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Methanol Metabolism by *Eubacterium limosum* B2: Effects of pH and Carbon Dioxide on Growth and Organic Acid Production

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Abstract. Growth of *Eubacterium limosum* B2 on methanol–CO_2 was dependent upon the pH; optimum growth rates were obtained at pH 7.3–7.4. Carbon dioxide, a necessary cosubstrate for methylotrophic growth on methanol, was a determining factor for both growth and the nature of the organic acid produced. The coefficient of affinity for CO_2 was not affected by variations in pH if calculated relative to the concentration of hydrogen carbonate (16 m*M*). Similarly the conversion rate for methanol into acetic acid and butyric acid remained constant regardless of the level of pH with a fixed concentration of hydrogen carbonate. The metabolism of *E. limosum* B2 grown on methanol–CO_2 was regulated by the HCO_3^- concentration in the medium.

The discovery of anaerobic acidogenic bacteria capable of growth on single carbon substrates as sole source of carbon and energy is relatively recent. Methanol has been shown to be utilized for anaerobic fermentative growth by *Butyribacterium methylotrophicum* [7, 8, 10, 14], *Eubacterium limosum* [3, 4], *Eubacterium limosum* B2 [11], *Acetobacterium woodii* [2], *Clostridium thermoautotrophicum* [12], and *Clostridium* CV-AA1 [1]. Carbon dioxide acts as an essential cosubstrate during this methylotrophic fermentation. *Butyribacterium methylotrophicum* and *E. limosum* produce butyric acid as well as acetic acid during growth. A study of the insertion order of C_1 compounds enabled Kerby et al. [5] to propose a model of assimilation in which the various C_1 substrates enter a common metabolic sequence at different positions to be ultimately transformed into organic acids via a common intermediate, acetyl CoA. The point of entry of each substrate correlated with the degree of reduction. Methanol was preferentially incorporated into the methyl group of acetyl CoA, while the carboxyl group was incorporated via CO_2, some of which originated from oxidation of methanol yielding reducing equivalents ($NADH_2$). Synthesis of butyric acid from acetyl CoA regenerates some of the NAD^+. Growth in culture conditions in which there is an excess of acetate and low concentrations of dissolved CO_2 produces butyric acid exclusively according to the following stoichiometry [4, 8, 11, 13, 14]:

$$10\ MeOH + 2\ CO_2 \rightarrow 3\ CH_3CH_2CH_2COOH$$

An increase in the concentration of dissolved CO_2 stimulates acetic acid production and represses synthesis of butyric acid [11].

The effects of carbon dioxide on both growth and organic acid production have been investigated in this study with particular emphasis being placed upon which form of dissolved carbon dioxide exerts a metabolic influence. Carbon dioxide exists in aqueous solution in several forms and at various concentrations, depending upon the pH, temperature, ionic strength of the medium, and the gaseous-phase composition. With the pH values investigated in this study, the carbonate ion (CO_3^{2-}) concentration would be negligible compared with that of dissolved CO_2 and hydrogen carbonate thus may be ignored. A balance between the two latter forms depends on the pH. Thus, to quantify the effect of pH and CO_2, it was necessary to investigate the effects of all three interrelated parameters (pH, dissolved CO_2, and HCO_3^-).

Materials and Methods

Strain. The strain was isolated by Samain [11] from a fermentation population growing on pea-processing wastes. It has been

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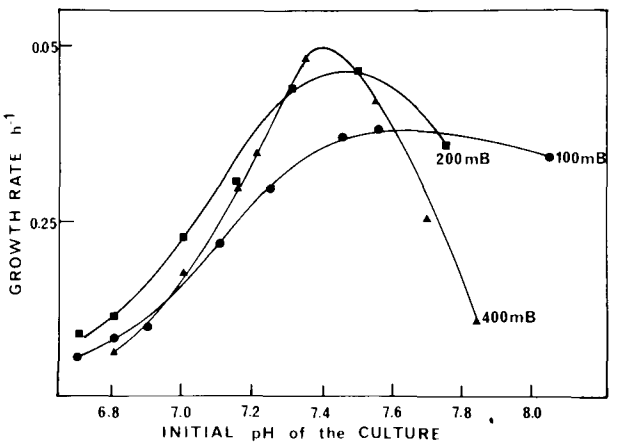

Fig. 1. Effects of pH and three partial pressure of CO_2 upon the growth rate of *Eubacterium limosum*.

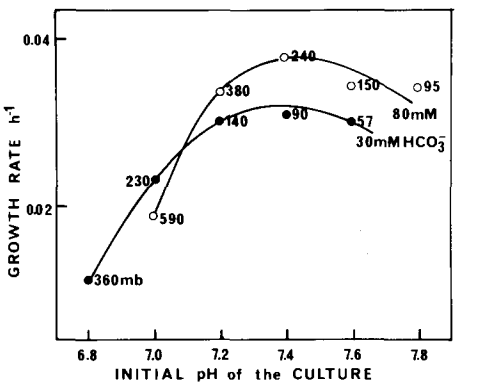

Fig. 2. Effects of pH on the growth rate when CO_2 is expressed as concentration of hydrogen carbonate (HCO_3^-). The given HCO_3 for pH was related with a partial pressure, indicated alongside each point.

classified as a member of the genus *Eubacterium* and has been named *Eubacterium limosum* B2.

Medium composition. The medium was composed of the following salts per liter of distilled water:

—*Macromineral solution* (KH_2PO_4, 6 g · liter^{-1}; $NaCl_2$, 12 g · liter^{-1}; $MgSO_4 \cdot 7H_2O$, 2.4 g · liter^{-1}; and $CaCl_2 \cdot 2H_2O$, 1.6 g · liter^{-1}), 50 ml.

—*Trace element solution* ($FeSO_4 \cdot 7H_2O$, 0.1 g · liter^{-1}; $MnCl_2 \cdot 4H_2O$, 0.1 g · liter^{-1}; $CaCl_2 \cdot H_2O$, 0.17 g · liter^{-1}; $CoCl_2 \cdot 2H_2O$, 0.1 g · liter^{-1}; $ZnCl_2$, 0.1 g · liter^{-1}; $CuCl_2$, 0.02 g · liter^{-1}; H_3BO_3, 0.01 g · liter^{-1}; Na_2MoO_4, 0.01 g · liter^{-1}; NaCl, 1 g · liter^{-1}; Na_2SeO_3, 0.017 g · liter^{-1}; $NiSO_4 \cdot 6H_2O$, 0.026 g · liter^{-1}; and nitilotriacetic acid, 12.8 g · liter^{-1} [neutralized at pH 6.5 with KOH]), 10 ml.

—*Vitamin solution* (biotin, 2 mg · liter^{-1}; pantothenic acid, 5 mg · liter^{-1}; and lipoic acid, 5 mg · liter^{-1}), 10 ml.

To this mixture were added yeast extract (0.5 g · liter^{-1}), NH_4Cl (1 g · liter^{-1}), and resazurine (0.0002% wt/wt) as a redox potential indicator. Methanol and sodium acetate, $3H_2O$, concentrations were, respectively, 2.4 g · liter^{-1} (75 m*M*) and 1.7 g · liter^{-1} (12.5 m*M*). Immediately before inoculation, cysteine–HCl (0.25 g · liter^{-1}) and Na_2S (0.25 g · liter^{-1}) were added. The potassium hydrogen-carbonate concentration was varied throughout the study.

Medium preparation. Anaerobic medium was prepared by a modification of the Hungate technique [6, 9].

Growth conditions. Growth medium was inoculated with a 48-h culture (5% vol/vol) prepared in the identical medium. Cultures were then incubated at 37°C and continually agitated.

Analytical methods. Culture density was determined by direct spectrophotometric evaluation of the tubes against a water blank at 660 nm. Growth rates were calculated from these measurements. Methanol and acetic and butyric acids were analyzed by gas chromatography after acidification with HCl and inclusion of an internal standard (propionic acid). The column (1 m) was Porapack QS (80–100 mesh), the injection temperature was 250°C, the oven temperature was 190°C, and the detector temperature was 300°C. Gases were analyzed using a Porapack Q (80–100 mesh, 1 m) column with an injection temperature of 70°C and a 40°C oven temperature. Detection was by flame ionization (acids) or catharometer (gases).

Evaluation of partial pressure of CO_2(pCO_2), HCO_3^-, and dissolved CO_2: A relative evaluation of the three forms of CO_2 with pH values close to the neutral was made possible by considering the following equations:

$$CO_2g \rightarrow CO_2d \text{ with } CO_2d = H \cdot pCO_2 \text{ (H is Henry's constant)} \quad (1)$$

$$CO_2d + H_2O \rightarrow H_2CO_3 \text{ with } (H_2CO_3) = CO_2d \cdot 10^{-3} \quad (2)$$

$$H_2CO_3 \rightarrow HCO_3^- + H^+ \text{ with } (HCO_3^-) = (H_2CO_3)/(H^+) \cdot 2.5 \cdot 10^{-4} \quad (3)$$

$$(HCO_3^-) = H \cdot 10^{pH} \cdot 2.5 \cdot 10^{-7} \cdot pCO_2 \quad (4)$$

The medium used here had a Henry's constant estimated to be 52.99 m*M* CO_2 · atm^{-1}.

Results and Discussion

By studying various values of both pH and pCO_2, the relative effects of the three closely linked parameters (pH, dissolved CO_2, and HCO_3^-) were elucidated. For a given pH, an increase in pCO_2 leads to a higher concentration of HCO_3^- within the medium. A pH series prepared with three different partial pressures of CO_2 (100, 200, and 400 mb) was made possible by changing the hydrogen-carbonate concentration in the media. It was possible therefore to obtain maximum growth rates or output rates in relation to the pH with either fixed pCO_2 (concentration of HCO_3^- varied), or with fixed concentrations of hydrogen carbonate ion, in which case the pCO_2 varied. An assessment of the sepa-

Table 1. Growth parameters associated with carbon dioxide metabolism by *Eubacterium limosum* while fermenting methanol–CO_2 mixtures at various initial pH values

pH	$\mu_m^* \cdot h^{-1}$	K_s $\%CO_2$	K_s m*M* HCO_3	K_{is} $\%CO_2$	K_{is} m*M* HCO_3
6.8	0.02	14	12	36	29
7.0	0.04	12	16	28	38
7.2	0.05	9	18	71	250
7.4	0.07	8	27	—	—
7.6	0.05	3	13	42	360

rate parameters was possible by combining and analyzing the various approaches.

Effect upon growth. Figure 1 represents the maximal growth rate relative to pH with three partial pressures of CO_2. The growth was sensitive to the pH, particularly with high pCO_2 values. The bacterium was more resistant to high pH values when the pCO_2 value was low.

The curves enabled variations in the growth optima relative to pH to be visualized for two fixed concentrations of hydrogen carbonate. The influence of pH on growth rates is shown in Fig. 2. Optimum growth rates were obtained at pH 7.4, at which value and with 40% gaseous CO_2 (133 m*M* HCO_3^-) maximum growth rate reached 0.05 h^{-1}. Growth was very sensitive to pH, whether CO_2 was expressed as CO_2 dissolved or hydrogen carbonate, whereas it was less affected by HCO_3^- or pCO_2.

Using Monod's model, the constants of affinity (K_s) and inhibition (K_{is}) of the cells for carbon dioxide were estimated. External conditions being constant, it is possible to postulate:

$$\mu_m = \mu_m^* \cdot \frac{(CO_2)}{K_s + (CO_2)} \cdot \frac{K_{is}}{K_{is} + (CO_2)}$$

where μ_m^* represents the maximum growth rate without taking into consideration the effect of the cosubstrate. A double reciprocal plot of $1/\mu_m$ against $1/(CO_2)$ gave K_s values for each pH value, while K_{is} values were obtained by a plot of $1/\mu_m$ against (CO_2) (see Table 1).

When the affinity constants (K_s) were expressed as dissolved CO_2, they varied according to the pH, while the corresponding values for HCO_3^- were independent of pH and were relatively constant. Growth of *E. limosum* B2 was thus directly affected by the concentration of hydrogen carbonate ions and not by dissolved CO_2. The affinity of *E. limosum* B2 for HCO_3^- was approximately 16 m*M*. High concentrations of hydrogen carbonate affected growth rates, inhibition beginning at 250–330 m*M* HCO_3^-. A possible alternative explanation for this phenomenon that should not be overlooked is the possible inhibitory effects of the associated potassium ion.

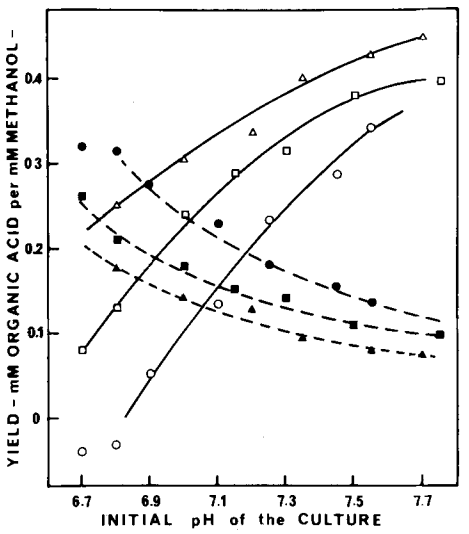

Fig. 3. Relation between yields of organic acids and pH with three partial pressures of CO_2. The open symbols indicate the acetic acid yield and the solid symbols indicate butyric acid yield: ●. 100 mb CO_2; ■, 200 mb CO_2; and ▲, 400 mb CO_2.

Effect upon output. Acetic and butyric acids were the only products to be detected during growth of *E. limosum* on methanol 1/CO_2. An indication of the rates of production of each acid may be obtained by relating the increase in concentration at the end of growth to the methanol consumed. As for growth (see above), production can be related to the pH either with constant pCO_2 (Fig. 3) or for fixed concentrations of HCO_3^- (Fig. 4).

For a given pCO_2 (Fig. 3), an increase in pH, and therefore enhanced HCO_3^- concentration, induced higher production of acetic acid and a corresponding reduction of butyrate production. Likewise with a fixed pH, increases in HCO_3^- made possible by changing the pCO_2 (100, 200, 400 mbar) brought about the same effect. With 10% gaseous CO_2 and pH levels lower than 6.8, i.e., low HCO_3^- concentrations, the acetate present in the medium was partly consumed and was visualized as a negative product of acetic acid. When hydrogen carbonate was kept constant (Fig. 4), acetic acid and butyric acid production was shown to be constant irrespective of pH or CO_2.

Organic acid production during growth on methanol–CO_2 depended on the concentration of

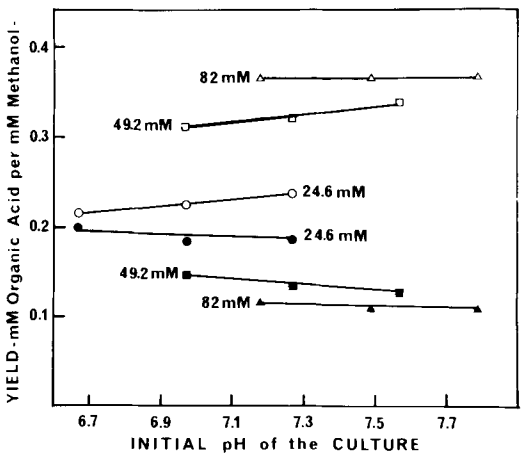

Fig. 4. Effects of pH upon the yields of organic acids for three concentrations of hydrogen carbonate. For symbols, see Fig. 3 legend.

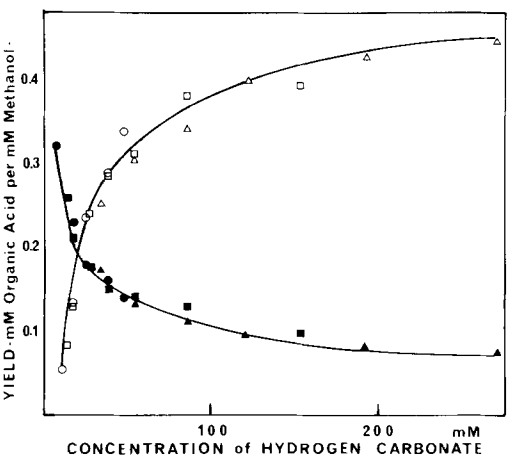

Fig. 5. The effect of hydrogen-carbonate concentration upon the production of acetic and butyric acids. For symbols, see Fig. 3 legend.

hydrogen carbonate in the medium and, all other conditions being equal, was not affected by pH or pCO_2. The acetic acid and butyric acid production can therefore be expressed as a function of the hydrogen-carbonate ion concentration (Fig. 5) and can be seen to vary. Increasing the HCO_3^- lowered butyrate and increased acetate production. With hydrogen-carbonate concentrations of less than 0.5 g · liter^{-1}, consumption of acetate was observed, while under the experimental conditions (75 m*M* methanol, 12.5 m*M* sodium acetate) production was limited by high concentrations of HCO_3^-. Acetic acid was limited to 0.84 g · g^{-1} (0.45 mol · mol^{-1}), while butyric acid was limited to 0.2 g · g^{-1} (0.07 mol · mol^{-1}). Butyrate production relative to acetate production was shown to be linear (Fig. 6); thus, carbon utilization for total acid production related to methanol consumption remained constant with different HCO_3^- concentrations. The value was 1.2 in accordance with the following stoichiometry:

10 methanol + $2CO_2$ →

x butyric acid + y acetic acid

The points of intersection of the line $R_B = f(R_A)$ with the axes (Fig. 6) relate the theoretical production limits for acetic and butyric acids (1.1 g · g^{-1}, 0.59 mol · mol^{-1} and 0.83 g · g^{-1}, 0.3 mol · mol^{-1}, respectively).

Interpretation of the above results based upon the assimilation model proposed by Kerby et al. [5] shows that the condensation of two single-carbon molecules yields acetyl CoA, precursor of both acetic acid and butyric acid. Total acid production for a certain quantity of C_1 compounds will be distributed between acetic acid and butyric acid, the ratio of products being inversely proportional. With low concentrations of HCO_3^- (less than 0.5 g · liter^{-1}) butyric acid was produced exclusively according to the following stoichiometry:

10 methanol + 2 CO_2 → 3 butyric acid

Increased concentrations of HCO_3^- resulted in coproduction of acetic and butyric acids until a maximum acetic acid production of 0.84 g · g^{-1} (see above) was reached corresponding to the following stoichiometry:

10 methanol + 2 CO_2 →

4.5 acetic acid + 0.75 butyric acid

The actual transformation of substrates to acids remains constant although the ratio of acetic acid to butyric acid altered. Carbon dioxide did not directly interfere with the synthesis of organic acid during methylotrophic growth, while its importance in regulating many enzymic reactions is known for some bacteria. It is thought probable that the metabolism of acid production was affected by the hydrogen-carbonate concentration although the mechanism is still unknown.

The importance of reducing equivalents in the metabolism of single-carbon compounds should be examined (Fig. 7). During growth on methanol–CO_2, some of the methanol is oxidized to CO_2, yielding reducing equivalents as $NADH_2$ in order to form acetyl CoA. To regulate the $NADH_2$–NAD^+ balance within the cell, some reducing reactions are necessary. A portion of the $NADH_2$ will be regenerated to NAD^+ during biomass formation, while

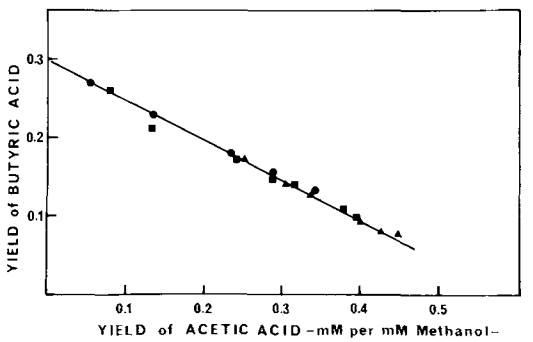

Fig. 6. Relationship between molar yields of butyric and acetic acids. For symbols, see Fig. 3 legend.

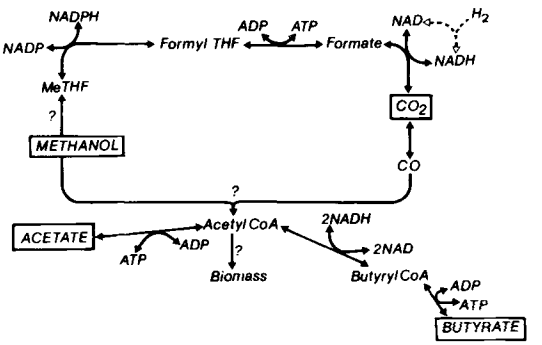

Fig. 7. Schematic representation of the carbon flux during growth of *Eubacterium limosum* on methanol–CO_2.

the other major metabolic sequence using $NADH_2$ during methylotrophic growth of *E. limosum* will be the synthesis of butyric acid. Production of acetic acid, while high in ATP production, does not involve the use of reducing equivalents. It is possible that HCO_3^- affects the ratio of acids produced by interfering with the efficiency of $NADH_2$ oxidation. Slower rates of growth may reflect less rapid utilization of the carbon sources and hence slower production of $NADH_2$. This may enable the organism to switch its production of overflow metabolites toward acetic acid with a resultant gain in ATP production.

Acetobacterium woodii growing on methanol–CO_2 produces only acetic acid, utilizing 2 methanol/CO_2. Only during growth on CO_2–H_2 does *E. limosum* produce only acetic acid and in this instance the necessity for butyric acid production is negated as formation of acetyl CoA does not yield $NADH_2$. Indeed, $NADH_2$ is consumed and thought to be regenerated via the action of hydrogenase. During methylotrophic growth on methanol–CO_2, a change in the amount of methanol oxidized via THF intermediates to CO_2 would affect the metabolite pool status. If high concentrations of HCO_3^- repress this sequence and acetyl CoA formation involves direct incorporation of CO_2 from the medium, a logical consequence would be a lowering of the butyric acid production, since $NADH_2$ reoxidation would no longer be as important. This interpretation does, however, imply a change in methanol–CO_2 consumption ratio from 5 : 1 to 4 : 1, 3 : 1, or 2 : 1, i.e., an increase in organic acid production relative to methanol consumption of 1.2 to 1.25, 1.33, or 1.5.

Experiments here do not support this stoichiometry variation and indicate a constant carbon output value of 1.2 regardless of HCO_3^- concentration.

Since the stoichiometry of acetyl CoA formation remains constant, the amount of $NADH_2$ would also remain constant. Any influence of HCO_3^- in promoting acetic acid production would therefore lead to an imbalance in the $NADH_2$–NAD^+ pool. Regeneration of NAD^+ essential for continued methanol utilization must be a result of an alternative regeneration process. One possible explanation is that the organism simply wastes its reducing equivalents by releasing hydrogen when growth conditions such as high hydrogen-carbonate ion concentration promote a diminished butyric acid synthesis. The balance of electrons during growth of anaerobic methylotrophs will need further investigation before the true role of factors such as HCO_3^- can be elucidated.

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