower maximum growth rate (μ = 0·075 h^{-1}) and co-production of acetate and butyrate was observed from the onset (Fig. 2). Again, a prolonged phase of decelerating growth was observed. Specific rates of substrate consumption were directly comparable with those obtained at similar growth rates in medium supplemented with acetate, i.e. during the decelerating growth phase.

Chemostat cultures were used to study carbon distribution under steady-state conditions. In the absence of acetate (other than that proved by the micro-organism), *E. limosum* showed behaviour which deviated significantly from that to be expected under carbon limitation (Fig. 3). In particular, the profile of biomass concentration and residual substrate concentrations were not as predicted from Monod-based kinetic models. The pseudo-linear increase in residual methanol concentration relative to dilution rate can be explained by either a nutritional limitation other than the carbon source, or by the inhibitory effect(s) of fermentation products.

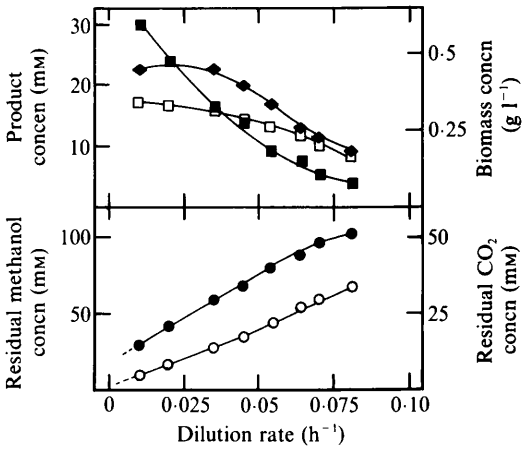

Fig. 3

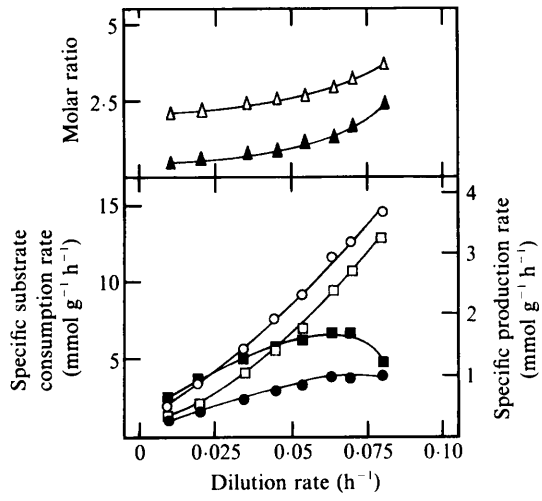

Fig. 4

Fig. 3. Chemostat steady-state values for growth of *E. limosum* at various dilution rates on a methanol and CO_2 mixture. ○, Residual methanol; ●, residual CO_2. □, Butyrate; ■, acetate; ◆, biomass.

Fig. 4. Specific rates of substrate consumption and organic acid production and the molar ratios of substrate consumption (methanol : CO_2) and organic acid production (butyrate : acetate) for data presented in Fig. 3. ○, Methanol; ●, CO_2; □, butyrate; ■, acetate. △, Methanol : CO_2 consumption ratio; ▲, butyrate : acetate production ratio.

Increasing the mineral salts component of the medium (either individually or globally) did not alter the steady-state characteristics at a growth rate of 0·06 h^{-1}. Washout occurred at dilution rates higher than 0·08 h^{-1}, a value comparable with the maximum rate of growth obtained in batch cultures lacking acetate. The product ratio varied with the growth rate, favouring butyrate rather than acetate at high rates of growth though homobutyric fermentations were never obtained. Bearing in mind the biomass profile, it is not surprising that organic acid concentrations in the medium decreased as the growth rate increased. It should be noted, however, that the specific rate of organic acid production remained constant relative to the specific substrate-consumption rate. Butyrate production was closely correlated with methanol consumption while acetate production aligned with CO_2 consumption (Fig. 4). Since the contribution of methanol to the total substrate used increased proportionally to the growth rate the accompanying shift towards butyrate production could be explained by the necessity to maintain a balanced fermentation relative to reducing equivalents. This situation was accentuated at growth rates in excess of 0·05 h^{-1} at which point specific rates of CO_2 consumption reached a maximum and did not increase when the CO_2 content of the medium was increased.

When the same experiment was repeated using chemostat cultures fed with medium containing acetate (50 mM) in addition to methanol and CO_2, acetate was consumed at growth rates as low as 0·04 h^{-1} and homobutyric fermentations were observed (Fig. 5). At this dilution rate the specific acetate consumption rate was 0·2 mmol g^{-1} h^{-1}. However, at higher growth rates no further increase in the specific acetate-consumption rate occurred. The critical dilution rate was estimated at 0·13 h^{-1} (similar to μ_m obtained in batch conditions) by extrapolation of the residual methanol curve. This was necessary since at growth rates above 0·07 h^{-1} the culture became heterogenous with wall growth and floc formation due to the excessive production of slime. Interestingly, this slime production was never observed in media lacking acetate. Furthermore, the induction of slime production at growth rates of 0·07 h^{-1} or above could not be stopped by lowering the dilution rate to values where the synthesis of this polymer did not normally occur. Under these conditions of low rates of growth accompanied by slime production the growth kinetics were noticeably different to those obtained for cultures not producing slime, particularly as regards CO_2 consumption and acetate production, both of which were higher than otherwise observed (results not shown).

The potential to use acetate as additional co-substrate to methanol and CO_2 allows this *E. limosum* to proliferate at growth rates significantly higher than would otherwise be possible. This organism has a relatively restrained ability to vary its product profile: only acetate and butyrate are normally produced though in the presence of high levels of saturated organic acids C_2-extension reactions have been reported (Lindley *et al.*, 1987). Unlike many acetogens (and indeed *E. limosum* on multi-carbon substrates) no capacity to produce H_2 occurs during methylotrophic metabolism and hence butyrate

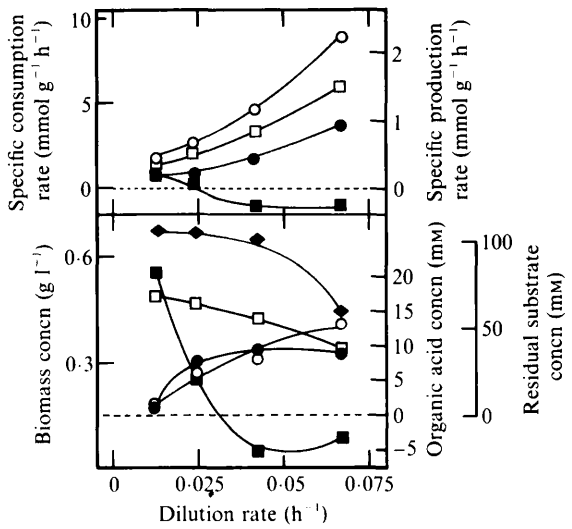

Fig. 5. Chemostat steady-state values for growth of *E. limosum* at various dilution rates on a methanol, CO_2 and acetate mixture. Negative values for acetate represent a shift from production to consumption. ○, Methanol; ●, CO_2; □, butyrate; ■, acetate; ◆, biomass.

offers the only route which can waste excess reducing equivalents. For such a metabolism the capacity to control the degree of reduction of the substrate mixture provides an alternative response enabling the bacterium to grow faster within the confines of a typical anaerobic environment. The homobutyric fermentation represents the limits of this organism's physiological flexibility but may be obtained with various theoretical substrate mixtures.

$$10 \text{ Methanol} + 2\ CO_2 \rightarrow 3 \text{ Butyrate}$$

$$6 \text{ Methanol} + 3 \text{ Acetate} \rightarrow 3 \text{ Butyrate}$$

If the role of acetate can be described as both source of carbon and electron acceptor under conditions in which consumption of CO_2 was limiting, certain aspects remain obscure. The nature of the rate-limiting step of CO_2 fixation needs to be ascertained. It is difficult to see how the consumption of acetate, which enters the metabolism as acetyl-CoA, could influence those carboxylation reactions situated further along the gluconeogenic pathway, so pyruvate synthase and pyruvate and/or PEP carboxylase(s) can be ignored (though possible candidates for major flux-limiting reactions when grown in the presence of acetate). The rate-limiting step(s) are therefore associated with the carboxyl-donor supply for acetyl-CoA formation. The consumption of acetate enables the limitation on CO_2 fixation to be overcome, but why the period of acetate consumption is relatively short-lived in batch cultures and what fixes the rate at which acetate consumption takes place remains obscure. The route of acetate assimilation is by the operation of the acetate kinase and phosphotransferase enzymes, the operation of which is reversible. The equilibrium constant of these enzymes favours acetate production from acetyl-CoA rather than acetate consumption (Schaupp & Ljungdahl, 1974). It is likely, therefore, that it is the acetate concentration that determines the manner in which this compound is consumed. It would be interesting to see what effect changing the initial acetate concentration in the medium would have on the kinetics, though biochemical phenomena effecting the intracellular acetate concentration should also be examined.

If the positive effects of acetate can be related to carbon- and energy-flow difficulties resulting from rate-limiting reactions of CO_2 fixation, the unusual kinetics of biomass formation needs to be further examined. This has been vaguely attributed to product-inhibition phenomena accentuated at high growth rates by the tendency towards homobutyric fermentations – butyrate being considerably more inhibitory than acetate (Pacaud *et al.*, 1986*b*). The biomass concentration relative to the dilution rate was modelled to see if butyrate concentration was directly responsible for the unusual biomass profile obtained above. The inhibition was assumed to be of a non-competitive nature and was modelled by

$$X = (\mu_m \cdot Y_x \cdot K_i)/(Y_p \cdot D) - (Y_x \cdot K_i)/Y_p$$

where Y_x is the biomass yield, Y_p is the inhibitory product yield and K_i the inhibition constant. If butyrate is inhibitory a plot of biomass concentration against the reciprocal of the dilution rate should be linear over the range in which inhibition influences the kinetic behaviour. This type of relationship was obtained at growth rates in excess of 0·04 h^{-1}. A critical dilution rate was predicted at 0·16 h^{-1} though this does not take into account other limiting reactions, e.g. CO_2 fixation. The biomass profile can therefore be attributed to butyrate inhibition and other maintenance energy calculations allow this phenomenon to be quantified.

Surprisingly, a linear relationship was obtained between the growth rate and the specific rate of combined substrate consumption (Fig. 6) implying that the maintenance coefficient was constant at 0·8 mmol substrate (g dry wt)$^{-1}$ h^{-1} and that no growth-rate-related maintenance (Pirt, 1982) took place. However, it must be remembered that in this case the substrate was a mixture of variable composition and that this would directly affect the energetic yield. It must also be born in mind that the ATP generated from the terminal steps of organic acid production is a major contribution to the overall energy budget of this organism, and this yield is also a function of the substrate mixture consumed. After applying this information to the experimental data it can be seen that the energetic yield relative to the growth rate

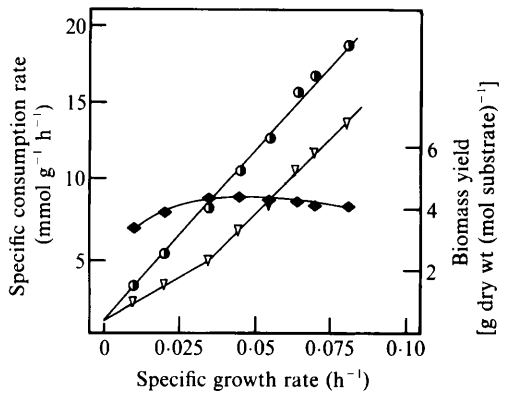

Fig. 6. Relationship between growth rate and specific substrate consumption rate (◑), calculated specific rate of ATP consumption (▽) and biomass yield (◆) for steady-state chemostat cultures shown in Fig. 3.

was not constant and that at growth rates above 0·045 h^{-1} the contribution of ATP to the maintenance became particulary important, explaining the fall in biomass concentrations at faster growth rates. At low growth rates, the growth-associated maintenance requirements are low and represent only 0·8 mmol ATP g^{-1} h^{-1} with an Y_{ATP} of 9 g biomass (mol ATP)$^{-1}$. At high growth rates the apparent Y_{ATP} is as low as 5 g biomass (mol ATP)$^{-1}$, indicating that a significant proportion of the generated ATP was spent on growth-unassociated functions. The generally accepted explanation for butyrate inhibition has been that as butyrate accumulates within the broth the intracellular concentrations will be considerably higher due to the cross-membrane pH gradient. This will result in a slowing down and eventually a complete halt to further production of butyrate and hence the incapacity of the bacterium to maintain a balanced fermentation and an accompanying lowering of all metabolic rates. More recently, an alternative explanation has been proposed based on the finding that intracellular butyrate concentrations were too low to result from passive diffusion (Loubière *et al.*, 1990). Instead, it would appear that a non-passive excretion of butyrate (but not acetate) takes place associated with an energetically expensive protonophore effect due to the tendency for free butyric acid to passively re-enter the cell. Such a futile-cycle phenomenon would consume large quantities of ATP and would result in an increased maintenance requirement at concentrations of butyrate considerably lower than might be expected to bring about chemical dissipation of the membrane potential by the weak acid. One effect of this would be to diminish the intracellular concentration of both acetate and CO_2 and hence the capacity of the microbe to consume either of these co-substrates. This in turn will result in reduced rates of methanol assimilation and a general slowing of specific metabolic rates. Further studies of the behaviour of anaerobic acetogens on substrate mixtures will enable the adaptive limits of their metabolism to be ascertained.

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