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Carbon and Energy Balances of Glucose Fermentation with Hydrogen-producing Bacterium *Citrobacter amalonaticus* Y19

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For the newly isolated H_2-producing chemoheterotrophic bacterium *Citrobacter amalonaticus* Y19, anaerobic glucose metabolism was studied in batch cultivation at varying initial glucose concentrations (3.5–9.5 g/l). The carbon-mass and energy balances were determined and utilized to analyze the carbon metabolic-pathways network. The analyses revealed (a) variable production of major metabolites (H_2, ethanol, acetate, lactate, CO_2, and cell mass) depending on initial glucose levels; (b) influence of NADH regeneration on the production of acetate, lactate, and ethanol; and (c) influence of the molar production of ATP on the production of biomass. The results reported in this paper suggest how the carbon metabolic pathway(s) should be designed for optimal H_2 production, especially at high glucose concentrations, such as by blocking the carbon flux *via* lactate dehydrogenase from the pyruvate node.

Keywords: Hydrogen production, carbon and energy balances, anaerobic glucose fermentation, *Citrobacter amalonaticus*, carbon-flux analysis

Hydrogen can be produced by photosynthetic or fermentative bacteria. Compared with photosynthetic bacteria, fermentative bacteria produce H_2 faster, but their H_2 yield on organic substrate is lower [21]. Fermentative H_2 production is carried out by many facultative and strictly anaerobic bacteria. Strict anaerobes such as *Clostridium butyricum* produce H_2 at a high rate and yield [1]. However, their H_2 production activity is so sensitive to O_2 that the presence of only a trace amount of dissolved O_2 often stops the H_2 production completely [17]. Facultative anaerobes usually give a lower H_2 yield than strict anaerobes. However, they are less sensitive to O_2, and sometimes recover the H_2 production activity from O_2 damage after they remove O_2 in culture broth by themselves. As a consequence, from a practical standpoint, facultative anaerobes are considered more advantageous than strict anaerobes [17, 20].

In most facultative anaerobic bacteria, the production of H_2 is accompanied by that of various organic acids. Depending on culture conditions, the H_2 production yield varies in the range of 0.5 to 2.0 mole H_2 per mole glucose. In general, the yield is low when lactate is produced extensively and high when acetate is produced as a major organic acid [4, 6, 8]. However, carbon-mass balances with the mixed-acid fermentation and the quantitative analysis on the relationship among the production yields of various organic acids and H_2 are available only for a few strains [4].

We have been studying a new H_2-producing chemoheterotrophic facultative bacterium, *Citrobacter amalonaticus* Y19 [9, 10, 12, 13, 22, 24]. This strain produces H_2 from various carbon sources, including carbon monoxide, by a water-gas shift type of reaction and many carbohydrates by anaerobic fermentation. Under anaerobic conditions with glucose as a carbon source, it grows fast with a maximum specific growth rate of 0.76 h^{-1} and produces H_2 in a wide range of pH (5–9) and temperature (25–40°C). The Y19 strain has also shown a high volumetric H_2 production rate of 16 mmol H_2/l-h with a stable production capability during a prolonged continuous fermentation for more than 20 days. Therefore, the Y19 strain has been suggested to be a potentially useful strain for the production of H_2 from carbon monoxide and various organic substrates [24].

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The purpose of this study was to quantitatively analyze the carbon and energy balances of glucose fermentation by Y19. First, batch experiments were carried out and the carbon-mass distributions were determined. Second, the metabolic network of anaerobic glucose fermentation was constructed based on the experiments, pathway database, and other publications. Finally, the relationship of the production yields for H_2, cell biomass, and various metabolites was analyzed. Experiments were conducted at varying initial glucose concentrations, since the distribution of carbon metabolic fluxes to various organic acids could be varied.

C. amalonaticus Y19 was isolated from a sludge digester [9, 24] and used in this study. For inoculum culture, PFN mineral salt medium [9] containing a fortified phosphate buffer (180 mM and pH 7.3), 0.1 g yeast extract/l (Becton, Dickinson and Company, Sparks, U.S.A.), and 9.5 g glucose/l (Sigma Chemical Co., U.S.A.) was used. The cultivation for H_2 production was conducted in the same PFN mineral salt medium but with an increased yeast extract concentration at 0.3 g/l and a varying glucose concentration in the range of 3.5–9.5 g/l.

Fermentation was performed in a batch mode in a reciprocating shaker (30°C and 100 strokes/min). Serum bottles of 165 ml with a working volume of 50 ml were used. After inoculation, the bottle was sparged with argon gas (99.999%) for 5 min to provide the anaerobic condition and then sealed with a 12-mm-thick butyl rubber septum and aluminum cap. The inoculum was cultivated in the same bottle and transferred anaerobically at the late-exponential phase using a sterile hypodermic disposable syringe [22].

Cell concentrations were measured using a spectrophotometer at 600 nm (Lambda 20, Perkin-Elmer, U.S.A.). Hydrogen and CO_2 concentrations were determined by a gas chromatograph equipped with a thermal conductivity detector. Various organic acids (succinate, lactate, formate, acetate, propionate, and pyruvate), ethanol, and glucose were analyzed using a liquid chromatograph equipped with a diode array and refractive index detectors. Detailed procedures and conditions have been described in our earlier reports [22, 23].

Glucose fermentation by *C. amalonaticus* Y19 was studied in a batch mode at varying initial glucose concentrations of 3.5 to 9.5 g/l (Fig. 1). A minimal medium complemented with a fixed amount of yeast extract at 0.3 g/l was used. As the glucose concentration increased, the biomass concentration increased from 0.27 to 0.44 g/l, whereas H_2 production yield decreased from 1.27 to 0.7 mol H_2/mol glucose. The yields of some extracellular metabolites varied depending on glucose concentration. For example, with increasing glucose concentration, the yield for lactate production increased whereas that for ethanol or acetate production decreased. On the other hand, the yields for succinate and formate remained practically constant.

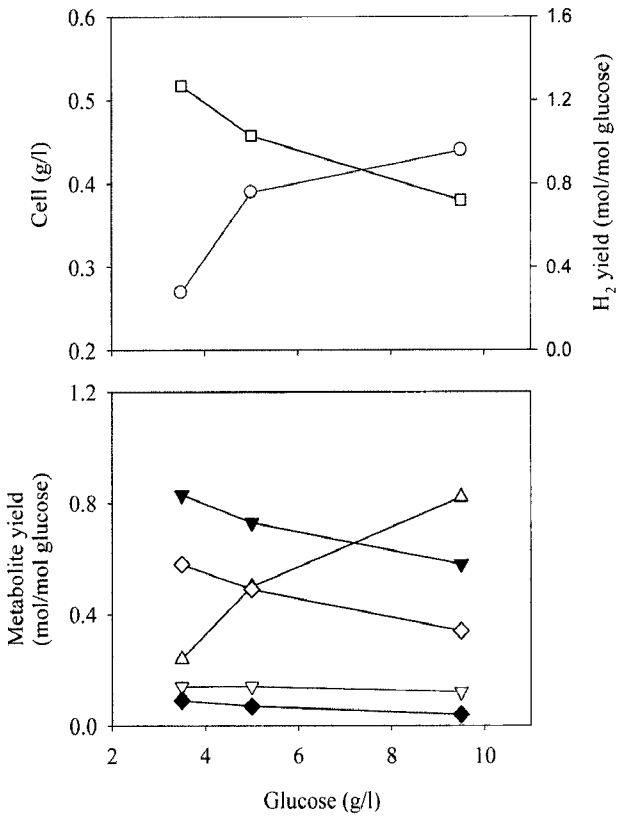

Fig. 1. Effect of glucose concentration on the cultivation of *C. amalonaticus* Y19.

Symbols: Cell (○), H_2 (□), lactate (△), ethanol (▼), succinate (▽), formate (◆), and acetate (◇). Y19 was cultivated for 48 h with initial pH and yeast extract concentration at 7.3 and 0.3 g/l, respectively. The glucose supplied was completely consumed irrespective of initial glucose concentrations. The pH after 48 h incubation was maintained in the range of 6.1–7.0. Each value is an average of three independent experiments.

The results described in Fig. 1 were quantitatively analyzed to examine the carbon-mass balance between the substrate used and the various metabolites produced (Table 1). The carbon content in the yeast extract was ignored since it was less than 0.1 g/l. The fractional distributions of the glucose carbons to various metabolites were as follows: lactate (12.7–41.7%), ethanol (20.1–29.8%), acetate (12.0–20.6%), CO_2 (10.6–13.8%), succinate (7.9–9.6%), and biomass (6.1–11.3%). The fractional carbons directed to propionate and formate were below 2.2% regardless of initial glucose concentration. Carbon recoveries as cell mass and various metabolites were 95.5–100.2% of the theoretical maximum, indicating that major metabolites in Table 1 were properly identified and quantified with a high accuracy. The composition of extracellular metabolites suggests that anaerobic glucose metabolism of Y19 is a typical mixed-acid fermentation, similar to that of other enterobacteria species such as *Escherichia coli* and *Enterobacter aerogenes* [4, 6, 15, 19].

Table 1 also shows the reduction degree balance analysis for the substrate and various metabolites. The reduction

Table 1. Carbon material balance and reduction degree balance for anaerobic metabolism of 1 mole of glucose by *C. amalonaticus* Y19 at varying initial glucose concentrations.[a]

	Glucose concentration (g/l)		
	3.5	5.0	9.5
Carbon material balance			
Reactant (C-mol)			
Glucose	6.00	6.00	6.00
Product (C-mol)			
Ethanol	1.71	1.53	1.18
Lactate	0.73	1.46	2.44
Acetate	1.18	0.99	0.70
Succinate	0.55	0.57	0.46
Formate	0.09	0.07	0.07
Propionate	0.03	0.03	0.06
Biomass	0.65	0.63	0.36
CO_2[b]	0.79	0.73	0.62
Total products	5.73	6.01	5.86
Error (%)	-4.5	0.2	-2.3
Product (%)[c]			
Ethanol	29.8	25.5	20.1
Lactate	12.7	24.3	41.7
Acetate	20.6	16.5	12.0
Succinate	9.6	9.5	7.9
Formate	1.6	1.2	1.2
Propionate	0.5	0.5	1.0
Biomass	11.3	10.5	6.1
CO_2	13.8	12.2	10.6
Reduction degree balance			
Reactant			
Glucose	24	24	24
Product			
Ethanol	9.96	8.76	6.96
Lactate	2.88	6.00	9.84
Acetate	4.64	3.92	2.72
Succinate	1.96	1.96	1.68
Formate	0.18	0.14	0.08
Propionate	0.14	0.14	0.28
Biomass	2.32	2.35	1.40
H_2	2.54	2.06	1.44
O_2[d]	-0.33	-0.33	-0.18
Total products	24.29	25.00	24.22
Error (%)	-1.2	-4.0	-0.9

[a]Calculated for the serum-bottle culture with the working volume of 50 ml. The culture was started with 45 ml of fresh medium and 5 ml of inoculum. The glucose supplied was completely consumed irrespective of initial glucose concentrations. Each value was measured after 48 h cultivation and was an average of three independent experiments.
[b]CO_2 in the liquid phase was ignored.
[c]Carbon distribution (%) from total products-carbon.
[d]Based on Eq. (16) in the Appendix.

degree (ε) represents the number of available electrons per unit carbon atom and is calculated from the following equation [4, 7]:

$$\varepsilon = 4\,C + H - 2\,O - 3\,N \quad (1)$$

where C, H, O, and N denote the atomic coefficient of the chemical formula of a compound. This expression includes the factor -3 N, which compensates for the electrons donated to the biomass from ammonia and thereby omits ammonium consumption in the calculation [see Eq. (16) in Appendix; 7]. Table 1 indicates that the reduction degrees are well balanced with the relative errors less than 5%. This supports that data in Table 1 are quantitatively consistent.

According to most literatures, H_2 fermentation is performed in the presence of a large amount of a complex nitrogen source, typically 10 g/l yeast extract [13, 20, 22]. This gives a fast cell growth and high H_2 production. However, because of its unknown composition, yeast extract causes a serious problem in the quantitative analysis of cellular metabolism. Furthermore, yeast extract has a considerable carbon content of 29–36% (w/w) [5, 22] and its negligence results in a significant overestimation of the glucose-to-H_2 yield as found in many literatures (~3.3 mol H_2/mol glucose [4, 14, 18, 26, 28]). In the present study, a very low yeast extract concentration of 0.3 g/l, an order of magnitude lower than the typical one, was adopted. Although the final cell density was not high, this concentration of yeast extract could support a reasonable growth and glucose metabolism of Y19. It should be noted that, even from batch experiments, quantitative analysis of carbon-mass balances is possible if the medium composition is carefully chosen.

Based on the experimental measurements, KEGG metabolic-pathways database [11; http://www.genome.jp/kegg] and literature data of mixed-acid fermentation by other Enterobacteriaceas including *E. coli* and *En. aerogenes* [4, 6], a brief model of the anaerobic metabolic pathway network for Y19 is suggested (Fig. 2). Detailed metabolic reactions are presented in the Appendix and their stoichiometric coefficients are given in Table 2. The cellular composition of Y19 was assumed to be constant as $CH_{1.67}O_{0.20}N_{0.27}$ [22], which was determined for the Y19 cells growing at the mid-log phase in the inoculum medium mentioned above. All product yields were given in C-mol for the consumption of 100 C-mol glucose. According to our model, glucose is directed to pyruvate *via* the Embden-Meyerhof (EM) pathway and pyruvate is converted to lactate by lactate dehydrogenase or acetyl-CoA by pyruvate-formate lyase with co-generation of formate. Formate is then split to H_2 and CO_2 by FHL [13]. These pathways leading to H_2 production are quite similar to those reported in most enteric bacteria. Some succinates were also produced and accumulated **via** anaplerotic reactions initiated by phosphoenolpyruvate carboxylase [16].

The NADH balances were determined based on the metabolic pathways model for the three different initial

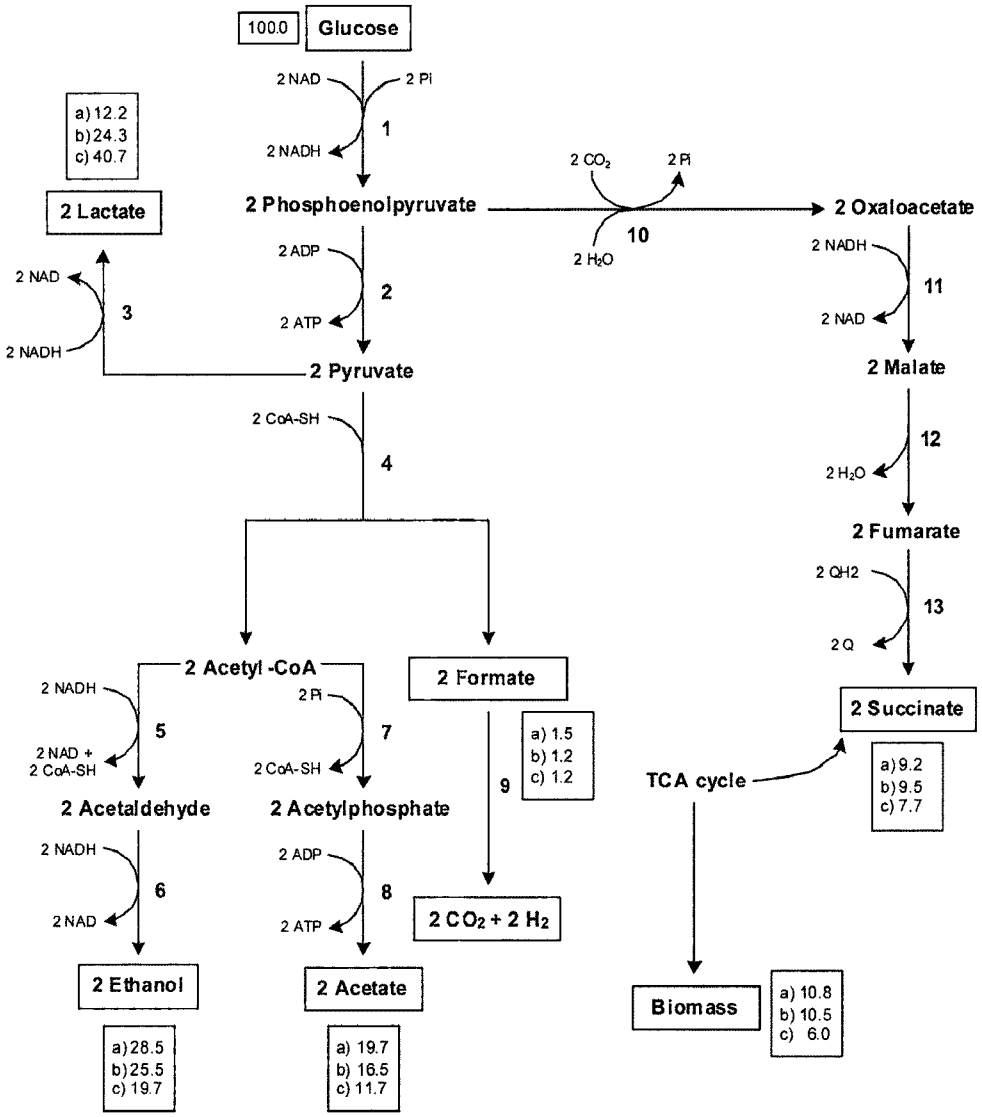

Fig. 2. Anaerobic glucose metabolism in *C. amalonaticus* Y19.

All metabolites are given in C-mol carbon and are referred to as the consumption of 100 C-mol glucose. Glucose concentration (g/l): a, 3.5; b, 5.0; c, 9.5. Enzymes: 1, enzymes of glycolysis; 2, pyruvate kinase; 3, lactate dehydrogenase; 4, pyruvate-formate lyase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, phosphotransacetylase; 8, acetate kinase; 9, formate-hydrogen lyase; 10, phosphoenolpyruvate carboxylase; 11, malate dehydrogenase; 12, fumarase; and 13, fumarate reductase. Detailed metabolic reactions are presented in the Appendix.

glucose concentrations. They were 1.94 mmole NADH (production) and 1.98 mmole NADH (consumption) at 3.5 g glucose/l, 2.77 mmole NADH (production) and 2.91 mmole NADH (consumption) at 5.0 g glucose/l, and 5.27 mmole NADH (production) and 5.54 mmole NADH (consumption) at 10 g glucose/l. This indicates that the production and consumption of NADH are generally well balanced and no NADH accumulation is expected during the glucose metabolism in the batch cultivation of Y19. The balance on NADH is a strict requirement for all anaerobic metabolisms [3], and this result is considered as a supporting evidence for the metabolic pathway network of Y19 suggested in Fig. 2.

Once the carbon-mass balance and metabolic pathway network are available, it is possible to estimate ATP yields during cell growth (Table 2). By using the equation on the overall anaerobic metabolism (Eq. (17) in the Appendix), ATP yields are calculated as follows (see Table 2 for the values of coefficients):

$$Y_{ATP/G}=2\alpha+\alpha_3+2\beta$$
$$Y_{X/G}=6\delta$$
$$Y_{ATP/X}=(2\alpha+\alpha_3+2\beta)/6\delta$$

As glucose concentration increased, $Y_{ATP/G}$ decreased gradually from 2.18 to 1.91 mol ATP/mol glucose. The ATP-to-cell yield ($Y_{ATP/X}$), on the other hand, was almost constant as 3.23±0.11 mol ATP/C-mol DCW at glucose concentrations of 3.5 and 5.0 g/l, and increased to 5.31 mol ATP/C-mol DCW at the higher glucose concentration of 9.5 g/l. The high $Y_{ATP/X}$ at 9.5 g glucose/l is comparable to the value

Table 2. Stoichiometric coefficients and products molar yield (mol/mol glucose) of anaerobic glucose metabolism by *C. amalonaticus* Y19 at varying initial glucose concentrations. Data refer to the consumption of 1 mole of glucose. See the Appendix for nomenclature and the calculation of the coefficients and molar yields.

	Glucose concentration (g/l)		
	3.5	5.0	9.5
Coefficients			
α	0.68	0.55	0.38
α_1	1.27	1.03	0.72
α_2	0.83	0.72	0.57
α_3	0.58	0.48	0.33
β	0.12	0.25	0.41
γ	0.07	0.07	0.06
δ	0.11	0.11	0.06
Molar yields			
$Y_{ATP/G}$	2.18	2.08	1.91
$Y_{X/G}$	0.66	0.66	0.36
$Y_{ATP/X}$	3.30	3.15	5.31

reported for *En. aerogenes* (6.3 mol ATP/C-mol DCW at 9 g glucose/l [4]). Although more studies are required, the different $Y_{ATP/X}$ at different glucose concentrations seem to be related to the assimilation rate of carbon substrate during the cultivation of Y19. According to Islam *et al.* [8] who have investigated the effect of substrate loading on H_2 production by *Clostridium* species, carbon catabolism is tightly coupled to anabolism, and a high $Y_{X/G}$ is resulted under carbon-limited conditions. In comparison, under carbon-excess conditions, they have observed a low $Y_{X/G}$ and high $Y_{ATP/X}$ due to "overflow metabolism", where carbon or electron flows are shifted to energetically less efficient pathways and/or "energy-spilling" reactions. Moreover, it has been reported that the maintenance energy is related to $Y_{ATP/X}$. From the continuous culture of *Klebsiella aerogenes*, Russell and Cook [25] have observed that the enhanced maintenance energy under carbon-sufficient condition increases $Y_{ATP/X}$.

According to the metabolic pathway network, it is also possible to explain the different carbon distributions toward various metabolites in Y19 at different initial glucose concentrations. For example, the higher production of lactate at a higher glucose concentration can be explained by the elevated level of intracellular pyruvate [3]. To maintain a fast glycolytic flux in the EM pathway and thus rapidly produce ATP and other precursors for cell biomass, the NAD^+ consumed in the EM pathway should be regenerated by depositing the reducing equivalents on partially oxidized metabolic intermediates such as lactate and ethanol. The elevated pyruvate concentration improves the expression of lactate dehydrogenase and allosterically activates the lactate dehydrogenase [27]. With improved lactate dehydrogenase activity, more carbon is directed to lactate rather than to ethanol in order to quickly regenerate NAD^+. The result shown in Fig. 2 suggests the presence of the same controlling mechanism by pyruvate in *C. amalonaticus* Y19.

Fig. 2 indicates that the H_2 yield in Y19 can be improved by reducing the carbon flux to lactate through deleting lactate dehydrogenase activity [2]. If carbon flow to lactate is completely diverted to ethanol and acetate *via* acetyl-CoA, the H_2 yield can be increased from 1.27 to 1.51 mol H_2/mol glucose when the initial glucose is 3.5 g/l, or from 0.72 to 1.54 mol H_2/mol glucose when the initial glucose is 9.5 g/l. This indicates that, from the analysis of carbon-mass balance and metabolic pathways network, it is possible to estimate the improvement in H_2 yield after genetic modification of H_2-producing organisms. It should be noted that the degree of improvement varies remarkably depending on fermentation conditions, especially on the glucose concentration.

In summary, the carbon and energy balances of glucose fermentation in the newly isolated *C. amalonaticus* Y19 were studied from carefully designed batch experiments. A brief metabolic pathways model was developed from the batch experiment and metabolic pathways database of similar microorganisms. Data consistency for the experiments and proposed metabolic pathways model was confirmed from the carbon-mass balances, NADH utilization, and reduction degree balances. Carbon distributions to cell mass and various metabolites suggest that H_2 metabolism in Y19 is highly interconnected with the cellular activities leading to the production of major metabolites. The low H_2 production yield at high medium glucose concentration is expected to be recovered by blocking lactate dehydrogenase.

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APPENDIX

Anaerobic metabolism of *C. amalonaticus* Y19

Pyruvate-formate lyase activity

$$\alpha\text{Glucose}+2\alpha\text{NAD}^+ +2\alpha\text{ADP}+2\alpha\text{Pi}\rightarrow 2\alpha\text{Pyruvate} +2\alpha\text{NADH}+2\alpha\text{ATP}+2\alpha\text{H}_2\text{O} \quad (1)$$

$$2\alpha\text{Pyruvate}+2\alpha\text{CoA-SH}\rightarrow 2\alpha\text{Acetyl-S-CoA} +2\alpha\text{Formate} \quad (2)$$

Net (Eqs. (1) and (2)):

$$\alpha\text{Glucose}+2\alpha\text{NAD}^+ +2\alpha\text{ADP}+2\alpha\text{Pi} +2\alpha\text{CoA-SH}\rightarrow 2\alpha\text{Formate}+2\alpha\text{NADH} +2\alpha\text{ATP}+2\alpha\text{H}_2\text{O}+2\alpha\text{Acetyl-S-CoA} \quad (3)$$

α_1 : Hydrogen production

$$\alpha_1\text{Formate}\rightarrow\alpha_1\text{CO}_2+\alpha_1\text{H}_2 \quad (4)$$

α_2 : Ethanol production

$$\alpha_2\text{Acetyl-S-CoA}+2\alpha_2\text{NADH}\rightarrow\alpha_2\text{Ethanol}+2\alpha_2\text{NAD}^+ +\alpha_2\text{CoA-SH} \quad (5)$$

α_3 : Acetate production

$$\alpha_3\text{Acetyl-S-CoA}+\alpha_3\text{Pi}\rightarrow\alpha_3\text{Acetylphosphate}+\alpha_3\text{CoA-SH} \quad (6)$$

$$\alpha_3\text{Acetylphosphate}+\alpha_3\text{ADP}\rightarrow\alpha_3\text{Acetate}+\alpha_3\text{ATP} \quad (7)$$

Net (Eqs. (6) and (7)):

$$\alpha_3\text{Acetyl-S-CoA}+\alpha_3\text{Pi}+\alpha_3\text{ADP}\rightarrow\alpha_3\text{Acetate}+\alpha_3\text{CoA-SH}+\alpha_3\text{ATP} \quad (8)$$

Net (Eqs. 3-5 and 8):

$$\alpha\text{Glucose}+(2\alpha_2-2\alpha)\text{NADH}+(2\alpha+\alpha_3)\text{ADP}+(2\alpha+\alpha_3)\text{Pi}\rightarrow(2\alpha-\alpha_1)\text{Formate}+\alpha_2\text{Ethanol}+\alpha_3\text{Acetate}+\alpha_1\text{H}_2+2\alpha\text{H}_2\text{O}+\alpha_1\text{CO}_2+(2\alpha_2-2\alpha)\text{NAD}^+ +(2\alpha+\alpha_3)\text{ATP} \quad (9)$$

The sum of mol acetate and mol ethanol due to pyruvate-formate lyase activity corresponds to the mol formate before its transformation into CO_2 and H_2

Lactate production

$$\beta\text{Glucose}+2\beta\text{NAD}^+ +2\beta\text{ADP}+2\beta\text{Pi}\rightarrow2\beta\text{Pyruvate}+2\beta\text{NADH}+2\beta\text{ATP}+2\beta\text{H}_2\text{O} \quad (10)$$

$$2\beta\text{Pyruvate}+2\beta\text{NADH}\rightarrow2\beta\text{Lactate}+2\beta\text{NAD}^+ \quad (11)$$

Net (Eqs. (10) and (11)):

$$\beta\text{Glucose}+2\beta\text{ADP}+2\beta\text{Pi}\rightarrow2\beta\text{Lactate}+2\beta\text{ATP}+2\beta\text{H}_2\text{O} \quad (12)$$

Succinate production

$$\gamma\text{Glucose}+2\gamma\text{NAD}^+ +2\gamma\text{Pi}\rightarrow2\gamma\text{Phosphoenolpyruvate}+2\gamma\text{NADH}+2\gamma\text{H}_2\text{O} \quad (13)$$

$$2\gamma\text{Phosphoenolpyruvate}+2\gamma\text{CO}_2+2\gamma\text{NADH}+2\gamma\text{QH2}\rightarrow2\gamma\text{Succinate}+2\gamma\text{Pi}+2\gamma\text{NAD}^+ +2\gamma\text{Q} \quad (14)$$

QH2, Ubiquinol; Q, ubiquinone

Net (Eqs. (13) and (14))

$$\gamma\text{Glucose}+2\gamma\text{QH2}+2\gamma\text{CO}_2\rightarrow2\gamma\text{Succinate}+2\gamma\text{Q}+2\gamma\text{H}_2\text{O} \quad (15)$$

Cell growth

$$\delta\text{Glucose}+1.62\delta\text{NH}_3\rightarrow6\delta\text{CH}_{1.67}\text{O}_{0.20}\text{N}_{0.27}+0.75\delta\text{O}_2+3.3\delta\text{H}_2\text{O} \quad (16)$$

Overall anaerobic metabolism

Net (Eqs. (9), (12), (15), (16)), where $\alpha+\beta+\gamma+\delta=1$:

$$\text{Glucose}+(2\alpha-2\alpha_2)\text{NAD}^+ -2\gamma\text{Q}+(2\alpha+\alpha_3+2\beta)\text{ADP}+(2\alpha+\alpha_3+2\beta)\text{Pi}+1.62\delta\text{NH}_3\rightarrow(2\alpha-\alpha_1)\text{Formate}+\alpha_2\text{Ethanol}+2\beta\text{Lactate}+\alpha_3\text{Acetate}+2\gamma\text{Succinate}+\alpha_1\text{H}_2+(2\alpha-2\alpha_2)\text{NADH}-2\gamma\text{QH2}+(\alpha_1-2\gamma)\text{CO}_2+(2\alpha+\alpha_3+2\beta+2\gamma+3.3\delta)\text{ATP}+(2\alpha+2\beta+2\gamma+3.3\delta)\text{H}_2\text{O}+0.75\delta\text{O}_2+6\delta\text{CH}_{1.67}\text{O}_{0.2}\text{N}_{0.27} \quad (17)$$