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Modulation of Carbon and Electron Flow in *Clostridium acetobutylicum* by Iron Limitation and Methyl Viologen Addition

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The metabolic flexibility of *Clostridium acetobutylicum* during growth on glucose with methyl viologen addition (1 mM) and/or iron limitation was examined in batch cultures at pH 5.5. The physiological effects of iron limitation and methyl viologen addition are additive, suggesting that they have different and complementary sites of action.

Decreasing the in vivo activity of the hydrogenase of *Clostridium acetobutylicum* has been shown to increase alcohol formation specifically. The in vivo activity of the hydrogenase has been decreased in several ways: (i) by an increase of the hydrogen partial pressure (4, 15); (ii) by carbon monoxide flushing (a reversible inhibitor of hydrogenase) (3, 8, 10); (iii) by iron limitation (1, 7); and (iv) by the addition of an artificial electron carrier such as methyl viologen (9, 11–14) or neutral red (5, 6). In this research, we investigated the combined effects of methyl viologen and iron on the electron and carbon flow distribution in batch cultures at pH 5.5. The results contribute to a better understanding of the effect of methyl viologen on the metabolism of *C. acetobutylicum.*

Organism and culture conditions. *C. acetobutylicum* ATCC 824 was grown at 35°C and pH 5.5 in the synthetic medium described previously (11), except iron sulfate concentrations ranged from 8 to 50 μM depending on the culture conditions.

Analytical procedures. Concentrations of cells, glucose, ethanol, butanol, acetone, acetoin, and acetic, butyric, and lactic acids were determined as previously described (2). The iron concentration was measured by atomic absorption spectrometry (model 3030 apparatus; Perkin-Elmer Cetus, Norwalk, Conn.).

Effects of iron and methyl viologen on growth of *C. acetobutylicum*. The maximal cell concentration was proportional to the iron concentration if the culture medium contained less than 25 μM iron and was independent of the iron concentration above that value. The maximal cell density was not further affected by methyl viologen supplements (1 mM) at any iron concentration.

Stoichiometric analysis of cultures at different initial iron concentrations with or without methyl viologen. The distribution of products was affected by iron limitation and methyl viologen addition. The curve of the butanol yield (Fig. 1a) related to glucose can be divided into two parts: one above 25 μM iron, in which the butanol yield was not dependent on initial iron concentration; and the other below 25 μM iron, in which the butanol yield was increased when the iron supply was decreased. With 1 mM methyl viologen and 8 μM iron, the butanol yield reached a value of 0.5 mol/mol of glucose. This value was five times higher than that obtained in the control experiment. The butyric acid (Fig. 1b) yield related to glucose did not vary with iron concentration when methyl viologen was not provided to the culture. On the other hand, with 1 mM methyl viologen, this yield decreased with iron concentration at all concentrations lower than 25 μM. The acetic acid yield was decreased by methyl viologen addition under conditions of iron excess, while under iron limitation it was not significantly different with or without methyl viologen addition (Fig. 1c).

The moles of NAD(P)H produced from reduced ferredoxin [MNADH] by ferredoxin-NAD(P)$^+$ reductases were calculated (Fig. 1d) from the difference between the moles of NAD(P)H consumed in product formation and the moles of NADH produced by the Emden-Meyerhof-Parnas pathway. When the iron supply was nonlimiting, the metabolism of *C. acetobutylicum* was normally acidogenic and the MNADH/Mglucose value was negative (−0.2), indicating that part of the NADH produced by the Emden-Meyerhof-Parnas pathway was used by the NADH-ferredoxin reductase to produce reduced ferredoxin and hence hydrogen. Under iron limitation, a reversed situation was observed: the MNADH/Mglucose value was positive and part of the reduced ferredoxin was used by the ferredoxin-NAD(P)$^+$ reductases to produce NAD(P)H, thereby diminishing the amount of hydrogen produced. When methyl viologen was present, the whole curve of MNADH/Mglucose as a function of iron supply was displaced towards a higher value of MNADH/Mglucose.

Kinetics analysis. Two iron-excess (50 μM) cultures (pH 5.5; with and without methyl viologen) and two iron-limited (8 μM) cultures (with and without methyl viologen) were chosen for a more detailed kinetic analysis. For the control experiment (Fig. 2a), growth stopped when glucose was totally consumed. The major products were acetic and butyric acids, with some butanol and ethanol, but no acetone was produced. Under iron limitation (Fig. 2b) in a first phase, consumption of iron led to iron limitation (data not shown) and growth stopped. After lysis of the cells, a second phase of growth occurred with only a slight increase in cell concentration. It was at the beginning of this second phase of growth that production of lactate and butanol started. Lactate was totally reconsumed (whereas no reconsumption of acetic and butyric acids was observed), and its reassimilation occurred in a cosubstrate with glucose. When the culture broth contained methyl viologen, with 50 μM iron,

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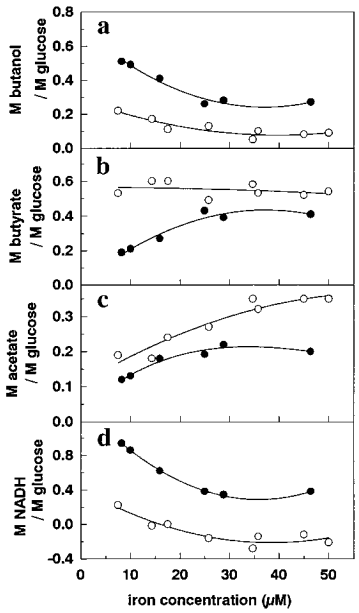

FIG. 1. Influence of initial iron concentration on molar yields (related to glucose) of batch cultures of *C. acetobutylicum* at pH 5.5 in the absence (○) or presence of 1 mM methyl viologen (●). (a) Butanol; (b) butyrate; (c) acetate; (d) pyridine nucleotides reduced (positive value) or oxidized (negative value) from ferredoxin.

all products were formed at the same time (Fig. 2c). The maximal biomass concentration was not affected, but the lag phase increased from 10 to 70 h. Production of both butanol and ethanol was increased compared with the control at the expense of acetic and butyric acids. Lactate was also produced but to a lesser extent than in the case of iron limitation, and it was only partly reassimilated. Though alcohol production was improved by the addition of methyl viologen, the fermentation remained predominantly acidogenic. Under iron limitation, when methyl viologen was added to the culture (Fig. 2d), no second phase of growth was observed as in the case of iron limitation alone. All products were synthesized simultaneously after a long lag phase, as in the iron-excess culture with methyl viologen (Fig. 2c). The main product was butanol and its concentration reached an inhibitory concentration of 175 mM, provoking a premature halt to the fermentation before all glucose had been consumed.

Conclusions. Iron limitation and methyl viologen are separately capable of causing metabolic shifts in favor of butanol formation. The data presented here demonstrate that when these two conditions are combined within the same culture their effects are additive. This suggests that methyl viologen and iron limitation operate at different sites in the metabolic pathway. Junelles et al. (7) had demonstrated that under iron limitation hydrogenase was either not synthesized or was synthesized but not in a functional form. On the other hand, methyl viologen was shown to be a better substrate than ferredoxin for the ferredoxin-$NAD(P)^+$ reductase and to increase its activity 60-fold, creating an artificial electron transport chain: pyruvate:ferredoxin oxidoreductase–methyl viologen–ferredoxin-$NAD(P)^+$ reductase–$NAD(P)^+$ (12). By both decreasing the hydrogenase activity and increasing the ferredoxin-$NAD(P)^+$ reductase activity, it is possible to obtain a high yield of butanol at a pH value that typically yields acidogenic cultures.

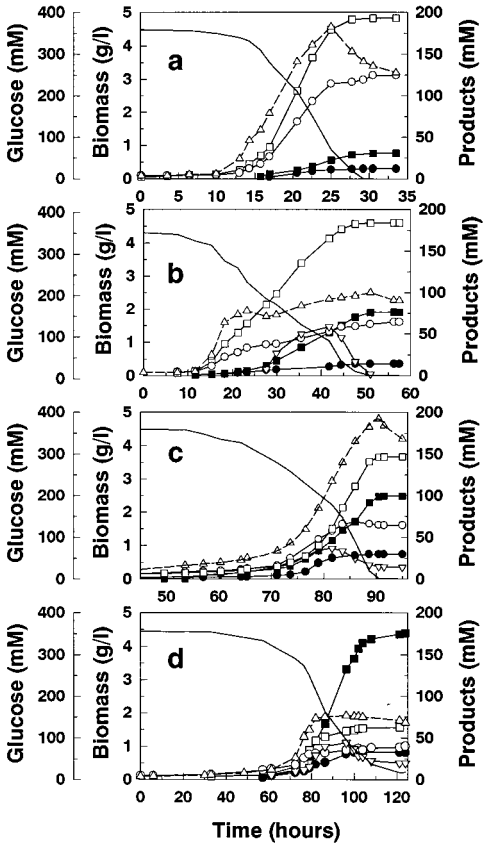

FIG. 2. Kinetics of batch fermentations of *C. acetobutylicum* at pH 5.5. (a) Control culture (50 μM iron); (b) iron-limited culture (8 μM iron); (c) iron-excess culture (50 μM) with 1 mM methyl viologen; (d) iron-limited culture (8 μM iron) in the presence of methyl viologen (1 mM). Symbols: △, biomass; ■, butanol; ●, ethanol; □, butyrate; ○, acetate; ——, glucose.

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