A Strategy for Improving the Yield of Hydrogen by Fermentation

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Abstract: It was theoretically shown that the yield of hydrogen by fermentation will be improved up to 10 mole of hydrogen from 1 mole of glucose. The maximal yield will be achieved by inhibiting the NADH dehydrogenase complex of the electron transport chain under aerobic condition. Aerobic condition is needed to produce large amount of NADH at TCA cycle and also to re-oxidize FADH2 through electron transport chain by oxygen or other electron acceptors. This method is effective to the facultative anaerobic bacteria or aerobic bacteria with hydrogenase.

keywords: hydrogen production, fermentation, *Enterobacter aerogenes*, TCA cycle, inhibition, electron transport chain

1. Introduction

Recently hydrogen production by fermentation has been widely noticed as the practical use of fuel cell vehicle becomes close at hand. Because, the huge amount of garbage exhausted in cities is a good substrate for the fermentative hydrogen production, and the estimated amount of garbage energy in Japan is more than 10% the Japan's gasoline consumption. The fermentative production of hydrogen is therefore expected as one of the onsite production methods of hydrogen. However, since the yield of hydrogen by fermentation is small as approximately 2 moles from 1 mole of glucose [1], improving the yield of hydrogen is an argent subjects to be solved.

In this paper, a new strategy for improving the yield of hydrogen by fermentation is proposed.

2. NADH pathway of hydrogen evolution and NADH production system

Many bacteria evolve hydrogen through NADH pathway by fermentation. 2 moles of NADH are produced by glucose assimilation as shown in Equation (1), and 2 moles of H_2 are evolved by an enzymatic reaction like Equation (2).

(1) $C_6H_{12}O_6 + 2NAD^+ \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2NADH + 2H^+$

(2) $2NADH + 2H^+ \rightarrow 2NAD^+ + 2H_2$

The yield of 2 was actuary reported in the butyrate fermentation [2,3], however, by a modification of fermentation pathway, we can only expect at most 4 mol in the ideal acetate fermentation as follows.

(3) $C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2$

Now, Turning our eyes to aerobic digestion of glucose, we can see 10 moles of NADH, i.e., aerobic and facultative anaerobic bacteria produce 8 moles of NADH through TCA cycle in addition to the 2 mol NADH from glycolysis like follows:

Glycolysis:

(4) $C_6H_{12}O_6 + 2NAD^+ (+ 2ADP) \rightarrow 2CH_3COCOOH + 2NADH + 2H^+ (+ 2ATP)$

TCA cycle:

(5) $2CH_3COCOOH + 8NAD^+ + 2FAD + 6H_2O (+ 2ADP) \rightarrow 6CO_2 + 8NADH + 8H^+ + 2FADH_2 (+ 2ATP)$

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(6) $C_6H_{12}O_6 + 10NAD^+ + 2FAD + 6H_2O (+ 4ADP) \rightarrow 6CO_2 + 10NADH + 10H^+ + 2FADH_2 (+ 4ATP)$

These total 10 mol NADH and 2 mol $FADH_2$ are usually re-oxidized by transporting electrons to oxygen through the electron transport chain as follows:

(7) $NADH + H^+ + 1/2O_2 \rightarrow NAD^+ + H_2O$

(8) $FADH_2 + 1/2O_2 \rightarrow FAD + H_2O$ $\Delta G_0' = -200$ kJ/mol, $K_0' = 1.1 \times 10^{35}$

Thus if we can use all these NADH and also $FADH_2$ as the source of H_2 evolution, we may expect maximum 12 mol for the yield of hydrogen from glucose.

Then, how can we produce H_2 under active TCA cycle.

3. What is the key to keeping TCA cycle active

It was reported by Tanisho that a facultative anaerobic bacterium instantly utilized O_2 when anaerobic condition was changed to aerobic condition [4]. This means that the TCA cycle and also the electron transport chain keep their properties even under anaerobic condition in the bacterium cell. Nevertheless, why the TCA cycle can't be active under anaerobic condition but the metabolism is carried by fermentation?

3.1 Free energy change of TCA cycle

As shown in **Figure 1**, TCA cycle starts from citrate production that is resulted from the reaction between acetyl-CoA and oxaloacetate. Since this is equimolar reaction between acetyl-CoA and oxaloacetate, pyruvate decomposition to acetyl-CoA will be restricted by the amount of oxaloacetate.

(9) Acetyl-CoA + oxaloacetate + $H_2O \rightarrow$ citrate + CoA-H + H^+ $\Delta G_0' = -32.2$kJ/mol

This reaction, however, has a strongly negative $\Delta G_0'$ at –32.2 kJ/mol, oxaloacetate must react immediately with acetyl-CoA, and consequently, concentration of oxaloacetate will be usually kept at nearly 0 M.

Free energy changes of TCA cycle after citrate production until succinate production, they are nearly 0 or strongly negative in all reactions. These reactions therefore must run easily to right side. However, reactions from succinate to fumarate and from malate to oxaloacetate have in contrast extremely positive $\Delta G_0'$ at +49.0 kJ/mol and +29.7 kJ/mol, respectively.

(10) Succinate + FAD $\rightarrow$ fumarate + $FADH_2$ $\Delta G_0' = 49.0$ kJ/mol, $K_0' = 2.58 \times 10^{-9}$

(11) Malate + $NAD^+ \rightarrow$ oxaloacetate + NADH + H^+ $\Delta G_0' = 29.7$ kJ/mol, $K_0' = 6.22 \times 10^{-6}$

Reactions having these extremely positive $\Delta G_0'$ easily stop running by reaching the equilibration due to very few amount of products. Reaction (11), therefore, may stop by very few amount of oxaloacetate. However, since oxaloacetate has been kept nearly 0 by the reaction (9), and NADH is easily oxidized by oxygen through electron transport chain under aerobic condition, consequently the concentration ratio of $[NADH]/[NAD^+]$ is kept small, this reaction proceeds perpetually from left to right. These are the reasons why the reaction (11) proceeds to the right.

The most notable matter for hydrogen production is that this reaction may run even under anaerobic condition. According to the mechanism of H_2 evolution through NADH pathway [x], protons receive electrons to be H_2 at the culture side of the cell membrane from NADH. Thus, if culture pH is kept low at around 5 to make redox potential of H_2/H^+ high, as shown in **Figure 2**, the concentration ratio of [Oxaloacetate]/[Malate] may be made large enough for running the cycle by H_2 production.

3.2 $[FADH_2]/[FAD]$ ratio as the key of fumarate evolution

In contrast, reaction (10) (from malate to fumarate) is a reaction FAD taking part in, and has distinctive properties as not only extremely small equilibrium constant at $K_0' = 2.58 \times 10^{-9}$ but also fairly high standard redox potentials at +0.033V and –0.21V for fumarate/succinate and FAD/$FADH_2$ pairs, respectively. Even if the concentration of $FADH_2$ becomes slightly high, for example $[FADH_2]/[FAD] = 1 \times 10^{-3}$, the ratio [fumarate]/[succinate] goes down to extremely small at 10^{-6}, and consequently the concentration of fumarate becomes quite small. Owing to this result, concentration of malate also becomes quite small and finally the TCA cycle stops to keep its function. $FADH_2$, therefore, should be oxidized rapidly and the concentration ratio $[FADH_2]/[FAD]$ must be kept quite small for the circulation. Organisms have solved this problem under aerobic condition by transporting electrons to oxygen which has very high standard redox potential at +0.818V.

(8) $FADH_2 + 1/2O_2 \rightarrow FAD + H_2O$ $\Delta G_0' = -200$ kJ/mol, $K_0' = 1.1 \times 10^{35}$

The ratio of [$FADH_2$]/[FAD] is exactly the key of circulation of the TCA cycle.

4. Electron transport chain and inhibition

From the above consideration, it has made clear that how to oxidize $FADH_2$ is the most important problem for utilizing TCA cycle. In the previous paper [4], the author proposed a scheme to oxidize $FADH_2$ by producing H_2 to keep cultural pH low at around 5, however, it seems difficult because the redox potential of succinate/fumarate pair is too high as seen in Figure 2. So the author proposes another scheme where $FADH_2$ is re-oxidized by oxygen while NADH is re-oxidized by H_2 production.

4.1 Where should we inhibit the electron transport chain

Electron transport chain is the enzyme system to re-oxidize $FADH_2$ and NADH by oxygen. The enzymes of the electron transport chain are not the same for all microorganisms but different organisms may have different enzyme systems, the mitochondrial electron transport chain is composed of 4 enzyme complexes as shown in **Figure 3**. Since NADH is re-oxidized by oxygen under the aerobic condition, the re-oxidation of NADH should be inhibited for H_2 production. Fortunately, the enzyme complex active to NAD is different from the enzyme complex active to FAD, therefore, we can easily recognize that the inhibiting site should be NADH dehydrogenase complex. Showing the over all reaction equation, we can see that the yield of hydrogen will be maximum 10 mol in this case as follows:

(12) $C_6H_{12}O_6 + 10NAD^+ + 2FAD + 4ADP + 6H_2O \rightarrow 10NADH + 10H^+ + 2FADH_2 + 6CO_2 + 4ATP$

(13) $2FADH_2 + O_2 + 4ADP \rightarrow 2FAD + 2H_2O + 4ATP$

(14) $10NADH + 10H^+ \rightarrow 10NAD^+ + 10H_2$

(15) $C_6H_{12}O_6 + 8ADP + O_2 + 4H_2O \rightarrow 10H_2 + 6CO_2 + 8ATP$

4.2 Phenomena that will appear when TCA cycle is active under inhibition

Many inhibitors acting on the enzyme complexes of electron transport chain have been reported as shown in Figure 3. Since inhibitions by these reagents were examined by using extracted enzymes, it is not definite whether they effect on living cell or not. Our experiments using these inhibitors under aerobic condition have not given good results yet, but we can imagine by thought experiment what will be observed if the TCA cycle is still running even under the condition that the NADH dehydrogenase is inhibited. Followings are the results:

1) Based from the Equation (15), since the productivity of ATP becomes higher than fermentation (from 2ATP to 8ATP), the consumption rate of glucose becomes small.
2) The growth yield of the cell from substrate becomes larger owing to the higher ATP productivity.
3) The gas evolution rate becomes faster, because the productivities of H_2 and also CO_2 become higher.
4) The yield of CO_2 becomes lager based from the Equation (15).
5) The yield of H_2 becomes larger, if the NADH dehydrogenase is not the enzyme participating H_2 production, since the yield of NADH becomes larger.
6) Concentration ratio between H_2 and CO_2 changes in the product gas.
7) Total amount of metabolic products decreases.

From the above consideration, we will be able to judge whether the hydrogen yield is developed by the inhibition on NADH dehydrogenase in an actual experiment or not.

5. Conclusion

The yield of hydrogen from glucose will be improved up to 10 mol-H_2/mol-glucose by using aerobic or anaerobic bacteria which have hydrogenase for H_2 production. To secure this maximal yield, the following operations should be required, that is to use high redox potential electron acceptors such as oxygen or so, inhibit NADH dehydrogenase complex of electron transport chain or screen mutants which loose the enzyme complex to re-oxidize NADH but have the enzyme complex to re-oxidize $FADH_2$ on the electron transport chain. By the above consideration, the strategy improving the yield of hydrogen by fermentation has been cleared.

References

[1] Tanisho, S. and Y. Ishiwata, Continuous hydrogen production from molasses by the bacterium Enterobacter aerogenes, *Int. J. Hydrogen Energy*、**19**、807-812 (1994).

[2] Wood, W.A., in The Bacteria, ed. I.C. Gunsalus & R.Y. Stanier, Fermentation of carbohydrates and related compounds, Academic Press, Vol. II, p.77, 1961.

[3] Miyake, J., X.-X. Mao and S.Kawamura, Hydrogen photoproduction from glucose by co-culture od a photosynthetic bacterium and Clostridium butyricum, J. Ferment. Technol., 62, 531 (1984).

[4] Tanisho, S., in BioHydrogen, ed. O. R. Zaborsky, Hydrogen Production by Facultative Anaerobe Enterobacter aerogenes, Plenum Press, p.273-279, 1998.

[5] Tanisho, S., N. Kamiya and N. Wakao, Hydrogen evolution of Enterobacter aerogenes depending on culture pH, *Biochim. Biophys. Acta*、**973**, 1-6 (1989).

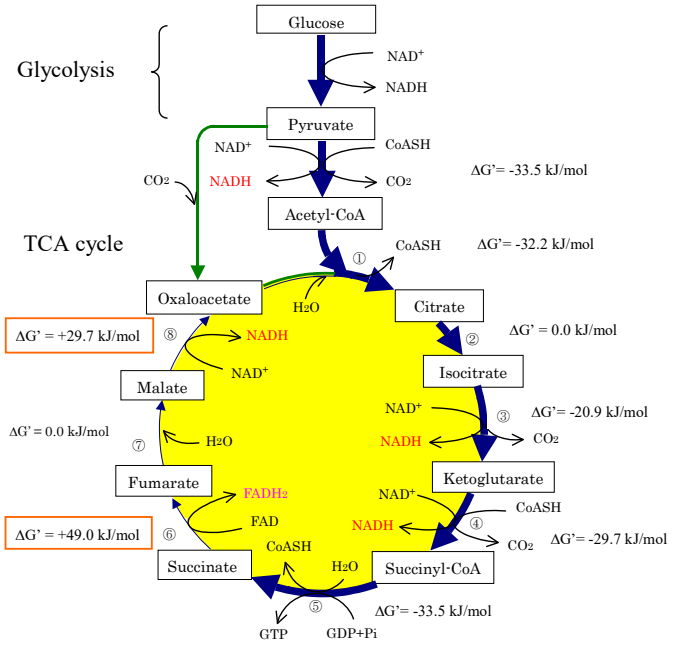

Fig.1 Glycolysis and Tricarboxylic acid (TCA) cycle

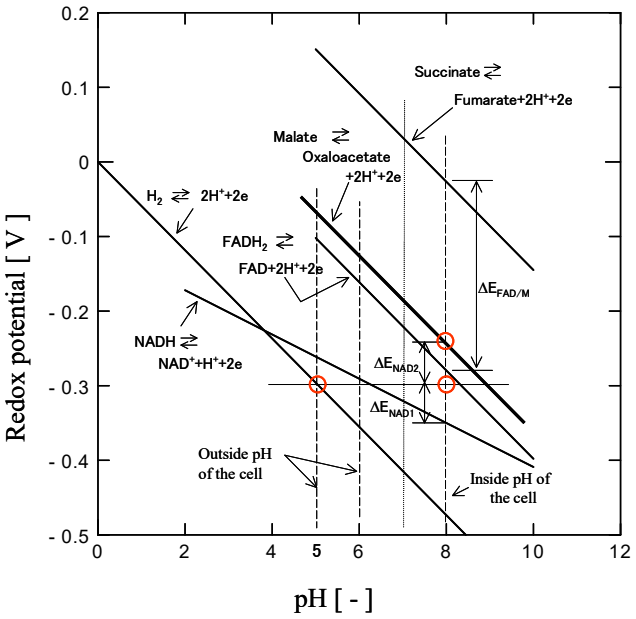

Fig. 2 Reduction-Oxidation potental vs. pH

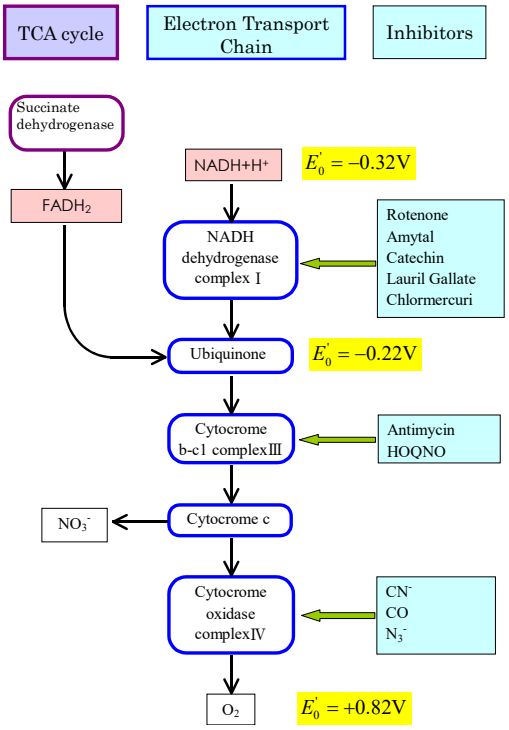

Fig.3 Electron transport chain of mitochondria