

# Equations and Calculations for Fermentations of Butyric Acid Bacteria

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A stoichiometric equation has been derived which describes the interrelations among the various products and biomass in fermentations of butyric acid bacteria. The derivation of the equation is based on an assumed ATP yield, two biological regularities, and the biochemistry of product formation of the fermentations. The equation obeys the constraints imposed on growth and product formation by thermodynamics and the biochemical topology. The validity of the equation is tested using a variety of fermentation data from the literature. The uses, improvements, limitations, and extensions of the equation are also discussed in detail. For example, the fermentation equation is used to calculate the maximal possible yields of the main fermentation products.

## INTRODUCTION

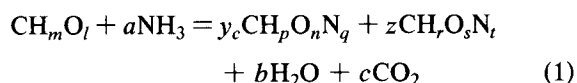
Despite the currently falling prices and plentiful supplies of crude oil, there is considerable evidence to suggest that renewable resources (mono- and oligosaccharides from biomass hydrolysis or from waste streams of manufacturing activities) will provide a significant percentage of the chemical industry's feedstocks in the future.<sup>1-4</sup> Ethanol, acetic acid, lactic acid, butanol, acetone, butanediol, and isopropanol are some of the main feedstocks which can be produced by saccharolytic fermentations.<sup>1-3</sup> Which, how, and when any of the above or other chemicals is or will be produced economically by fermentation depends upon a sequence of complex socioeconomical and technological factors,<sup>2</sup> which change drastically with geography and time. Very conservatively, we can say that these fermentation processes appear in principle as attractive alternatives to current petrochemical processes and therefore deserve some decent investment for technological improvements. The latter means that fermentations must be switched from batch to continuous or semi-continuous, reactor configurations and instrumentation should be advanced, product yields and tolerances of microorganisms should be improved by recombinant DNA or classical genetic techniques, and more efficient separation processes must be employed. If the case of ethanol fermentation can be a guiding example, even modest research and development investments can bring about significant technological and economical improvements.

Saccharolytic clostridia grow anaerobically on a variety of substrates, can produce a large number of useful products,<sup>5-7</sup> and thus appear to be very promising bacteria for production of organic chemicals from mono-, oligo-, and polysaccharides. Butyric acid bacteria (clostridia) in particular, can anaerobically ferment a variety of sugars (hexoses, pentoses, and oligosaccharides)<sup>5,8,9</sup> to produce a variety of organic solvents (butanol, acetone, ethanol, isopropanol, acetoin), carboxylic acids (acetic, butyric, lactic, and formic acids) and hydrogen.<sup>5,6,10-13</sup>

The economic feasibility of any such saccharolytic fermentation will be determined by the maximal possible: 1) conversion of the sugar(s), 2) product yield and selectivity, 3) reactor productivity, and 4) product separation and purification efficiency. Ethanol and acetic acid can be produced with an equal or better biochemical efficiency by yeast or other bacteria fermentations, as sole products;<sup>5-7</sup> overall, thus, they can be produced more efficiently by those other fermentations than by the mixed-product fermentations of butyric acid bacteria. On the other hand, butanol has a significantly higher market price per mole than acetone, butyric acid, isopropanol, or acetoin. Therefore, economically, the most attractive fermentations of butyric acid bacteria are those with the highest selectivity for butanol, which thus optimize substrate utilization and reduce separation costs.<sup>14,15</sup> The ideal fermentation would then be the one that could convert the sugar units to butanol to the highest allowable degree, and thus produce none or very little of the other fermentation products. The maximal allowable butanol (or any other product) yield is determined by both thermodynamic constraints and the biochemical topology. The establishment of the thermodynamic and biochemical constraints which determine the theoretically highest yield for each product and the calculation of these maximal yields would be of both fundamental and practical importance. They would allow us to establish rationally the upper bounds for the productivity of the fermentations, which in turn can be used as a guide in feasibility studies, and experimentation for genetic and bioreactor-productivity improvements. Similarly, it would be desirable to establish any possible interrelations among the amounts and the rates of appearance

or disappearance of the various fermentation products, biomass and substrate. This would allow us to check the consistency of the experimental data and possibly identify sources of experimental error.

With current technology, butanol-acetone fermentations operate in the batch mode, with the solvents produced toward the end of the rather long and thus inefficient process.<sup>14,15</sup> It is conceivable and desirable however, that the fermentations can be operated continuously or semicontinuously<sup>15-21</sup> for improved reactor productivity. Because of the complex (and largely unelucidated) nature of solvent production,<sup>15</sup> reactor operation policies for optimal product yields would be conceivable only with the help of a highly instrumented, computer-controlled fermentation system.<sup>15</sup> A major problem in the employment and usefulness of the computer controlled fermentation systems is the on-line measurement of the fermentation parameters, for lack of fast and accurate sensors.<sup>22,23</sup> This is a particularly acute problem in multiproduct fermentations. A possible resolution of the problem is the use of "gateway sensors,"<sup>22,24</sup> whereby certain significant fermentation parameters (e.g., biomass and product concentrations) can be extracted from a combination of available sensors. Measurable quantities which are frequently used for correlation purposes are the concentrations of the various gases (O<sub>2</sub>, CO<sub>2</sub>, CH<sub>4</sub>, etc.) in the feed and exit streams, biomass, rates of nutrient addition, and redox potential.<sup>22</sup> The rest of the fermentation parameters for process control must be then extracted from overall stoichiometric equations representing the fermentation processes.<sup>22</sup> The coefficients of the various species of this stoichiometric equation are related through equations representing carbon and nitrogen elemental balances. For an anaerobic fermentation, for example, a stoichiometric balance equation for carbon, hydrogen, oxygen, and nitrogen can be written in the form,



where CH<sub>m</sub>O<sub>l</sub>, CH<sub>p</sub>O<sub>n</sub>N<sub>q</sub>, and CH<sub>r</sub>O<sub>s</sub>N<sub>t</sub> denote the elemental compositions of the organic substrate(s), microbial biomass, and extracellular products, respectively. The values of subscripts *m*, *l*, *r*, *s*, and *t* are known from the molecular formulae of the organic substrate and products. The values of *p*, *n*, and *q* can be found from the elemental analysis of the biomass. Elemental carbon and nitrogen balances for eq. (1) then yield, respectively,

$$y_c + z + c = 1 \quad (2)$$

and

$$y_c q + z t = a \quad (3)$$

Because of the presence of the water in the right-hand side of eq. (1), the production rates of which cannot be typically measured in fermentation systems, elemental balances for oxygen or hydrogen do not provide any additional information. However, additional information can be derived

from what has been known for quite some time as an *oxidation reduction (O/R balance)*<sup>5,6,25</sup> or balance of the *number of available hydrogens*<sup>6</sup> or more recently as an *available electron balance*.<sup>26-28</sup> The three balances are equivalent to each other. In the oxidation reduction balance, the O/R value of formaldehyde and multiples thereof are arbitrarily taken to be zero. Each H in excess contributes  $-0.5$  to the O/R value of the "compound"; a lack of H contributes  $+0.5$  and each excess N contributes  $+1.5$  to the O/R value of the "compound." Units of CH<sub>2</sub>O and H<sub>2</sub>O can be added or subtracted from the "compound" for convenience. Thus, the O/R values of CH<sub>m</sub>O<sub>l</sub>, CH<sub>p</sub>O<sub>n</sub>N<sub>q</sub>, CH<sub>r</sub>O<sub>s</sub>N<sub>t</sub>, and CO<sub>2</sub> are  $(l - 0.5m)$ ,  $(n + 1.5q - 0.5p)$ ,  $(s + 1.5t - 0.5r)$ , and 2, respectively. The O/R values of NH<sub>3</sub> and H<sub>2</sub> are zero and the O/R balance for eq. (1) yields (after multiplication by two):

$$2l - m = y_c(2n + 3q - p) + z(2s + 3t - r) + 4c \quad (4)$$

The number of available hydrogens is obtained<sup>6</sup> from oxidation of the "compound" to CO<sub>2</sub> and NH<sub>3</sub> with water (CH<sub>m</sub>O<sub>l</sub> + (2 - *l*) H<sub>2</sub>O → (4 + *m* - 2*l*) H + CO<sub>2</sub>; CH<sub>p</sub>O<sub>n</sub>N<sub>q</sub> + (2 - *s*) H<sub>2</sub>O → (4 + *r* - 2*s* - 3*t*) H + CO<sub>2</sub> + *t*NH<sub>3</sub>). Thus, the balance of the number of available hydrogens for eq. (1) yields,

$$4 + m - 2l = y_c(4 + p - 2n - 3q) + z(4 + r - 2s - 3t) \quad (5)$$

The available electrons of a "compound" are the electrons that would be transferred to oxygen upon oxidation of the "compound" to CO<sub>2</sub>, H<sub>2</sub>O, and NH<sub>3</sub>. The balance of available electrons is most conveniently written in terms of the degrees of reductance ( $\gamma$ ) of the compounds, defined as the number of equivalents of available electrons per atom of carbon in the "compound."<sup>26,27</sup> Thus, the reductance degrees of CH<sub>m</sub>O<sub>l</sub>, CH<sub>p</sub>O<sub>n</sub>N<sub>q</sub>, and CH<sub>r</sub>O<sub>s</sub>N<sub>t</sub> are  $\gamma_s = 4 + m - 2l$ ,  $\gamma_b = 4 + p - 2n - 3q$ , and  $\gamma_p = 4 + r - 2s - 3t$ , respectively. The available electron balance for eq. (1) can then be written as,

$$\gamma_s = y_c \gamma_b + z \gamma_p \quad (6)$$

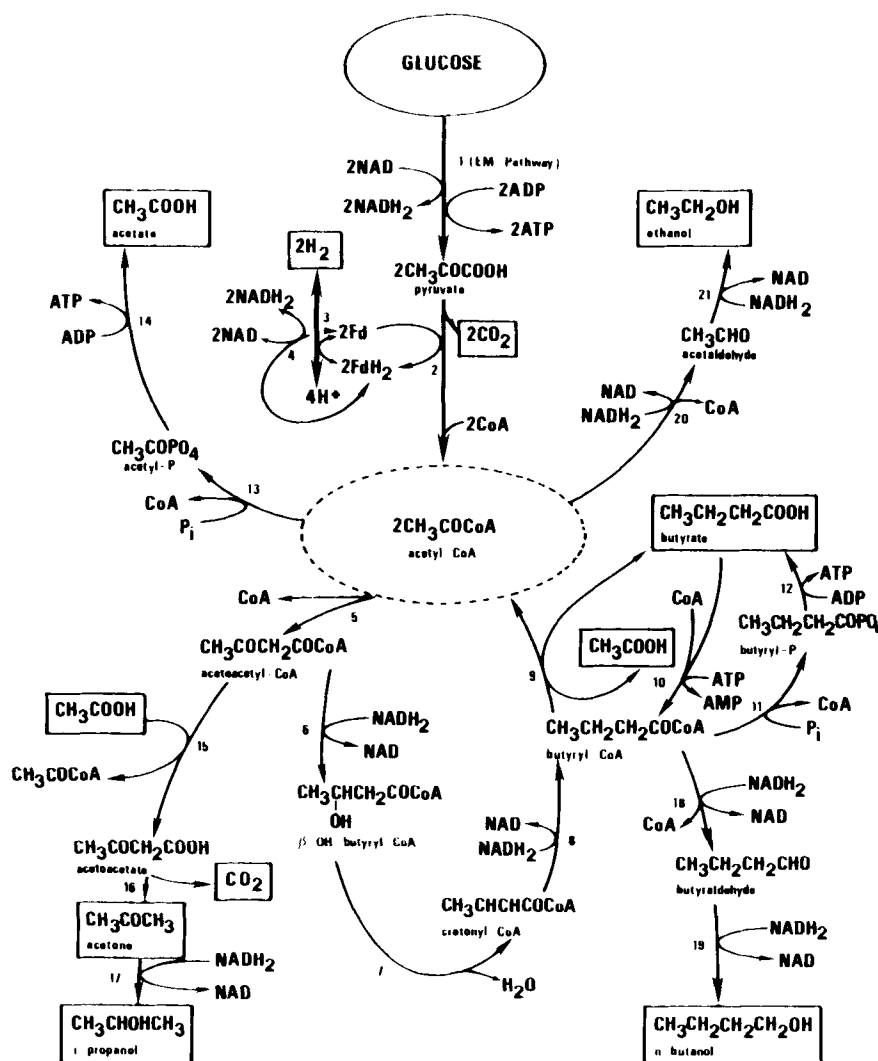
which is obviously the same as eq. (5). Equation (4) also reduces to eq. (5) using eq. (2) and, thus, the three balances are equivalent. So long as the elemental formulae of biomass and products of eq. (1) are based on organic carbon, hydrogen, oxygen, and nitrogen, eq. (5) is rigorously and not merely approximately valid. In summary, eq. (5) provides additional information for the fermentation system and can be used in conjunction with the carbon and nitrogen balances, eqs. (2) and (3), as "gateway sensors." However, no information is provided by the above three equations regarding the interrelations among the various products in multiproduct fermentations like those of butyric acid bacteria. Such additional information can possibly only be provided by the biochemical topology of the particular fermentation. The objective of this article is to derive, for fermentations of butyric acid bacteria, an equation (we will deliberately call it a "fermentation equa-

tion"), which will incorporate all this additional information in a simple and convenient form. First, we shall review the biochemistry of butyric acid fermentations on which the fermentation equation will be based. The equation will then be derived and its validity tested using a variety of literature data. Finally, uses, limitations and extensions of the equation will be discussed.

## BIOCHEMISTRY OF FERMENTATIONS OF BUTYRIC ACID BACTERIA

Figure 1 shows the sequence of biochemical reactions with their enzymes which lead to the production of sol-

vents, carboxylic acids and hydrogen from glucose by butyric acid bacteria. The figure has been drawn on the basis of the most recent available information in the literature.<sup>5,6,12,14,15,29-41</sup> Compounds in boxes in Figure 1 indicate possible extracellular products. It should be noted that the reactions shown in Figure 1 do not occur in all butyric acid bacteria. For example, a number of butyric acid bacteria do not possess an isopropanol dehydrogenase (enzyme 17 in Fig. 1) and, thus, acetone is typically a final product rather than isopropanol. The two possible biochemical routes of acetoin production from pyruvate are not shown in Figure 1, but they seem to be quite well established and understood.<sup>5,6</sup> The production of formate



**Figure 1.** Biochemical pathways of glucose fermentation by butyric acid bacteria. The pathways or enzyme systems catalyzing the reactions shown are as follows: (1) phosphoenolpyruvate phosphotransferase system and the Embden-Meyerhof pathway, (2) pyruvate-ferredoxin oxidoreductase, (3) hydrogenase, (4) NADH-ferredoxin oxidoreductase, (5) acetyl-CoA-acetyl transferase, (6) L(+)- $\beta$ -hydroxybutyryl-CoA dehydrogenase, (7) L-3-hydroxyacyl-CoA hydrolase, (8) butyryl-CoA dehydrogenase, (9) CoA transferase, (10) butyryl-CoA synthetase (ATP/ AMP), (11) phosphotransbutyrylase, (12) butyrate kinase, (13) phosphotransacetylase, (14) acetate kinase, (15) CoA transferase, (16) acetoacetate decarboxylase, (17) isopropanol dehydrogenase, (18) butyraldehyde dehydrogenase, (19) butanol dehydrogenase, (20) acetaldehyde dehydrogenase, and (21) ethanol dehydrogenase.

from pyruvate<sup>5,6,42</sup> or the incorporation of carbon dioxide into formate and eventually acetate<sup>5,6,42</sup> are not shown either in Figure 1, as we see very little need for that in our present calculations. Most complete fermentation data (for butyric acid bacteria) in the literature<sup>12</sup> show very little formate as an extracellular product, while there appears to be very little carbon dioxide incorporation into formate in fermentations of butyric acid bacteria.<sup>5,42</sup> Incorporation, however, of this information into the fermentation equation is certainly possible, as we shall subsequently discuss.

The CoA-transferase reactions (9) and (15) of Figure 1 have not been unambiguously established in butyric acid bacteria, although they play a very important role in the biochemistry and regulation of solvent production.<sup>33</sup> There is no doubt, yet, that their overall energetics (namely the requirements or production of ATP and/or reduction energy) will be as shown in Figure 1. Similarly, there is no evidence whatsoever that enzymatic reaction (10) of Figure 1 operates in butyric acid bacteria, although it is a distinct possibility. These ambiguities, in any case, do not affect significantly either the derivation of the fermentation equation or the calculations as we shall see shortly. The rest of the enzymatic reactions of Figure 1 have been qualitatively established in many bacteria of interest, although the extent of their operation is not known.

It is well known that all enzymatic reactions can operate in either of their two directions under proper conditions. Most of them however operate *in vivo* in one direction in most situations. For our specific case, for example, it is entirely unlikely that the sequence of reactions (15), (16), and (17) of Figure 1 will operate in reverse direction to produce acetoacetyl-CoA from isopropanol under normal fermentation conditions. Other reactions however can operate in reverse direction at different stages of a single fermentation. Such reactions are clearly indicated in Figure 1 using double arrows. The most interesting of these reactions are the NADH<sub>2</sub>-ferredoxin oxidoreductase reaction (4) and the hydrogenase reaction (3) of Figure 1. Both reactions can be readily reversed,<sup>30,34</sup> making possible, for example, the production of NADH<sub>2</sub> from H<sub>2</sub>, and vice versa. The Gibbs free-energy of H<sub>2</sub> formation is provided by the oxidation of FdH<sub>2</sub> to Fd.

## DERIVATION OF A FERMENTATION EQUATION

Since the landmark contribution of Bauchop and Elsdon,<sup>43</sup> it has become evident<sup>44-46</sup> that the ATP yield of a catabolic reaction rather than the free-energy change is an accurate index of the amount of biologically useful energy that is produced in that reaction. It has been widely confirmed indeed,<sup>44,45,47</sup> that the amount of cell material that can be produced from an *available* amount of ATP is a constant for every microorganism. This led to the useful concept of the ATP yield,  $Y_{ATP}$  (g dry biomass produced/mol ATP utilized). Since it is possible to estimate the amount of ATP produced by catabolic processes and to

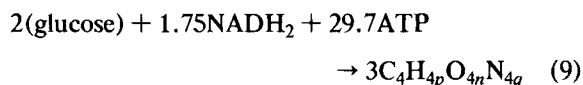
measure the biomass yield, values of  $Y_{ATP}$  have been calculated for a large variety of microorganisms.<sup>44,45,47</sup> These calculations have found a mean  $Y_{ATP}$  value of 10.5 g/mol for microorganisms growing on glucose. Different values have been found for other substrates. These values seem to indicate that the energy derived from catabolic processes is used with approximately the same efficiency for cell material biosynthesis by a wide variety of microorganisms. Reducing energy (in the form of NADH<sub>2</sub>, NADPH<sub>2</sub>, or other reduced coenzymes) is another form of biological energy which must be balanced between reactions that produce it and reactions that require it.<sup>48-50</sup> Reducing energy can be converted to ATP by either oxidative or substrate level phosphorylation. In a number of instances however, the supply of reducing energy rather than ATP may be limiting the fermentation.<sup>49,50</sup> Thus, balances of both ATP and reducing energy appear necessary if the yields of biomass and of the various fermentation products are to be assessed for a specific fermentation of a given substrate. Calculations based on such balances have proved useful in cases of bacterial growth where biomass and CO<sub>2</sub> are almost exclusively produced.<sup>48-50</sup> Naturally, these calculations require a fairly accurate knowledge of the biochemistry of carbon assimilation for the particular substrate(s). As we have discussed in the preceding section, the qualitative biochemistry of butyric acid bacteria is fairly well established. It would be possible then to calculate yields for the various fermentation products and to study their interrelations by employing ATP, NADH<sub>2</sub>, FdH<sub>2</sub>, and key intermediate balances in addition to the carbon, nitrogen and available electron balances. This is the objective of the following development.

We will let  $C_4H_{4p}O_{4n}N_{4q}$  represent the elemental composition of microbial biomass. In the absence of any specific knowledge on this elemental composition and for generality, we shall use two well accepted regularities from studies of biomass composition with a large variety of microorganisms. These regularities state that the weight fraction of carbon in the biomass,  $\sigma_b$ , and the reductance degree of biomass,  $\gamma_b$ , are relatively constant with coefficients of variation 5 and 4%, respectively,<sup>26,51</sup> namely:

$$\sigma_b = 0.462 \pm 0.023 \quad (7)$$

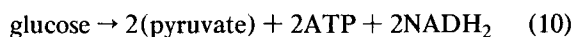
$$\gamma_b = 4 + p - 2n - 3q = 4.291 \pm 0.172 \quad (8)$$

The ATP needs of biomass synthesis can be estimated from the appropriate  $Y_{ATP}$ , which is defined as the amount of dry biomass produced in grams per mole ATP used in biosynthesis. Although the maximal  $Y_{ATP}$  for growth on glucose has been calculated to be 28.8,<sup>46</sup> the actual  $Y_{ATP}$  for most bacteria is widely accepted to be 10.5 g/mol,<sup>44,45</sup> from which and using eq. (7) we calculate that 29.7 mol of ATP are required for biomass synthesis for every 2 mol of glucose incorporated into biomass. Using eq. (8) next, we find that 1.75 mol of NADH<sub>2</sub> are needed for every 2 mol of glucose incorporated into biomass, to bring glucose to the oxidation level of  $C_4H_{4p}O_{4n}N_{4q}$ . Thus, biomass synthesis from glucose can be represented by the overall scheme:

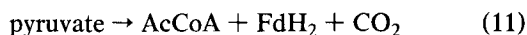


For simplicity and generality we have omitted the nitrogen source in the left-hand side of eq. (9). For simplicity we have also omitted the oxidized form of NADH<sub>2</sub> and ADP in eq. (9), and have made no attempt at this stage to balance the oxygen and hydrogen atoms. Any deviations of the actual  $Y_{\text{ATP}}$  and chemical formula of cell material from those used here will have only a small effect on the final calculations regarding the production of solvents, carboxylic acids, and hydrogen. This is because less than 10% of the substrate carbon is typically converted into biomass in actual clostridia fermentations, and also, as we shall see below, these fermentations do not appear to be limited by the ATP supply.

Next, we set out to write equations which represent single reactions or unique strings of reactions depicted in Figure 1, toward the production of final fermentation products. Again, these equations are only balanced for carbon, ATP, and reducing energy (NADH<sub>2</sub>, FdH<sub>2</sub>). The free form of coenzyme A (CoA) and the oxidized forms of NADH<sub>2</sub> and FdH<sub>2</sub> are not shown in these equations either. For the production of pyruvate from glucose by the phosphoenolpyruvate phosphotransferase system and the Embden-Meyerhof pathway (reaction string 1 in Fig. 1), we write



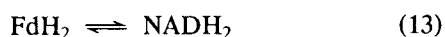
For the production of acetyl-CoA (AcCoA) from pyruvate [reaction (2) in Fig. 1], we can write



For the readily reversible<sup>34</sup> production of H<sub>2</sub> through FdH<sub>2</sub> [reaction (3) in Fig. 1], we can write,



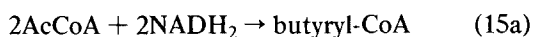
The NAD reduction by FdH<sub>2</sub> and the readily operating reverse reaction<sup>30</sup> [reaction (4) in Fig. 1] can be represented by



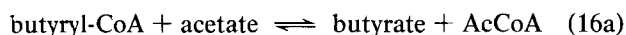
The production of acetate from acetyl-CoA through the phosphotransacetylase and acetate kinase [reactions (13) and (14) in Fig. 1] can be represented by



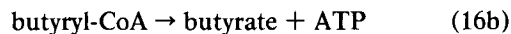
The formation of butyryl-CoA through the string of reactions (5)–(8) in Figure 1 can be represented by



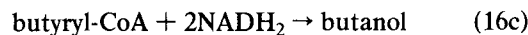
Reversible reaction (9) in Figure 1 for butyrate production from butyryl-CoA and acetate can be represented by



If butyrate production from butyryl-CoA proceeds via phosphotransbutyrylase and butyrate kinase [reactions (11) and (12) in Fig. 1], we should write



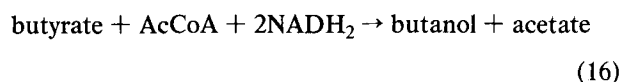
which, in view of eq. (14), is equivalent to eq. (16a). Note that although reaction (10) in Figure 1 has not been definitely demonstrated in butyric-acid *Clostridia*, it can still be represented (for the present calculations) by eq. (16a) in view of eq. (14). The *n*-butanol production from butyryl-CoA catalyzed by the two dehydrogenases [reactions (18) and (19) in Fig. 1] can be represented by



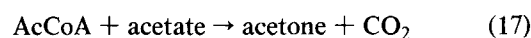
In view of the reversibility of the reaction represented by eq. (16a), butyryl-CoA can be eliminated from eqs. (15a), (16a), and (16c) to yield



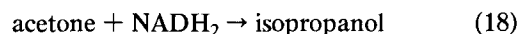
and



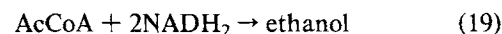
Equation (16) should not be interpreted to imply that butanol is necessarily produced through butyrate. Acetone production through the string of reactions (5), (15), and (16) in Figure 1 can be represented by



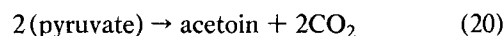
Isopropanol production from acetone [reaction (17) in Fig. 1] can be represented by



Ethanol production from acetyl-CoA [reactions (20) and (21) in Fig. 1] can be represented by



Acetoin production, although not specifically studied in butyric acid clostridia, appears to proceed by two possible mechanisms (which have been demonstrated in other bacteria):<sup>5,6</sup> through acetolactate, summarized by the reaction



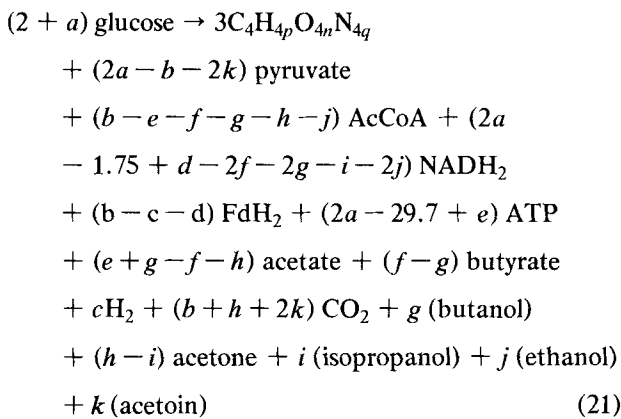
or through diacetyl, which can be represented by



In view of eqs. (11) and (13), however, eqs. (20) and (20a) are equivalent; we will use eq. (20).

Before we proceed further, some comments on the preceding equations are in order. A careful look at the reaction scheme of Figure 1 should reveal that these fermentations produce an excessive amount of ATP through glycolysis and acetate production, while they use none for production of solvents or butyrate. Some of the ATP will be used for biomass formation [eq. (9)], but since typically there is relatively little biomass produced, most of it will be hydrolyzed, for no other reason perhaps but to regenerate ADP for the glycolysis pathway. This implies that a very active ATPase system should be operating during both the growth and solvent production phases. Unless some ATP is necessary for the transport into the cells of some early fermenta-

tion products (carboxylic acids), ATP hydrolysis would appear then as an utter but necessary energy waste. In writing all the equations above, we have used  $\text{NADH}_2$  and  $\text{FdH}_2$  as the only forms of reduction energy generation and requirements. It is well known yet, that some of the reactions of Figure 1<sup>5,6,30,32</sup> and biomass synthesis require  $\text{NADPH}_2$ . It has been calculated, however, that  $\text{NADH}_2$  conversion to  $\text{NADPH}_2$  by transhydrogenases requires very little or no ATP energy in bacteria.<sup>46</sup> Also, in view of the surplus ATP production discussed above, the simplification of using only  $\text{NADH}_2$  appears well justified. Finally, note that although some  $\text{CO}_2$  may be produced through general decarboxylation enzymes not shown in Figure 1, and although some  $\text{CO}_2$  may be fixed into biomass and/or fermentation products,<sup>42</sup> lack of specific information and experimental data make it impossible or unnecessary for us to incorporate these reactions in our present development. Our own calculations, however, have estimated these contributions to be insignificant under normal fermentation conditions; comparisons of experimental data with the results of present calculations, as are reported below, also confirm that these contributions are not usually significant. The overall fermentation equation is now derived by multiplying eqs. (9)–(20) by 1,  $a$ ,  $b$ ,  $c$ ,  $d$ ,  $e$ ,  $f$ ,  $g$ ,  $h$ ,  $i$ ,  $j$ , and  $k$ , respectively, and adding the resulting equations to obtain:



Since AcCoA,  $\text{NADH}_2$ ,  $\text{FdH}_2$  and, in most cases, pyruvate do not accumulate in the fermentation broth, their coefficients in eq. (21) should be set equal to zero, yielding thus the following four equations:

$$2a - b - 2k = 0 \quad (22)$$

$$b - e - f - g - h - j = 0 \quad (23)$$

$$2a - 1.75 + d - 2f - 2g - i - 2j = 0 \quad (24)$$

$$b - c - d = 0 \quad (25)$$

Strictly speaking, eqs. (22)–(24) should be valid during continuous steady-state fermentations, during balanced fermentations, at the end of most batch fermentations and whenever the pseudo-steady-state hypothesis for the corresponding four chemical species can be invoked. The latter is equivalent to stating that the rate of accumulation of the above four species in the fermentor (aqueous phase

and biomass phase) is very small or almost zero compared to the *rate of growth* or the *rates of production and uptake* of the four species. Although the concentrations of the four species may vary with time during the various phases of a (transient) batch fermentation, for example, simple calculations would nevertheless confirm that their rate of accumulation is indeed very small compared to the rate of growth or their rates of production or uptake.

From all the calculations we have performed for fermentations of butyric acid bacteria, we have found that more (very often much more) ATP is produced by glycolysis and acetate formation than is necessary for biomass synthesis. Thus, the coefficient of ATP in eq. (21) cannot be put equal to zero. In fact,  $y = 2a - 29.7 + e$  will give the amount of ATP produced in excess, which must be hydrolyzed for ADP regeneration, as we have said earlier. Thus, the rest of the coefficients of the right-hand side of eq. (21) should be greater than or equal to zero, i.e.,

$$2a - 29.7 + e \geq 0 \quad (26)$$

$$e + g - f - h \geq 0 \quad (27)$$

$$f - g \geq 0 \quad (28)$$

$$b + h + 2k \geq 0 \quad (29)$$

$$h - i \geq 0 \quad (30)$$

$$c \geq 0 \quad (31)$$

$$g \geq 0 \quad (32)$$

$$i \geq 0 \quad (33)$$

$$j \geq 0 \quad (34)$$

$$k \geq 0 \quad (35)$$

In addition, and by the derivation of eq. (21), we must have that

$$a > 0 \quad (36)$$

$$b > 0 \quad (37)$$

and

$$e \geq 0 \quad (38)$$

From eqs. (28), (30), (32), and (33) we also have that  $f \geq 0$  and  $h \geq 0$ . Finally, note that because of the reversibility of the reaction of eq. (13),  $d$  can be positive, negative, or zero, corresponding to production of  $\text{NADH}_2$  from  $\text{FdH}_2$ ,  $\text{FdH}_2$  from  $\text{NADH}_2$ , or no production of  $\text{NADH}_2$  from  $\text{FdH}_2$ , respectively. Thus, the coefficients of eq. (21) are related through four equality conditions [eqs. (22)–(25)] and thirteen inequality conditions [inequalities (26)–(38)]. Equation (21), whose coefficients will be understood from now on to satisfy conditions (22)–(38), is what we shall call the fermentation equation for butyric acid bacteria. Next we show that eqs. (22)–(25) contain both the carbon balance and the available electron balance equations. The carbon balance for eq. (21) (with no accumulation of pyruvate, AcCoA,  $\text{NADH}_2$ ,  $\text{FdH}_2$ , or ATP in the fermentation broth) is given by

$$6(2 + a) = 12 + 2(e + g - f - h) + 4(f - g) \\ + b + h + 2k + 4g + 3(h - i) \\ + 3i + 2j + 4k$$

which, after simplification, gives

$$6a = 2(e + g + f + h + j + 3k) + b \quad (39)$$

Equation (39) can be obtained by multiplying eq. (22) by three and adding it to eq. (23) multiplied by a factor of 2. Similarly, the available electron balance for eq. (21) and using eq. (8) reduces to

$$12a = 1.75 + 4(e + f + h) + 6(g + j) + 10k + i + c \quad (40)$$

which can be obtained by multiplying eqs. (22) and (23) by factors of 10 and 4, respectively, and adding the resulting equations to eq. (24) and then to eq. (25). When an appropriate nitrogen source is introduced to the left-hand side of eq. (21), a nitrogen balance equation can be added to the set of eqs. (22)–(25). Thus, this latter set provides *two more equations* (to be used as “gateway sensors,” for example) than the carbon, nitrogen and available electron balances do.

## CALCULATIONS

### Validity of Fermentation Equation

A major problem is evaluating the validity of eq. (21) in the absence of recent, complete fermentation data. The best complete fermentation data for butyric acid bacteria date back to 1930–1956.<sup>12</sup> As such, some of the employed experimental and analytical methods may render some of the data suspicious. Unfortunately, to the best of our knowledge, all more recent data are incomplete. Smaller components of the fermentation products and gas data are typically missing or are obviously inconsistent. We therefore, had to rely on older data to check the validity of eq. (21). For each particular *Clostridium* fermentation, we set equal to zero all the coefficients of the products not produced, and use the data on the mathematically smallest possible number of products to calculate the rest of fermentation products. Results are shown on Tables I–VII. The values marked by an *a* are those used for the calculations. Carbon recovery was often recalculated to be consistent with the rest of the reported data. The rest of the carbon was assumed to have been incorporated into biomass according to eq. (9). For each fermentation then, we can write a specific equation with explicit numerical coefficients, where all C, N, O, and H atoms can be balanced by the addition of the correct number of NH<sub>3</sub> and H<sub>2</sub>O moles. This is left to the reader.

An easy test of data consistency is the CO<sub>2</sub> test. The numbers of moles of CO<sub>2</sub> produced per mole of the various fermentation products are well established and accepted to be those shown in Figure 1. For example, 2 mol CO<sub>2</sub> are produced per 1 mol butanol, 3 mol CO<sub>2</sub>/mol acetone, and

**Table I.** Fermentation of glucose with *C. acetobutylicum*; Data are from van der Lek.<sup>10</sup>

	Amount (mol/100 mol glucose fermented)				
	Experimental	Calculated			
Butyrate	4.3	4.3 <sup>a</sup>	3.6	4.3 <sup>a</sup>	4.3 <sup>a</sup>
Acetate	14	12.5	14 <sup>a</sup>	16.5	14 <sup>a</sup>
H <sub>2</sub>	139	136	137	139 <sup>a</sup>	139 <sup>a</sup>
CO <sub>2</sub>	221	222	222	221 <sup>a</sup>	222
Butanol	56	56 <sup>a</sup>	56 <sup>a</sup>	55.2	55.2
Acetone	22.4	22.4 <sup>a</sup>	22.4 <sup>a</sup>	21.2	22.5
Ethanol	9.3	9.3 <sup>a</sup>	9.3 <sup>a</sup>	9.3 <sup>a</sup>	9.3 <sup>a</sup>
Acetoin	6.3	6.3 <sup>a</sup>	6.3 <sup>a</sup>	6.3 <sup>a</sup>	6.3 <sup>a</sup>
Isopropanol	0	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Carbon recovery (as glucose)	100.2	99.9 <sup>a</sup>	99.9 <sup>a</sup>	99.9 <sup>a</sup>	99.9 <sup>a</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	51	50	48	48
Excess ATP	—	238	238	240	239
Apparent Y <sub>ATP</sub>	—	0.06	0.06	0.06	0.06
CO <sub>2</sub> consistency test	224/221	—	—	—	—
Δγ [eq. (41)]	−16	—	—	—	—
H <sub>2</sub> /CO <sub>2</sub>	0.63	0.61	0.62	0.63	0.63

<sup>a</sup>Data used in calculations.

1 mol CO<sub>2</sub>/mol acetate. The CO<sub>2</sub> test consists of checking how close to unity is the ratio of moles of CO<sub>2</sub> calculated from the data on the rest of the fermentation products over the experimental moles of CO<sub>2</sub>. Tables I–VII also show how much FdH<sub>2</sub> is produced from NADH<sub>2</sub> or vice versa by treating the experimental data according to eq. (21). As we mentioned earlier, a positive value for *d*, which results in a positive entry in Tables I–VII, indicates a net production of NADH<sub>2</sub> from FdH<sub>2</sub>, while a negative value for *d*, and thus a negative entry in Tables I–VII, indicates a net production of FdH<sub>2</sub> from NADH<sub>2</sub>. Despite the higher negative standard redox potential of ferredoxin, small changes in the reaction conditions can readily reverse the electron flow to have Fd reduction by NADH<sub>2</sub>, as has been shown experimentally.<sup>30</sup>

We have also mentioned above that in all of our calculations the coefficient of ATP in eq. (21) comes out to be always positive, indicating an amount of ATP in excess of what we think is necessary for the fermentations. Obviously, this ATP amount is either used for an unknown to us cellular function, or it is hydrolyzed without performing any cellular function. This ATP amount, called “excess ATP” for lack of a better term, is also shown in Tables I–VII. The phenomenon of excessive ATP production by glycolysis is not new and, in connection with yeast fermentation, had even engaged Meyerhof’s curiosity.<sup>53</sup> The ATP yield calculated on the basis of total ATP produced, called apparent ATP yield here, is a measure of how much excess ATP is produced in the fermentation and is shown in Tables I–VII, as well. Finally, the consistency of the experimental data can be checked on the basis of the available electron balance alone, by calculating the difference of the degrees of reductance between the sub-

**Table II.** Fermentation of glucose with *C. acetobutylicum*; Data are from van der Lek.<sup>10</sup>

Column	Amount (mol/100 mol glucose fermented)						
	Experimental	Calculated			Experimental	Calculated	
		1	2	3		4	5
Butyrate	36.4	36.4 <sup>a</sup>	35.8	54	54 <sup>a</sup>	55.7	
Acetate	24.1	22.8	24.1 <sup>a</sup>	30.7	34.2	30.7 <sup>a</sup>	
H <sub>2</sub>	169	169	170	186	181	177	
CO <sub>2</sub>	206	209	209	194	187	187	
Butanol	28.9	28.9 <sup>a</sup>	28.9 <sup>a</sup>	10.4	10.4 <sup>a</sup>	10.4 <sup>a</sup>	
Acetone	10	10 <sup>a</sup>	10 <sup>a</sup>	0	0 <sup>a</sup>	0 <sup>a</sup>	
Ethanol	14.6	14.6 <sup>a</sup>	14.6 <sup>a</sup>	13.6	13.6 <sup>a</sup>	13.6 <sup>a</sup>	
Acetoin	5.4	5.4 <sup>a</sup>	5.4 <sup>a</sup>	5.1	5.1 <sup>a</sup>	5.1 <sup>a</sup>	
Isopropanol	0	0 <sup>a</sup>	0 <sup>a</sup>	0	0 <sup>a</sup>	0 <sup>a</sup>	
Carbon recovery (as glucose)	99.4	99.4 <sup>a</sup>	99.4 <sup>a</sup>	93.4	93.4 <sup>a</sup>	93.4 <sup>a</sup>	
NADH <sub>2</sub> from FdH <sub>2</sub>	—	19.3	18	—	-4.22	-0.73	
Excess ATP	—	259	260	—	177	188	
Apparent Y <sub>ATP</sub>	—	0.35	0.35	—	3.7	3.6	
CO <sub>2</sub> consistency test	210/206	—	—	183/194	—	—	
Δγ [eq. (41)]	0.54	—	—	14.9	—	—	
H <sub>2</sub> /CO <sub>2</sub>	0.82	0.81	0.81	0.96	0.97	0.95	

<sup>a</sup>Data used in calculations.**Table III.** Fermentation of glucose with *C. butyricum*; Data are from Kluyver.<sup>11</sup>

	Amount (mol/100 mol glucose fermented)		
	Experimental	Calculated	
Butyrate	75.4	75.4 <sup>a</sup>	75.6
Acetate	42.7	43.2	42.7 <sup>a</sup>
H <sub>2</sub>	231	235	234
CO <sub>2</sub>	196	194	194
Butanol	0	0 <sup>a</sup>	0 <sup>a</sup>
Acetone	0	0 <sup>a</sup>	0 <sup>a</sup>
Ethanol	0	0 <sup>a</sup>	0 <sup>a</sup>
Acetoin	0	0 <sup>a</sup>	0 <sup>a</sup>
Isopropanol	0	0 <sup>a</sup>	0 <sup>a</sup>
Carbon recovery (as glucose)	97	97 <sup>a</sup>	97 <sup>a</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	-41	-40
Excess ATP	—	268	268
Apparent Y <sub>ATP</sub>	—	1.5	1.5
CO <sub>2</sub> consistency test	193.5/196	—	—
Δγ [eq. (41)]	69	—	—
H <sub>2</sub> /CO <sub>2</sub>	1.18	1.21	1.21

<sup>a</sup>Data used in calculations.

strate and the fermentation products (biomass and extracellular products). On the basis of 100 mol glucose, this difference is

$$\Delta\gamma = 2400 - 6.4365(100 - x) - 8y_{Ac} - 20y_{By} - 2y_{H_2} - 24y_{Bu} - 16y_{Aco} - 18y_{Is} - 12y_{Et} - 20y_{Aci} \quad (41)$$

where  $x$  is the number of moles of glucose recovered in products and  $y_{Ac}$ ,  $y_{By}$ ,  $y_{H_2}$ ,  $y_{Bu}$ ,  $y_{Aco}$ ,  $y_{Et}$ , and  $y_{Aci}$  are the number of moles of acetate, butyrate, hydrogen, butanol,

acetone, isopropanol, ethanol, and acetoin, respectively, per 100 mol glucose fermented. Again, all glucose not converted to extracellular products is assumed to go into biomass.

Table I shows data and calculations for a glucose fermentation by *Clostridium acetobutylicum*. This is a predominantly solvent fermentation with 100% carbon recovery. The calculated values agree well with the experimental data, for all combinations of data used in the calculations. The calculations show a net production of NADH<sub>2</sub> from FdH<sub>2</sub> and an impressively low apparent  $Y_{ATP} = 0.05$ . As a straightforward error propagation analysis would show, the relative error in the predicted values does not only depend on the experimental error of the data used in the calculations, but also on the magnitude of the predicted value. For example, the percent error in the prediction of the high gas values is very small, as Table I shows, while the error in the prediction of the very low values of butyrate and acetate is relatively higher. This observation will be demonstrated even better in the following tables.

Table II shows data and calculations for two glucose fermentations by *C. acetobutylicum* which produce more carboxylic acids. The fermentation of columns 4-6 is a predominantly carboxylic acid fermentation. Despite the somewhat poorer CO<sub>2</sub> tests, the predictions agree quite well with the experimental data. Thus, eq. (21) seems to be valid for acid, solvent, or mixed fermentations, and independent of the percentage of carbon recovery (columns 4-6) or the H<sub>2</sub>/CO<sub>2</sub> ratio. The acid fermentation of columns 4-6 shows a net production of FdH<sub>2</sub> from NADH<sub>2</sub>. In fact, a comparison of Tables I and II shows that as the amount of carboxylic acids increases, less NADH<sub>2</sub> is produced from FdH<sub>2</sub> until the process is reversed. Acid fermentations also show higher apparent  $Y_{ATP}$  values.

**Table IV.** Fermentation of glucose with *C. falsineum*; Data are from van der Lek.<sup>10</sup>

	Amount (mol/100 mol glucose fermented)			
	Experimental	Calculated	Experimental	Calculated
Butyrate	40.4	42.1	40.4 <sup>a</sup>	50
Acetate	36	36 <sup>a</sup>	36 <sup>a</sup>	38.3
H <sub>2</sub>	186	188	185	179
CO <sub>2</sub>	208	204	204	200
Butanol	25	25 <sup>a</sup>	26.7	19
Acetone	8.3	8.3 <sup>a</sup>	8.3 <sup>a</sup>	1.3
Ethanol	6.7	6.7 <sup>a</sup>	6.7 <sup>a</sup>	14.3
Acetoin	1	1 <sup>a</sup>	1 <sup>a</sup>	1
Isopropanol	0	0 <sup>a</sup>	0 <sup>a</sup>	0
Carbon recovered (as glucose)	97.3	97.3 <sup>a</sup>	97.3 <sup>a</sup>	98
NADH <sub>2</sub> from FdH <sub>2</sub>	—	5.5	8.9	—
Excess ATP	—	241	239	—
Apparent Y <sub>ATP</sub>	—	1.5	1.5	—
CO <sub>2</sub> consistency test	200/208	—	—	196.5/200
Δγ [eq. (41)]	81	—	—	54
H <sub>2</sub> /CO <sub>2</sub>	0.89	0.92	0.91	0.9

<sup>a</sup>Data used in calculations.**Table V.** Fermentation of glucose with *C. lactoacetophilum*; Data are from Bhat and Barker.<sup>52</sup>

	Amount (mol/100 mol glucose fermented)	
	Experimental	Calculated
Butyrate	73	73 <sup>a</sup>
Acetate	28	33.5
H <sub>2</sub>	182	204
CO <sub>2</sub>	190	179
Butanol	0	0 <sup>a</sup>
Acetone	0	0 <sup>a</sup>
Ethanol	0	0 <sup>a</sup>
Acetoin	0	0 <sup>a</sup>
Isopropanol	0	0 <sup>a</sup>
Carbon recovery (as glucose)	89.7	89.7 <sup>a</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	-24.5
Excess ATP	—	133
Apparent Y <sub>ATP</sub>	—	5.62
CO <sub>2</sub> consistency test	174/190	—
Δγ [eq. (41)]	286	—
H <sub>2</sub> /CO <sub>2</sub>	0.96	1.14

<sup>a</sup>Data used in calculations.

Table III shows data and calculations for an exclusively acid fermentation by *C. butyricum*. The agreement between predicted and experimental values is very good. An even higher net production of FdH<sub>2</sub> from NADH<sub>2</sub> is indicated, which is accompanied by a higher H<sub>2</sub>/CO<sub>2</sub> ratio.

Table IV shows data and calculations for two mixed fermentations by *C. falsineum*, where relatively high amounts of carboxylic acids are produced. Calculated values agree quite well with data despite poorer CO<sub>2</sub> tests. Very small amounts of NADH<sub>2</sub> are produced from FdH<sub>2</sub>, in accordance with the high amounts of carboxylic acids produced.

Table V shows data and calculations for an exclusively acid fermentation by *C. lactoacetophilum* and seems to indicate that the poorer the CO<sub>2</sub> test the poorer the agreement between calculated and experimental data. This fermentation shows a relatively high apparent Y<sub>ATP</sub> value in accordance to our preceding observations.

Table VI shows data and calculations for two solvent fermentations by *C. butylicum* (currently labelled *C. beijerinckii*<sup>54</sup>). Predictions agree quite well with experimental data except for hydrogen for which the calculated values are higher than the experimental values. In fact, this overprediction of the hydrogen values was observed for all the data from Osburn and co-workers.<sup>25</sup> Our speculation is that these higher values are due to a systematic experimental error in the hydrogen measurement. This seems to be confirmed by calculations based on data of fermentations by the same strain but from different investigators, as shown, for example, in Table VII for another solvent fermentation by *C. butylicum*. The calculations of Table VII show also more clearly the effects of both the magnitude of the predicted values and the choice of the data used for calculations on the relative error between the predicted and the experimental values, as we discussed earlier. Most strikingly, the set of values shown in column 5 of Table VII, when used for calculations according to eq. (21), produce a negative value for acetate, which has a very small experimental value of 2.8. In fact, calculations using different combinations of experimental values may be used for detecting possible experimental errors in the data, as it will be discussed in the following section.

### Uses of the Fermentation Equation

The comparisons in the previous section between experimental data and predicted values on the basis of fermenta-

**Table VI.** Fermentation of glucose with *C. butylicum*; Data are from Osburn and co-workers.<sup>25</sup>

	Amount (mol/100 mol glucose fermented)					
	Experimental		Calculated		Experimental	
	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated
Butyrate	14.5	14.5 <sup>a</sup>	15.1	17.2	17.2 <sup>a</sup>	16.9
Acetate	20.3	21.4	20.3	17.2	16.6	17.2 <sup>a</sup>
H <sub>2</sub>	111.1	120	119	77.6	100.6	101
CO <sub>2</sub>	207	205	205	203.5	204.5	204.5
Butanol	50.2	50.2 <sup>a</sup>	50.2 <sup>a</sup>	58.6	58.6 <sup>a</sup>	58.6 <sup>a</sup>
Acetone	0	0 <sup>a</sup>	0 <sup>a</sup>	0	0 <sup>a</sup>	0 <sup>a</sup>
Ethanol	0	0 <sup>a</sup>	0 <sup>a</sup>	0	0 <sup>a</sup>	0 <sup>a</sup>
Acetoin	0	0 <sup>a</sup>	0 <sup>a</sup>	0	0 <sup>a</sup>	0 <sup>a</sup>
Isopropanol	18	18 <sup>a</sup>	18 <sup>a</sup>	12.1	12.1 <sup>a</sup>	12.1 <sup>a</sup>
Carbon recovery (as glucose)	93.4	93.4 <sup>a</sup>	93.4 <sup>a</sup>	96.2	96.2 <sup>a</sup>	96.2 <sup>a</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	67	68	—	92	91
Excess ATP	—	142.7	142.1	—	182	182
Apparent Y <sub>ATP</sub>	—	4.3	4.3	—	2.5	2.5
CO <sub>2</sub> consistency test	204/207	—	—	205/203.5	—	—
Δγ [eq. (41)]	154	—	—	115	—	—
H <sub>2</sub> /CO <sub>2</sub>	0.54	0.59	0.58	0.38	0.49	0.49

<sup>a</sup>Data used in calculations.**Table VII.** Fermentation of glucose with *C. butylicum*; Data are from van der Lek.<sup>10</sup>

Column	Amount (mol/100 mol glucose fermented)				
	Experimental		Calculated		
	1	2	3	4	5 <sup>b</sup>
Butyrate	1.2	1.2 <sup>a</sup>	1.2 <sup>a</sup>	1.2 <sup>a</sup>	1.2 <sup>a</sup>
Acetate	2.8	2.8 <sup>a</sup>	2.8 <sup>a</sup>	6.84	<sup>b</sup>
H <sub>2</sub>	81.2	84.8	75.4	81.2 <sup>a</sup>	<sup>b</sup>
CO <sub>2</sub>	220	224	221	220 <sup>a</sup>	
Butanol	67.5	64.3	67.5 <sup>a</sup>	66.2	67.5 <sup>a</sup>
Acetone	0	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Ethanol	12.3	12.3 <sup>a</sup>	12.3 <sup>a</sup>	12.3 <sup>a</sup>	12.3 <sup>a</sup>
Acetoin	0	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Isopropanol	25.9	25.9 <sup>a</sup>	22.8	22	25.9 <sup>a</sup>
Carbon recovered (as glucose)	100.5	99 <sup>a</sup>	99 <sup>a</sup>	99 <sup>a</sup>	99 <sup>a</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	113	123	117	<sup>b</sup>
Excess ATP	—	213	210	213	<sup>b</sup>
Apparent Y <sub>ATP</sub>	—	0.68	0.69	0.68	<sup>b</sup>
CO <sub>2</sub> consistency test	230/220	—	—	—	—
Δγ [eq. (41)]	-43	—	—	—	—
H <sub>2</sub> /CO <sub>2</sub>	0.37	0.38	0.34	0.37	<sup>b</sup>

<sup>a</sup>Data used in calculations.<sup>b</sup>This data combination produces a negative acetate concentration.

tation eq. (21) seem to indicate that the equation is valid for a wide variety of fermentations by a wide variety of butyric acid bacteria. The validity of the equation, by virtue of its derivation, is essentially testing the assumptions made in the derivation of the equation, namely the assumptions about the microbial biochemistry, the ATP yield and the assumptions of validity of the biological regularities of eqs. (7) and (8). For batch fermentations, the degree of accuracy of the latter two equations and the as-

sumed value of the ATP yield seem to have a minimal effect on the validity of fermentation eq. (21), because of the high percentage of substrate-carbon recovery in fermentation products. Therefore, the validity of eq. (21) for a good variety of batch fermentations seem to indicate that the assumptions made about the fermentation biochemistry are valid. It is most unlikely that these assumptions would not be valid in other batch or continuous fermentations of butyric acid bacteria, certainly not for bacteria

with which the equation has been tested. Pending further testing and potential improvements of the equation, we shall assume in what follows that eq. (21) is valid and use it to demonstrate its potential usefulness.

On the basis of eq. (21), we can calculate the maximal possible yield for each of the main fermentation products, for an assumed carbon recovery in products. Sample calculations with a 95% carbon recovery in fermentation products are shown in Table VIII. Table VIII shows that acetone, isopropanol, butyrate, and acetate can be produced as sole products of the fermentation, save for hydrogen and carbon dioxide. For a 95% carbon recovery into products, most of the product carbon can be recovered in the form of butanol (93.9%), but a small percentage must be converted to acetate for lack of reduction energy ( $\text{NADH}_2$ ) to produce more butanol. Acetate formation requires no reducing energy. For acetone, acetate, and isopropanol fermentations, a significant amount of  $\text{NADH}_2$  reduction energy is converted to  $\text{FdH}_2$  and is eventually released in the form of molecular hydrogen. Finally, the fermentation with the highest possible butanol yield, produces no hydrogen and the *least* amount of excess ATP. The results of Table VIII indicate the good potential of the fermentations of butyric acid bacteria for industrial applications.

Equation (21) can be also used to determine possible sources of experimental error in a set of data. Let's assume that in a complete set of fermentation data, there is a significant experimental error in the value of one product. If the experimental value of this product is used to calculate other product values, the agreement between all the calculated and the experimental values will be most probably poor. If, from the other hand, presumably correct experimental values of other products are used for calculations, good agreement between calculated and experimental values should be obtained, except for the product whose value contains the experimental error. Tables IX and X

demonstrate the methodology more clearly. In Table IX, all combinations of data used in calculations which contain the experimental butanol value produce poor predictions or are inadmissible (columns 2 and 3). If the experimental butanol value is not used in calculations, the predictions are good (columns 4-6). Thus, it is very probable that the butanol value contains an experimental error. In the case of the data of Table X, the error appears to be in the value of hydrogen, which when used for calculations (columns 6-8) produces poor results, while when it is not used for calculations (columns 2-5), the predictions are good, except, of course, for hydrogen.

Finally, eq. (21) can be used as a "gateway" sensor for calculating values of fermentation products which cannot be measured directly. This can be best illustrated by considering a fermentation with fewer products, an acid fermentation similar to the fermentations of Tables III and V, for example. Such batch, continuous, or fed batch fermentations can be monitored and controlled by measuring the off gases (hydrogen and carbon dioxide) and the amount of substrate consumed. Then, the concentrations of biomass, butyrate and acetate can be continuously computed using eq. (21).

## DISCUSSION AND CONCLUSIONS

Fermentation eq. (21), by its derivation, obeys all constraints imposed on growth and product formation by thermodynamics and the biochemical topology. The thermodynamical constraints are contained in the biological regularities of eqs. (7) and (8), the value of  $Y_{\text{ATP}}$  used in the calculations and eqs. (10)-(20), which express the production or requirements of ATP and  $\text{NADH}_2$  for the enzymatic reactions of product formation. Equations (10)-(20) also contain the constraints imposed by the biochemical topology. In principle, then, fermentations with one or two only solvent and acid products, similar to those shown

**Table VIII.** Computed theoretically maximal yields for primary products of glucose fermentations with butyric acid bacteria. A 95% carbon recovery in products was assumed.

	Amount (mol/100 mol glucose fermented)				
	Butanol <sup>a</sup>	Acetone <sup>a</sup>	Butyrate <sup>a</sup>	Acetate <sup>a</sup>	Isopropanol <sup>a</sup>
Butyrate	0	0	95	0	0
Acetate	2.2	0	0	190	0
H <sub>2</sub>	0	376	186	376	281
CO <sub>2</sub>	190	285	190	190	285
Butanol	93.9	0	0	0	0
Acetone	0	95	0	0	0
Ethanol	0	0	0	0	0
Acetoin	0	0	0	0	0
Isopropanol	0	0	0	0	95
Carbon recovery (as glucose)	95	95	95	95	95
NADH <sub>2</sub> from FdH <sub>2</sub>	190	-186	4.4	-186	-91
Excess ATP	118	211	211	306	211
H <sub>2</sub> /CO <sub>2</sub>	0	1.32	0.98	1.98	0.99

<sup>a</sup>This represents the product of maximal yield.

**Table IX.** Fermentation of glucose with *C. butylicum*; Data are from van der Lek.<sup>10</sup>

Column	Amount (mol/100 mol glucose fermented)					
	Experimental			Calculated		
	1	2 <sup>a</sup>	3	4	5	6
Butyrate	1.94	1.94 <sup>b</sup>	1.94 <sup>b</sup>	1.94 <sup>b</sup>	1.94 <sup>b</sup>	1.94 <sup>b</sup>
Acetate	3.04	a	3.04 <sup>b</sup>	3.04 <sup>b</sup>	3.55	5.95
H <sub>2</sub>	95	a	74	94	95 <sup>b</sup>	95 <sup>b</sup>
CO <sub>2</sub>	224.5	a	219.5	226	226	224.5 <sup>b</sup>
Butanol	68.3	68.3 <sup>b</sup>	68.3 <sup>b</sup>	61.8	61.5	61.9
Acetone	0.6	0.6 <sup>b</sup>	0.6 <sup>b</sup>	0.6 <sup>b</sup>	0.6 <sup>b</sup>	0.6 <sup>b</sup>
Ethanol	11.4	11.4 <sup>b</sup>	11.4 <sup>b</sup>	11.4 <sup>b</sup>	11.4 <sup>b</sup>	11.4 <sup>b</sup>
Acetoin	0	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>	0 <sup>b</sup>
Isopropanol	27.5	27.5 <sup>b</sup>	21	27.5 <sup>b</sup>	27.5 <sup>b</sup>	25.9
Carbon recovered (as glucose)	103	99 <sup>b</sup>	99 <sup>b</sup>	99 <sup>b</sup>	99 <sup>b</sup>	99 <sup>b</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	a	124	104	103	103
Excess ATP	—	a	210	216	217	218
Apparent Y <sub>ATP</sub>	—	a	0.69	0.68	0.67	0.67
CO <sub>2</sub> consistency test	239/224.5	—	—	—	—	—
Δγ [eq. (41)]	-134	—	—	—	—	—
H <sub>2</sub> /CO <sub>2</sub>	0.42	a	0.34	0.42	0.42	0.42

<sup>a</sup>This data combination produces a negative acetate concentration.<sup>b</sup>Data used in calculations.**Table X.** Fermentation of glucose with *C. butylicum*; Data are from Osburn and co-workers.<sup>25</sup>

Column	Amount (mol/100 mol glucose fermented)							
	Experimental				Calculated			
	1	2	3	4	5	6	7	8
Butyrate	18.2	18.2 <sup>a</sup>	16.3	18.4	18.2 <sup>a</sup>	18.2 <sup>a</sup>	31.8	18.2 <sup>a</sup>
Acetate	12.1	8.3	12.1 <sup>a</sup>	12.1 <sup>a</sup>	12.1 <sup>a</sup>	25.7	12.1 <sup>a</sup>	22.6
H <sub>2</sub>	121.2	93	96	94	100	121.2 <sup>a</sup>	121.2 <sup>a</sup>	121.2 <sup>a</sup>
CO <sub>2</sub>	200	202	202	200 <sup>a</sup>	202	200 <sup>a</sup>	200 <sup>a</sup>	202
Butanol	54.6	54.6 <sup>a</sup>	54.6 <sup>a</sup>	54.6 <sup>a</sup>	52.7	48	41	47.5
Acetone	0	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Ethanol	2.9	2.9 <sup>a</sup>	2.9 <sup>a</sup>	2.9 <sup>a</sup>	2.9 <sup>a</sup>	2.9 <sup>a</sup>	2.9 <sup>a</sup>	2.9 <sup>a</sup>
Acetoin	0	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>	0 <sup>a</sup>
Isopropanol	15.1	15.1 <sup>a</sup>	15.1 <sup>a</sup>	13	15.1 <sup>a</sup>	13	13	15.1 <sup>a</sup>
Carbon recovered (as glucose)	93.5	93.5 <sup>a</sup>	93.5 <sup>a</sup>	93.5 <sup>a</sup>	93.5 <sup>a</sup>	93.5 <sup>a</sup>	93.5 <sup>a</sup>	93.5 <sup>a</sup>
NADH <sub>2</sub> from FdH <sub>2</sub>	—	94	91	93	87	66	66	66
Excess ATP	—	132	134	134	136	147	147	146
Apparent Y <sub>ATP</sub>	—	4.4	4.4	4.4	4.4	4.16	4.16	4.16
CO <sub>2</sub> consistency	206/200	—	—	—	—	—	—	—
Δγ [eq. (41)]	38	—	—	—	—	—	—	—
H <sub>2</sub> /CO <sub>2</sub>	0.61	0.46	0.48	0.47	0.50	0.61	0.61	0.6

<sup>a</sup>Data used in calculations.

in Table VIII, should be feasible. Whether the internal biochemical control mechanisms would make it possible to achieve such industrially attractive fermentations is open to discussion and experimentation. In any case, biochemical control mechanisms do not necessarily have thermodynamic or topological constraints as reasons of existence. Biochemical control mechanisms can be genetically altered by mutations and DNA recombination. Be-

sides, a careful look at experimental data provides some exciting hints about the possibilities of achieving ideal fermentations similar to those of Table VIII. For example, the data of column 4 of Table II indicate that butanol production needs not be accompanied by acetone production in *C. acetobutylicum*. The data in Tables VII and IX also indicate that little or no carboxylic acids need be produced for good solvent production.

In our present calculations, the maintenance requirements of the cells were not taken into account, first because of lack of specific information and second because of the apparent excess supply of ATP. Modifications to take the maintenance requirements into account can be easily made to produce an equation which will be growth-rate dependent. The modified equation will be perhaps useful in continuous production of solvents. Another weak point of the present calculations is the assumption that biomass is produced only from the glucose not converted into extracellular products. It is certain, however, that, in many instances, a good percentage of biomass and/or product carbon derives from the yeast extract in the medium. This however, would not detract from the fact that more than 95% of substrate carbon can be converted into extracellular fermentation products even in totally synthetic media.<sup>55</sup> The calculations of Table VIII, therefore, are truly realistic and perhaps practical.

The low values of the apparent  $Y_{ATP}$  of Tables I-VII, IX, and X should not be misconstrued to imply that the actual efficiency of biomass production is that low or that these values contradict our discussion in their derivation of eq. (9). These low apparent  $Y_{ATP}$  values imply that under the experimental conditions or assumptions of the data or calculations of Tables I-X, an excessive amount of ATP is used for an unknown cellular process, or that ATP is hydrolyzed without performing any cellular function. Some ATP yields considerably higher than 10.5 g/mol have been, in fact, measured in continuous fermentations.<sup>17</sup>

Improvements in eq. (21) can result from using more accurate information regarding the elemental composition of microbial biomass to replace eqs. (7) and (8), and a measured value for the ATP yield that would apply to the fermentation of interest. It is also quite probable that some general carboxylation enzymes incorporate  $CO_2$  into biomass and that general decarboxylation enzymes release more  $CO_2$  than predicted by eq. (21). Quantitative information on these last two processes could be used to improve the accuracy of eq. (9) and thus the accuracy of eq. (21). Another correction to the equation may be necessary for fermentations where a significant percentage of product carbon is derived from the yeast extract. Typically, it is expected that some amino acid carbon from the yeast extract will be incorporated into biomass. This effect can be accounted for by a higher  $Y_{ATP}$  value for the sugar substrate(s).<sup>46</sup> If, however, carbon from the yeast extract appears in the fermentation products, an overall balance must be written for the derivation of AcCoA, pyruvate or other intermediates from the amino acids of the yeast extract, so that this effect can be incorporated into a modified version of eq. (21). In any case, we are now working in our laboratory on the validity of eq. (21) (with the possible modifications discussed presently) using our own data from both batch and continuous fermentations. The validity of eq. (21) in continuous fermentation is of considerable practical importance to be left to rational speculation.

As we have mentioned earlier, fermentation eq. (21) and its extensions can provide two more equations than

the carbon, nitrogen, and available electron balances for relating the amounts of the various fermentation products, biomass and/or intracellular rates. Of these two equations, one is used to calculate  $d$ , which gives the amount of  $NADH_2$  converted to  $FdH_2$ , or vice versa. In essence then, fermentation eq. (21) provided one additional equation to relate the extracellular products and biomass, which, although not a small accomplishment in itself, is only one of the practical contributions. Equation (21) allows the calculation of (i.e., becomes a "gateway sensor" for) a number of physiological parameters, which characterize the state of the fermentation and cannot be measured directly. Such parameters are, for examples, the amount of  $FdH_2$  converted to  $H_2$  versus  $NADH_2$ , the amount of excess ATP and the percentage of AcCoA which is converted to acetate. This idea of the gateway sensor for measuring important physiological parameters will probably have practical applications in the control and optimization of fermentations. Finally, eq. (21) is useful for establishing the maximal possible yields of the various fermentation products (Table VIII), which thermodynamics and the biochemical topology will allow.

The procedure used for the derivation of eq. (21) can be applied to derive fermentation equations for other anaerobic and aerobic fermentations, provided that sufficient biochemical information exists. In fact, it is possible to generalize the procedure to be able to derive a fermentation equation from accurate data of fermentations for which little or no biochemical information exists. Our efforts on both the above two areas will appear in forthcoming reports.

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## NOMENCLATURE

$a, b, c, d, e,$	
$f, g, h, i, j, k$	stoichiometric coefficients, in eqs. (1) and (21)
AcCoA	acetyl-CoA
Fd, $FdH_2$	oxidized and reduced forms of ferredoxin
$Y_{ATP}$	ATP yield, g dry biomass per mole ATP utilized in biomass synthesis
$y_c$	stoichiometric coefficient, in eq. (1)
$\gamma_b, \gamma_p, \gamma_s$	reductance degrees of biomass, product(s) and substrate(s), respectively
$\sigma_b$	weight fraction of carbon in biomass

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