Regulation of Clostridium acetobutylicum Metabolism as Revealed by Mixed-Substrate Steady-State Continuous Cultures: Role of NADH/NAD Ratio and ATP Pool

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Glycerol-glucose-fed (molar ratio of 2) chemostat cultures of Clostridium acetobutylicum were glucose limited but glycerol sufficient and had a high intracellular NADH/NAD ratio (I. Vasconcelos, L. Girbal, and P. Soucaille, J. Bacteriol. 176:1443–1450, 1994). We report here that the glyceraldehyde-3-phosphate dehydrogenase, one of the key enzymes of the glycolytic pathway, is inhibited by high NADH/NAD ratios. Partial substitution of glucose by pyruvate while maintaining glycerol concentration at a constant level allowed a higher consumption of glycerol in steady-state continuous cultures. However, glycerol-sufficient cultures had a constant flux through the glyceraldehyde-3-phosphate dehydrogenase and a constant NADH/NAD ratio. A high substitution of glucose by pyruvate [P/(G + P) value of 0.67 g/g] provided a carbon-limited culture with butanol and butyrate as the major end products. In this alcohologenic culture, the induction of the NADH-dependent butyraldehyde and the ferredoxin-NAD(P) reductases and the higher expression of alcohol dehydrogenases were related to a high NADH/NAD ratio and a low intracellular ATP concentration. In three different steady-state cultures, the in vitro phosphotransbutyrylase and butyrate-kinase activities decreased with the intracellular ATP concentration, suggesting a transcriptional regulation of these two genes, which are arranged in an operon (K. A. Walter, R. V. Nair, R. V. Carry, G. N. Bennett, and E. T. Papoutsakis, Gene 134:107–111, 1993).

Clostridium acetobutylicum, a strictly anaerobic spore-forming bacterium, usually shows a biphasic batch fermentation pattern. After producing acetate and butyrate during exponential growth, the organism switches to the formation of acetone, butanol, and ethanol shortly before entering the stationary phase. The mechanisms responsible for the onset of solventogenesis are currently the focus of much scientific research.

In batch cultures, the initiation and sustained production of solvents are associated with a low extracellular and intracellular pH and a high undissociated butyric acid concentration (7, 15, 20). In continuous culture, ATP and NAD(P)H availabilities appear to play a key role in product selectivity. High ATP concentration related to low ATP demand or high efficiency of ATP generation would lead to enhanced solvent production (i) for glucose-sufficient cultures at a low pH with biomass recycling (12, 13); (ii) for iron-, nitrogen-, or phosphate-limited cultures (1, 2, 19); and (iii) during shifts induced on phosphate-limited cultures by lowering the pH or adding organic acids (6). Ethanol and butanol productions were associated with increased availability of reducing power (i) when the in vivo activity of the hydrogenase was decreased by CO gasping (5, 10, 13, 14) or by adding methyl viologen (6); (ii) during a shift in solvent production by lowering the pH when acetyl coenzyme A (CoA) was first converted to acetone (a pathway consuming no reducing energy), creating a redox imbalance (6); (iii) when an NADH pressure was provided by culturing the microorganism on glucose and a more reduced substrate (21). Under such mixed substrate (glucose plus glycerol) growth, the culture was reported to be glucose but not glycerol limited. In this study, it is shown that glycerol catabolism was limited at the level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by a high intracellular NADH/NAD ratio. Furthermore, the relationship between product selectivity, enzymatic activities, and either NADH or ATP levels was established for steady-state continuous culture of C. acetobutylicum on glucose-glycerol-pyruvate mixtures.

MATERIALS AND METHODS

Organism and growth conditions. The organism used was C. acetobutylicum ATCC 824. A stock culture in spore form was stored at −20°C, in the synthetic medium optimized by Monot et al. (16), and modified as described by Vasconcelos et al. (21). Continuous steady-state cultures were realized in the culture vessel previously described (21). The cell concentration was controlled by phosphate limitation, but in some of the steady-state cultures, the culture was also carbon limited. The feed medium for the continuous culture contained (per liter of distilled water) the following: KH₂PO₄, 0.1 g; KCl, 0.65 g; MgSO₄·7H₂O, 0.2 g; FeSO₄·7H₂O, 0.028 g; NH₄Cl, 1.5 g; CoCl₂·6H₂O, 0.01 g; biotin, 0.04 mg; p-aminobenzoic acid, 8 mg; stevクトol (antifoam), 0.1 g; and a constant total carbon concentration of 995 mM, with 163 mM glycerol and various concentrations of glucose and pyruv acid as specified in the text. Vitamins and pyruvic acid were sterilized by filtration. The dilution rate was 0.05 h⁻¹, and the temperature was controlled at 35°C. The pH was automatically maintained at 6.5 by the addition of NH₄OH (6 M).

Analysis. Biomass concentration was measured by cell dry weight determination. The concentrations of glucose, glycerol, lactate, acetoin, solvents, and acids were determined by high-
pressure liquid chromatography as described by Vasconcelos et al. (21). The fermentor effluent gas was measured by a gas flow meter and analyzed by gas chromatography (21). Protein determination was performed by the method of Bradford (3), because this assay is not subject to interference by thiol reagents.

Enzyme assays. The cell-free extracts were prepared by the strictly anaerobic procedures of Vasconcelos et al. (21). Unless otherwise indicated, all enzyme activities were determined in the physiological direction at 30°C.

Glycerol dehydrogenase (GDH) was measured spectrophotometrically by monitoring glycerol-dependent NADH formation in a solution of 0.1 M potassium bicarbonate (pH 9), 30 mM ammonium sulfate, 0.1 M glycerol, 2 mM dithiothreitol, and 0.5 mM NAD.

All the other assays for the following activities were adopted from the methods of Vasconcelos et al. (21): hydrogenase in both hydrogen uptake and hydrogen evolution directions; ferredoxin-NAD(P) reductase; NAD(P)H-ferredoxin reductase; NADH- and NADPH-dependent ethanol, butanol, acet-aldehyde, and butyraldehyde dehydrogenases; CoA transferase and acetateacetoacetate decarboxylase; phosphotransacetylase and phosphotransbutyrylase (PTB); acetate kinase in the physiological and nonphysiological directions; butyrate kinase (BK) in the nonphysiological direction; pyruvate dehydrogenase; GAPDH; thiolase in the nonphysiological direction and in air; and β-hydroxybutyryl-CoA dehydrogenase. Apart from CoA-transferase, acetateacetoacetate decarboxylase, and thiolase, which were assayed in the presence of air, all other activities were measured under strictly anaerobic conditions.

The effect of the NADH/NAD ratio on GAPDH activity was determined in the presence of arsenate, since it competes with phosphate and forms an unstable intermediate which rapidly decomposes. Under these conditions, 1,3-biphosphoglycerate never accumulates and a significant reverse reaction is not possible (11).

Determination of nucleotide pools. Intracellular concentrations of ATP, ADP, NAD(P), and NAD(P)H were determined after extraction of a culture broth sample as reported by Vasconcelos et al. (21): ATP and ADP levels were measured with a bioluminescence assay, and NAD(P)⁺ and NAD(P)H levels were measured with a fluorimetric enzyme assay.

Calculations. The parameters $q_{\text{NADHpro}}$, $q_{\text{NADHconc}}$, and $Y_{\text{ATP}}$ can be calculated from the specific production and consumption rate $q$ (in millimoles per gram per hour), according to the product distribution described earlier by Papoutsakis (18):

\[
q_{\text{NADHpro}} = q_{\text{ethanol}} + q_{\text{acetate}} + q_{\text{lactate}} + 2q_{\text{acetoin}} + 2q_{\text{butanol}} + 2q_{\text{butyrate}} + 2q_{\text{acetone}} + q_{\text{glycerol}} - q_{\text{pyruvate}}
\]

\[
q_{\text{NADHconc}} = 2q_{\text{ethanol}} + q_{\text{lactate}} + 4q_{\text{butanol}} + 2q_{\text{butyrate}}
\]

\[
Y_{\text{ATP}} = \frac{1000}{D - q_{\text{ATP}}}
\]

$Y_{\text{ATP}}$ is in grams of cell per mole of ATP produced, and $D$ is the dilution rate (in hours⁻¹).

Chemicals. Enzymes and coenzymes were purchased from Sigma Chimie (St Quentin Fallavier, France). All other chemicals were of analytical grade.

RESULTS

Effect of the NADH/NAD ratio on the activity of GDH and GAPDH. It was previously reported (21) that a steady-state continuous culture, in which biomass concentration was limited by the phosphate content of the medium, with a constant amount of carbon (995 mM) in the feed medium, was also carbon limited on glucose but carbon sufficient on a glucose-glycerol mixture (residual glycerol). The high intracellular NADH/NAD ratio reported during such mixed-substrate growth might inhibit some of the key enzymes leading to pyruvate and thereby limit the microorganism capacity to consume glycerol. To investigate this hypothesis further, the effect of the NADH/NAD ratio on GAPDH, an enzyme known to be affected by the NADH/NAD ratio in other clostridia (11), and on GDH, the first enzyme of glycerol metabolism that uses NAD as a cofactor, was examined (Fig. 1).

Significant inhibition of GAPDH by the NADH/NAD ratio was observed with no measurable activity at a NADH/NAD ratio greater than 1. During glucose-glycerol culture, the NADH/NAD ratios has been shown to be as great as 0.75, a level at which 90% inhibition of activity might be expected. On the other hand, GDH was not significantly affected by the NADH/NAD ratios.

Carbon and energy flux on glucose-glycerol-pyruvate mixtures. In order to further confirm that a high NADH/NAD ratio limits glycerol consumption at the level of GAPDH, chemostat cultures were established under the same conditions as those used by Vasconcelos et al. (21), except that part of the glucose was replaced by pyruvate (while maintaining a constant glycerol concentration and total amount of carbon in the feed medium), a more oxidized substrate that enters central metabolism downstream of the GAPDH. Two different pyruvate/glucose + pyruvate) ratios [this ratio will be called $P/(G + P)$ and is expressed in grams per gram], 0.33 and 0.67, were used. It is apparent from Fig. 2A that the specific glycerol consumption rate $q_{\text{glycerol}}$ increased with glucose substitution by pyruvate. For a $P/(G + P)$ value of 0.67, the enhanced $q_{\text{glycerol}}$ led to glycerol limitation. The in vivo flux through the GAPDH was constant for the two glycerol nonlimited cultures but decreased for the carbon-limited culture (Fig. 2B).

Carbon distribution in the end products was only slightly
The $q_{\text{NAD(P)H}}$ from $P$, the specific rate of NAD(P)H production by the ferrodoxin-NAD(P) reductases (the difference between $q_{\text{NAD(P)H,cons.}}$, the specific NAD(P)H consumption rate to form end products, and $q_{\text{NAD(P)H,prod.}}$, the specific NADH generation rate during substrate conversion to pyruvate) was higher for the pyruvate-substituted cultures, indicating a more efficient deviation of the electron flow from reduced ferrodoxin to NAD(P)H formation at the expense of $H_2$ formation. Analysis of the nucleotide pools showed that the NADH/NAD ratio was constant for the glycerol excess cultures but de-

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>Value for following data set$^c$:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution rate ($h^{-1}$)</td>
<td>0.05</td>
</tr>
<tr>
<td>Biomass concn (g/liter)</td>
<td>1.057</td>
</tr>
</tbody>
</table>

Fed substrate concn (mM)
- Glycerol: 163, 163, 163
- Glucose: 83, 56, 28
- Pyruvate: 0, 57, 114

Residual concn (mM)
- Glycerol: 65, 33.5, 0
- Glucose: 0, 0, 0
- Pyruvate: 0, 0, 0

Specific production rate$^d$ (mmol/h/g)
- Ethanol: 1.3, 1.656, 0.714
- Butanol: 3.859, 4.594, 3.92
- Acetate: 0.416, 0.437, 0.506
- Butyrate: 0.658, 0.91, 2.04
- Lactate: 0, 0.046, 0.223
- Acetoin: 0.119, 0.083, 0.062
- CO$_2$: 11, 13.14, 13.5
- CO$_2$/H$_2$: 7.24, 6.29, 7.92
- H$_2$: 1.52, 2.1, 1.7

Product yield$^d$
- Butanol: 41.1, 41.6, 34
- Ethanol: 6.9, 7.5, 3.1
- Acetate: 2.2, 1.5, 2.2
- Butyrate: 7, 8.3, 17.7

* Cultures were at a pH of 6.5, with a feed concentration of carbon of 995 mM.
* Data sets correspond to different P(G + P) values: I, 0 g/g; II, 0.33 g/g; and III, 0.67 g/g.
* Determined with an average accuracy of ±5%.
* Expressed as percent values of carbon recovery on a molar basis. Average accuracy, ±5%.

rate from Table 1, footnote $b$.
$^b$ Cultures were at a pH of 6.5, with a dilution rate of 0.05 $h^{-1}$.
$^c$ The values reported are from Vasconcelos et al. (21).

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Conc (pmol/g [dry cell mass]) (SD) for following data set $^a$ ($n = 4$):</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1.6 (0.15)</td>
</tr>
<tr>
<td>ADP</td>
<td>1.8 (0.2)</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>6.7 (1.9)</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>0.63 (0.2)</td>
</tr>
<tr>
<td>NADH</td>
<td>5.05 (1.3)</td>
</tr>
<tr>
<td>NADPH</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>NADH/NAD</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* See Table 1, footnote $b$.

- FIG. 2. Influence of the P(P + G) value in continuous phosphate-limited steady-state cultures of *C. acetobutylicum* (pH 6.5; dilution rate, 0.05 h$^{-1}$). (A) Influence on the specific substrate consumption rates of glycerol, glucose, and pyruvate and on the $Y_{ATP}$ value; (B) Influence on the specific NADH consumption rate, $q_{\text{NADH,cons.}}$; the specific NADH production rate, $q_{\text{NADH,prod.}}$; the $q_{\text{NADH,prod.}}$ value (difference $q_{\text{NADH,cons.}} - q_{\text{NADH,prod.}}$); and on the flux through the GAPDH.

- Table 1. Fermentation parameters for continuous phosphate-limited steady-state cultures of *C. acetobutylicum*

- Table 2. Influence of different P(G + P) values$^a$ on nucleotide levels of continuous phosphate-limited steady-state cultures of *C. acetobutylicum*
The activities of enzymes that were not statistically different when part of the glucose was substituted by pyruvate are listed below: hydrogenase, ferredoxin-dehydrogenases, NADH and NADPH-dependent acetaldehyde dehydrogenases, NADH- and NADPH-dependent ethanol dehydrogenases, NADH- and NADPH-dependent butanol dehydrogenases, CoA-transferase, GAPDH, thiolase, and NADH- and NADPH-dependent 3-hydroxybutyryl-CoA dehydrogenases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sp act (μmol/min/mg) (SD) for following data set (n = 6):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
</tr>
<tr>
<td>Solventogenic</td>
<td></td>
</tr>
<tr>
<td>Butyraldehyde dehydrogenase</td>
<td>0.016 (0.006) 0.028 (0.012)</td>
</tr>
<tr>
<td>NADH</td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.28 (0.07) 0.029 (0.01)</td>
</tr>
<tr>
<td>Acetocacate decarboxylase</td>
<td></td>
</tr>
<tr>
<td>Acidogenic</td>
<td></td>
</tr>
<tr>
<td>Phosphotransacetylase</td>
<td>0.16 (0.07) 1.75 (0.2)</td>
</tr>
<tr>
<td>Acetate kinase</td>
<td></td>
</tr>
<tr>
<td>Physiological direction</td>
<td>1.7 (0.4) 3.49 (0.5)</td>
</tr>
<tr>
<td>Reverse direction</td>
<td>5.6 (0.8) 11.6 (0.9)</td>
</tr>
<tr>
<td>PTB</td>
<td>2.62 (0.23) 10.9 (1.4)</td>
</tr>
<tr>
<td>BK, reverse direction</td>
<td>0.48 (0.1) 3.03 (0.8)</td>
</tr>
<tr>
<td>Central axis</td>
<td></td>
</tr>
<tr>
<td>Pyruvate ferredoxin reductase</td>
<td>2.7 (1.2) 6.6 (2)</td>
</tr>
</tbody>
</table>

* The activities of enzymes that were not statistically different when part of the glucose was substituted by pyruvate are listed below: hydrogenase, ferredoxin-dehydrogenases, NADH and NADPH-dependent acetaldehyde dehydrogenases, NADH- and NADPH-dependent ethanol dehydrogenases, NADH- and NADPH-dependent butanol dehydrogenases, CoA-transferase, GAPDH, thiolase, and NADH- and NADPH-dependent 3-hydroxybutyryl-CoA dehydrogenases.

a At a pH of 6.5 and a dilution rate of 0.05 h⁻¹.

b Values are from Vasconcelos et al. (21).

creased for the culture that was carbon limited (Table 2). The $Y_{ATP}$ (grams of dry biomass per mole of ATP produced) increased by 8 and 17% for P/(G + P) values of, respectively, 0.33 and 0.67 (Fig. 2A). Since the biomass level was similar during the three cultures, changes in $Y_{ATP}$ were directly dependent on ATP production. For a P/(G + P) value of 0.33, ATP synthesis during substrate conversion to pyruvate was reduced by 15%. For a P/(G + P) value of 0.67, this diminution was about 26%, but it was slightly counterbalanced by the increase of ATP generation by butyrate production. Pyruvate addition and the related increase in glycerol consumption were associated with a lower ATP production. Furthermore, when the glucose substitution by pyruvate was increased, the measured concentration of intracellular ATP was diminished while ADP increased (Table 2).

**Levels of key metabolic enzyme activities.** The levels of key enzyme activities were measured for a P/(G + P) value of 0.67 and compared with the values obtained previously by Vasconcelos et al. (21) for a P/(G + P) value of 0 (Table 3). A lot of enzymes presented statistically the same level for the two cultures, and in order to simplify Table 3, they are not presented.

(i) **Hydrogenases and coupling enzymes.** The hydrogenase activities measured in both directions are not statistically different for the two cultures. The ratios of H₂ uptake to H₂ evolution are 12 and 16 for cultures on glucose-glycerol and glucose-glycerol-pyruvate, respectively.

Both cultures show the same level and therefore the same type of regulation for the ferredoxin-oxidoreductase activities: high ferredoxin-NAD and ferredoxin-NADPH reductase activities and only a low NADH-ferredoxin reductase activity.

On both glucose-glycerol-pyruvate and glucose-glycerol mixtures, the reoxidation of reduced ferredoxin generated during pyruvate conversion to acetyl-CoA will preferentially occur via the ferredoxin-NAD(P) reductase pathway rather than via hydrogenase activity.

(ii) **Enzymes associated with solvent production.** All alcohol and aldehyde dehydrogenases present a higher activity with NADH as a cofactor than with NADPH.

The NADH-dependent acetaldehyde dehydrogenase activity remained constant when glucose is substituted by pyruvate, while a slightly higher level of expression of butyraldehyde dehydrogenase (for both NADH and NADPH as a cofactor) was recorded. The levels of NADH-dependent ethanol and butanol dehydrogenases were not statistically different for the two cultures. The lack of acetone production for both cultures was related to low CoA-transferase and acetococatalate decarboxylase activities compared with an acetone-producing culture (21). However, the acetococatalate decarboxylase level decreased by a factor of 10 when pyruvate was added.

(iii) **Enzymes associated with acid formation.** The phosphotransacetylase and acetate kinase were measured for a P/(G + P) value of 0 (Table 3). A lot of enzymes presented statistically the same level for the two cultures, and in order to simplify Table 3, they are not presented.

(iv) **Other enzyme activities.** Pyruvate ferredoxin oxidoreductase activity was increased by a factor of 2.4 when glucose was substituted with pyruvate. On the other hand, GAPDH, thiolase, and β-hydroxybutyryl-CoA dehydrogenase levels were not statistically affected by pyruvate addition.

**DISCUSSION**

In a previous study (21), a steady-state continuous culture of *C. acetobutylicum*, with a constant amount of carbon (995 mM) in the feed medium, had been shown to be carbon limited on glucose but carbon sufficient on a glucose-glycerol mixture (residual glycerol). In this study, it is reported that the GAPDH was inhibited by high NADH/NAD ratios while the
GDH was unaffected. As a high NADH/NAD ratio was measured on glucose-glycerol-fed chemostat cultures, it is tempting to speculate that high NADH/NAD ratios limited glycerol consumption by an inhibition of the GAPDH (Fig. 4). In a steady-state continuous culture, when part of the glucose was substituted by pyruvate (while maintaining constant glycerol concentration and total amount of carbon in the feed medium), with a \( P/(G + P) \) value of 0.33, the microorganism consumed more glycerol to finally reach the same flux through the GAPDH and the same NADH/NAD ratio. A \( P/(G + P) \) value of 0.67 led to a carbon-limited culture with a lower flux through the GAPDH and a lower NADH/NAD ratio. This suggests that for this degree of pyruvate substitution the microorganism would be able to consume more glycerol if provided in the feed before reaching the maximum flux capacity of the GAPDH fixed by the NADH/NAD ratio. This carbon-limited culture produced butanol and butyrate as the major end products, did not produce acetone, and had a high positive \( q_{\text{NADH/NAD}} \) from \( F_{\text{d}} \) value. As previously described for glucose-glycerol-grown chemostat cultures of \( C. \) acetobutylicum (21), this pattern of product formation and distribution of the electron flow was also associated to the induction/derepression of NADH-dependent butyraldehyde and alcohol dehydrogenases and ferredoxin-NAD(P) reductases and also with low-level expression of NADH-ferredoxin reductase, CoA-transferase, and acetoacetate decarboxylase. Although already low on glucose-glycerol cultures (compared with acetone-producing cultures [21]), addition of pyruvate further decreased the level of acetoacetate decarboxylase by a factor of 10. Janati-Idrissi et al. (8) reported a low acetoacetate decarboxylase biosynthesis during batch cultures on pyruvate as a carbon source at pH 4.8.

Higher butyric acid production in the glucose-glycerol-pyruvate culture compared with that grown on glucose-glycerol was associated with a higher level of expression of the PTB and BK. Under steady-state conditions, the in vitro PTB and BK activities (i.e., enzyme concentration) decreased linearly with the intracellular ATP concentration. As these two genes form an operon (4, 22), it is tempting to speculate that ATP regulates their level of expression (Fig. 4). Obviously, as soon as an in vitro transcription system is available, this will need to be verified. From a physiological point of view, this might be a good way for the cells to modulate an alternative pathway of ATP production as a function of ATP level. However, butyrate production was also controlled by other factors, since cultures grown on glucose alone (21) produced three times more butyric acid than did cultures grown on glucose-glycerol-pyruvate but had lower activities of the two enzymes.

Meyer and Papoutsakis (13) reported several correlations between the ATP and NADH levels and end product selectivity in various continuous cultures of \( C. \) acetobutylicum. In glucose-limited cultures, acidogenic metabolism was associated with low ATP and NADH levels, less than 0.64 and 0.33 \( \mu \text{mol/g} \) of cell mass, respectively. On the other hand, under glucose-sufficient conditions, solventogenic metabolism was related to a high ATP pool (5 \( \mu \text{mol/g} \) of cell mass) and a
relatively low NADH level (0.36 μmol/g of cell mass). Moreover, high levels of intracellular ATP and NADH were seen in solventogenic continuous cultures: (i) for glucose-limited cultures sparged with CO (13); (ii) when the pH was lowered or after organic acids or methyl viologen was added (6); (iii) in glucose-glycerol mixed-substrate experiments (21). These results from the literature support the hypothesis proposed by Papoutsakis (17) that biosynthetic energy (ATP) could be a key factor determining product selectivity: a low ATP concentration would be related to acidogenesis, and a high ATP level would relate to solventogenesis, with the NADH level contributing or not to the increase of solvent production. However, our results with continuous cultures grown at neutral pH on glucose-glycerol-pyruvate mixtures clearly illustrate that alcohol production can occur under conditions in which a high NADH/NAD ratio but a low ATP are found (Fig. 4).

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REFERENCES