HYDROGEN PRODUCTION BY FERMENTATION AND A TRIAL FOR IMPROVEMENT ON THE YIELD OF HYDROGEN

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Abstract

Continuous hydrogen production by *Enterobacter aerogenes* strain E.82005 was examined under cultivation with urethane foam as flock's supporter. The evolution rate of hydrogen from molasses was ca. 13 mmol/l-culture.h^{-1} which is a little faster than the speed obtained under batch cultivation. Metabolite compositions in the waste of culture liquid were very different from that of batch cultivation as pointed out in a previous paper. Lactate was the main metabolite. The yields of hydrogen from substrate were ca. 2 mol-H_2/mol-sub. on the average and 3.5 mol-H_2/mol-sub. at the maximum estimating in terms of sucrose. These results were fairly better than the previous result (1.5 mol-H_2/mol-sub. on the average) obtained from a continuous cultivation without urethane foam.

Another experiments were tried aiming at an improvement on the yield of hydrogen from substrates. The hydrogen evolving pathway of *E. aerogenes* E.82005 was known from balance analyses of metabolites to be the NADH pathway, an increase of NADH yield was considered to cause the increase of the yield of hydrogen. Since ten moles of NADH are produced under aerobic degradation of glucose and re-oxidized at the electron-transport chain, it was tried to inhibit the electron-transport chain with CN^- ion under aerobic cultivation. Then, speeds of gas evolution (H_2 and CO_2) were measured just after the inhibition. Valuable effects on the gas evolution speed were obtained from the inhibition.

1. INTRODUCTION

Hydrogen production by fermentation has some merits compared with the photohydrogen production as:
(1) capable to produce not only in the daytime but also in the nighttime,
(2) independent to weather and climatic conditions.
These merits are very important factors for industrial production of hydrogen. Having a high speed on hydrogen production is also a very important factor for
the industrial production. *Enterobacter aerogenes* st. E.82005 which was found by Tanisho et al.[1] is a facultative anaerobe and evolved hydrogen at speeds of 17 mmol/(g•h) in batch cultivation [2] and 36 mmol/(l-culture•h) in continuous cultivation [3].

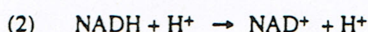
The reason for having shown such a high speed in continuous cultivation was pointed out that the density of bacteria became high as compared with that of batch cultivation because of the formation of flocks on the wall of the culture vessel. Keeping higher density of bacteria, therefore, was expected to lead higher speed of hydrogen production. For this purpose, some of the immobilizing methods, such as calcium alginate, carrageenan, poly-acrylamide and ager methods, were examined and their effects on hydrogen evolution were measured. However, owing to supporter's prevention on the supply of substrate to bacteria and the transfer of hydrogen from bacteria, we could not have favourable results from these immobilizing methods.

It is also very important to be large the yield of hydrogen molecule from substrates, because it evaluates the efficiecy of the utilization of hydrogen atoms in the substrate. The following reaction which produces acetate by glucose fermentation, for example,

(1) $$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH\ COOH + 4H_2 + 2CO_2$$

will have 4 moles of hydrogen as the yield from glucose, if any other reactions except this reaction do not proceed in the same time. In general, as shown in Table 1, some reactions producing various products proceed in parallel with each other during fermentation [2-4]. Therefore, it is difficult to get large yield such like Eq.(1). Thus, to develop certain methods for getting large yield has been an important subject.

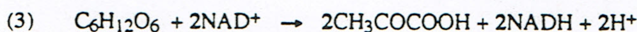
Roughly speaking, there are 3 pathways on the hydrogen evolution by bacteria as shown in Fig. 1. The pathway of hydrogen evolution of *Clostridium*, of which species generally show large yield of hydrogen as seen in Table 1, has been considered to be the NADH pathway as follows [5];



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

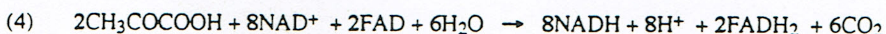
Enterobacte aerogenes st. E.82005 is also considered to evolve hydrogen through the NADH pathway by calculating NADH balances from its metabolites [6,7]

In anaerobic cultivation, i.e. fermentation, only two moles of NADH are produced through glycolysis;



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

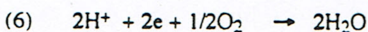
In aerobic cultivation, on the other hand, another 8 moles of NADH are produced through TCA (Tricarboxilic Acid) cycle;



(4) $$2CH_3COCOOH + 8NAD^+ + 2FAD + 6H_2O \rightarrow 8NADH + 8H^+ + 2FADH_2 + 6CO_2$$

These NADH's are transferred to the electron transport chain and re-oxidized to NAD^+ releasing electrons and protons to resolved oxygen making H_2O;

(5) $$NADH + H^+ \rightarrow NAD^+ + 2H^+ + 2e$$



(6) $$2H^+ + 2e + 1/2O_2 \rightarrow 2H_2O$$

Thus, it may be an idea to utilize TCA cycle for obtaining large yield of hydrogen from substrate.

In this paper, we report some results and informations obtained from the following experiments basing on the above considerations. At first, we tried a continuous cultivation with small pieces of porous urethane foam as supporters for bacterial flocks to decrease such preventions of supporter on substrate supply to bacteria and hydrogen transfer from bacteria. In the next, for the purpose of developing the hydrogen yield by using TCA cycle, batch cultivations with CN^- ion or amobarvital were tried to inhibit the electron transport chain under aerobic conditions.

2. MATERIALS AND METHODS OF CONTINUOUS CULTIVATION

Strain and Cultivation

Enterobacter aerogenes strain E.82005 which is one of the *Enterobacteriaceae*, a facultative anaerobe, was isolated from leaves of *Milabilis jalapa*[1] and periodically inoculated on ager culture at our laboratory. Molasses from a sugar refinery factory was used for the feed of pre-cultivation and continuous cultivation. Normal molasses containing about 51 % sugars was diluted with water to about 2 % sugars. The sugar composition is shown in Table 2. Several colonies of E.82005 grown

on an ager culture were inoculated onto the 250 ml of the diluted molasses in a 300 ml volume of fermenter and cultivated anaerobicaly for about 24 hours at 38°C under perpetual stirring with a magnetic stirrer. Then, continuous cultivation was started by dropping sterilized feed with a peristaltic pump on the pre-cultivated fluid. During the cultivation, pH of the culture was automatically kept at 6.0 with 5 % KOH.

Analytical Method

30 ml cultures were sampled for centrifugation. The supernatants were analyzed by liquid chromatography with RI meter. The separation column was a packed column prepared for organic acid analyses (GL-C610H, Hitachi Kasei Co. LTD). Carrier liquid was solution of 0.1 % phosphic acid.

The presipitates were dried and weighed to determine dry cell weights grown on the feed molasses.

The gas components were analyzed by two gas chromatographies[2]. Hydrogen and carbon dioxide were just the components of gaseous products. Evolution rate of hydrogen was measured periodically by means of a liquid-gas exchange method using 25 % KOH as the exchange liquid.

3. RESULTS OF CONTINUOUS CULTIVATION

Figure 2 shows the feed rate and the up-take rate of sugars calculated in terms of sucrose. After the 9th day's measurement, the flow rate of feed was increased from 50ml/h to 85ml/h. Then, after the 16th day's measurement, the flow rate again was decreased to 50ml/h. Consequently, the feed rate of sugars increased from c.a. 2.6 mmol/h to c.a. 4.2 mmol/h, then decreased to 2.6 mmol/h. However, the up-take rate was around 1.5 mmol/h through 21 days cultivation and did not change so clear as the feed rate changed.

Figure 3 shows the production rate of hydrogen and the concentration of cell mass containing in the waste liquid of fermentation. The production rate of hydrogen was at first more than 3 mmol-H_2 /h under the feed rate of 2.6 mmol-sugar/h. Then, the production rate decreased to ca. 2.5 mmol-H_2/h and the rate was maintained from the 6th to the 9th days of cultivation. The rate also increased to ca. 3.3 mmol-H_2/h with the increase of feed rate and decreased again to ca. 2.5 mmol-H_2/h with the decrease of feed rate. Expressing these production rates in terms per culture volume, 2.5 and 3.3 mmol-H_2/h are equal to 10 and 13 mmol/(l-culture•h), respectively.

The cell concentration in the waste liquid of fermentation was almost constant at ca. 0.9 g-dry cell/l all through cultivation days, and was not subject to the feed rate change. Estimating the cell concentrations in the fermenter from this bacteria's capability of hydrogen evolution [2], they were about 0.9 and 1.2 g-dry cell/l respectively depending on the evolution rate of 2.5 and 3.3 mmol-H_2/h. These cell concentrations are nearly equal to the cell concentration in the waste liquid of fermentation. Thus, almost any flocks might adhere on the urethane foam in opposition to our expectation.

Table 3 shows the yield of metabolites from molasses calculated in terms of glucose. Lactate is the main metabolite in continuous cultivation while ethanol is the main one in batch cultivation[3,8]. Compositions of metabolites resemble strongly between the continuous cultivation with urethane foam and without urethane foam, therefore, metabolism in continuous cultivation should be different from that in batch cultivation.

Figure 4 shows the daily yield of hydrogen from sugars estimated in terms of sucrose. Blank squares show experimental results of the yield calculated with the amount of hydrogen and the amount of uptaken sugar,and black rhombuses show the results calculated from the NADH balance of metabolites which are shown in Table 4. The maximum yield of experimental results was ca. 3.5 mol-H_2/mol-sugar, where the sugar was estimated in terms of sucrose. The yield always is the most important subject of fermentative hydrogen production as mentioned in the introduction. This bacteria has an ability of producing 2.5 mol-H_2 from one mole of sucrose under batch cultivation [9], and had shown only 1.6 mol-H_2/mol under continuous cultivation [3]. Therefore, 3.5 mol-H_2/mol is extraordinarily large yield as for expected yield. On the other hand, the mean yield was ca. 2 mol-H_2 /mol-sugar. This yield, however, was a little better than the previous yield obtained under continuous

cultivation. Thus, the cultivation with pieces of urethane foam might have some good effects on the development of the yield.

4. A TRIAL ON DEVELOPING HYDROGEN YIELD

Since, the two expressions of yield, experimental and calculated, showed very good agreement each other, it should be acceptable to discuss the yield of hydrogen from the point of NADH production. Under aerobic cultivation, this bacteria grows lively using dissolved oxygen as an electron acceptor. The pathway is as follows that glucose is decomposed to pyruvate and NADH through glycolysis under anaerobic condition, then, this pyruvate is fully decomposed to CO_2 and NADH at the TCA cycle, and these NADH's move toward the electron transport chain to pomp out protons from inside to outside of the cell through its re-oxidation and electron release to oxygen. It is well known that these two pathways appear only under aerobic condition and do not under anaerobic condition, and that the electron transport chain is easily inhibited by certain substances [10] as shown in Fig. 5. If the electron transport chain is inhibited during aerobic cultivation, where can NADH go to re-oxidize itself. This is a very interesting question. To make clear this question, we tried to inhibit the electron transport chain using $K_3Fe(CN)_6$ as for CN^- ion.

Experimental Method

Several mg's of cell obtained after pre-cultivation in synthetic culture were inoculated on 6 cultivaters with 150 ml of fresh synthetic culture. The composition of the synthetic culture was 0.5% glucose, 0.5% peptone, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.1% citrate•$2H_2O$, and 0.02% $MgSO_4$•$7H_2O$ in weight percent. Each volume of the cultivaters was ca.300 ml. Culture liquid was stirred by a magnetic stirrer. Cultivation temperature was 38°C. After 2 hours of aerobic cultivation, 3 samples were injected 1 ml of the solution of potassium ferri-cyanide on the culture to be 0.1 mM in CN^- concentration. Then, all samples were cultivated anaerobically collecting gases in upside-down cylinders contained with 1 M of H_2SO_4. These samples were periodically exposed to air babbles to keep TCA cycle and electron transport chain active. Couples of the samples, one was cultivated with CN^- and the other cultivated without CN^-, were injected air babbles for 1 min, 3 min and 5 min to every 30 minutes.

Results

The results are shown in Figs. 6 and 7. Figure 6 shows accumulated volumes of evolved gas under anaerobic cultivation after 2 hours of aerobic cultivation. Blank squares show the evolved gas from the cultivation without CN^- and black rhombuses show that from the cultivation with CN^-. These are the mean volume of three samples. The curve of blank squares shows a certain delay time to be steady speed of gas evolution, while the curve of black rhombuses shows a nearly straight line. Therefore, cultvation with CN^- seems to develop bacteria's ability of gas evolution compared to cultivation without CN^-.

Figure 7 shows accumulated volumes of evolved gas after the periodic aeration in which squares show the results of the 1 minute aeration after 29 minutes anaerobic cultivation and rhombuses show the results of the 3 minutes aeration after 27 minutes anaerobic cultivation. Blank symbols are cultivations without CN^- and black symbols are these with CN^-. The curves without CN^- have approximately equal slope to each other and these with CN^- also have approximately equal slope at steady state evolution. Slopes of black symbols are steeper than these of blank symbols. These results seem to show that cultivation with CN^- also develops speed of gas evolution compared to cultivation without CN^-.

5. CONCLUSION

Main product of the continuous cultivation was lactate. This is very different from that of the batch cultivation. Since lactate is a metabolite which do not contribute NADH production but utilize

NADH, low yield of hydrogen may be due to this lactate production. Effects of the co-cultivation with urethane foam appeared as a development on the yield of hydrogen. The yield, however, was a little smaller than the yield obtained at batch cultivation, the reason still remains as a question.

Inhibition of the electron-transport chain seems to take some effects on the speed of gas evolution of facultative anaerobic bacteria. It is still a problem whether this inhibition brings good effect on a development of the yield of hydrogen or not. The component of the gas evolved also has to be analysed in the near future.

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TABLE I. REPRESENTATIVE METABOLITES OF SOME MICRO-ORGANISMS [2,3,4]

microorganism	[amounts formed in mol/100 mol glucose fermented]								
	Formic	Acetic	Lacic	Butyric	Acetone	Butanol	Ethanol	CO_2	H_2
Yeast pH 3.0	0.36	0.52	0.82	0.13	-	-	171.5	180.0	-
pH 7.6	0.49	15.1	1.37	0.21	0.19	-	129.9	148.5	-
Clostridium									
butyricum	-	42	-	76	-	-	-	188	235
lactoaceto-philium	-	28	-	73	-	-	-	190	182
perfingens	-	60	33	34	-	-	26	176	214
aceto-butylicum	-	14.2	-	4.3	22.4	56	7.2	221	135
butylicum	-	17.2	-	17.2	-	58.6	-	203.5	77.6
Escherichia coli									
pH 6.2	2.43	36.5	79.5	-	-	-	49.8	88.0	75.0
pH 7.8	86.0	38.7	70.0	-	-	-	50.5	1.75	0.26
E. aerogenes st. E.82005									
molasses									
batch	-	29.2	25.9	19.7	-	-	73.7	*	124
Continuous	-	22.4	126.5	18.5	-	-	10.5	*	81

- ; negligiblly small, * ; not measured

TABLE II: SUGAR COMPOSITION OF DILUTED MOLASSES

Sugar	[g/l]	[wt.%]
Sucrose	13.8	79.3
Glucose	1.5	8.6
Fructose	2.1	12.1

TABLE III: YIELDS OF METABOLITES FROM MOLASSES ESTIMATED IN TERMS OF GLUCOSE

Cultivation	[amounts formed in mol/100 mol glucose fermented]					
	Lactate	Acetate	Fumarate	Butanediol	Ethanol	Butyrate
Continuous						
with Urethane Foam						
3(day)	27.5	34.9	5.15	-	29.0	57.0
13	108.9	26.2	1.87	-	-	31.5
without Urethane Foam [3]						
9	126.5	22.4	3.61	-	10.5	18.5
23	122.7	18.3	2.43	1.75	5.89	23.6
Batchwise [3]	25.9	29.2	5.11	13.4	73.7	19.7
Neish et al.[8] (from glucose)						
pH 7.0	25.0	42.4	-	10.8	57.7	0.68
pH 6.6	7.59	12.9	-	38.8	55.2	0.44
pH 6.0	3.35	7.96	-	47.5	57.4	0.00

TABLE IV. NADH BALANCE OF METABOLITE

Main pathway of glycolysis

$$C_6H_{12}O_6 + 2NAD^+ \rightarrow 2CH_3CO\cdot COOH + 2NADH + 2H^+$$

Pyruvate decomposition

Lactic acid	$CH_3CO\cdot COOH + NADH + H^+ \rightarrow CH_3CHOH\cdot COOH + NAD^+$
Butyric acid	$2CH_3CO\cdot COOH \rightarrow CH_3(CH_2)_2\cdot COOH + CO_2$
Fumaric acid	$CH_3CO\cdot COOH + CO_2 + NADH + H^+ \rightarrow (CH\cdot COOH)_2 + H_2O + NAD^+$
Formic acid	$HCOOH \rightarrow H_2 + CO_2$
Acetic acid	$CH_3CO\cdot COOH + N_2O \rightarrow CH_3\cdot COOH + HCOOH$
Butanediol	$2CH_3CO\cdot COOH + NADH + H^+ \rightarrow CH_3(CHOH)_2CH_3 + 2CO_2 + NAD^+$
Ethanol	$CH_3CO\cdot COOH + 2NADH + 2H^+ \rightarrow CH_3CH_2OH + HCOOH + 2NAD^+$
Acetone	$2CH_3CO\cdot COOH + H_2O + 2NAD^+ \rightarrow CH_3CO\cdot CH_3 + 3CO_2 + 2NADH + 2H^+$
Butanol	$2CH_3CO\cdot COOH + 2NADH + 2H^+ \rightarrow CH_3(CH_2)_2CH_2OH + 2CO_2 + H_2O + 2NAD^+$

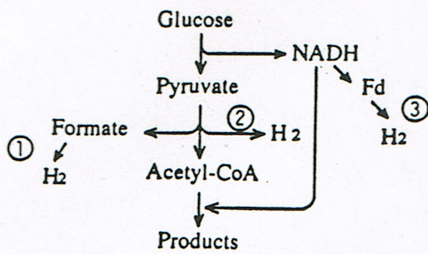

Fig. 1 Evolution Pathway of Hydrogen

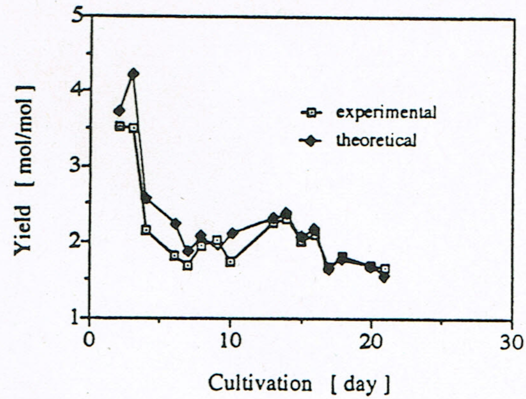

Fig. 4 Daily Yield of Hydrogen from Molasses Cultivated with Urethane Foam

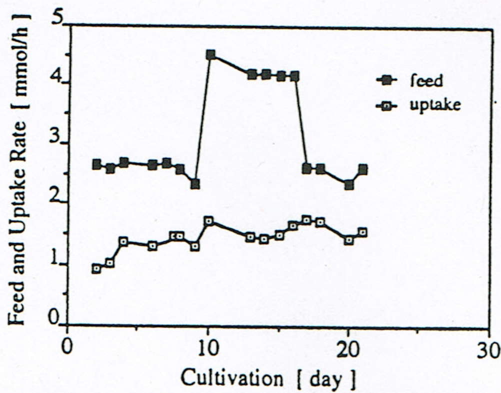

Fig. 2 Feed and Uptake Rate Estimated in Terms of Sucrose

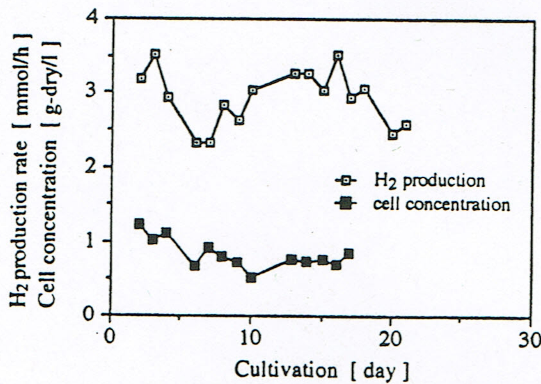

Fig. 3 Daily Rate of Hydrogen Production and Cell Concentration

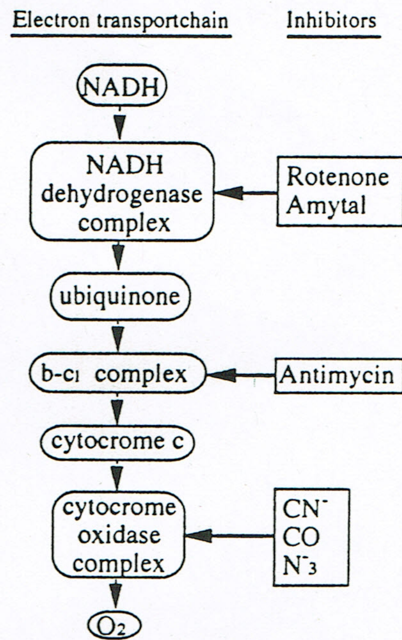

Fig. 5 Electron Transport Chain and Cites of Action of Inhibitors

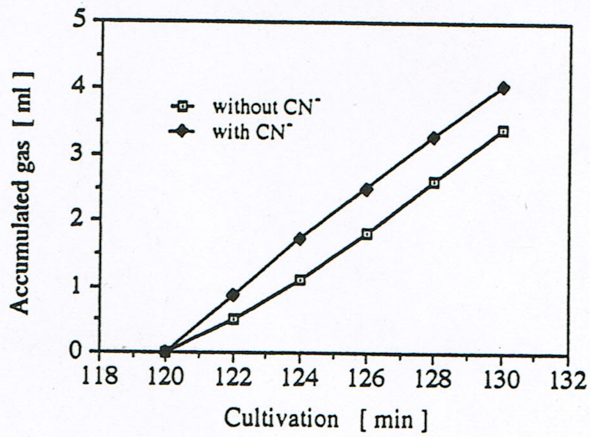

Fig. 6 Accumulated Volume of Gas Evolved from Cultivation with CN- and without CN-

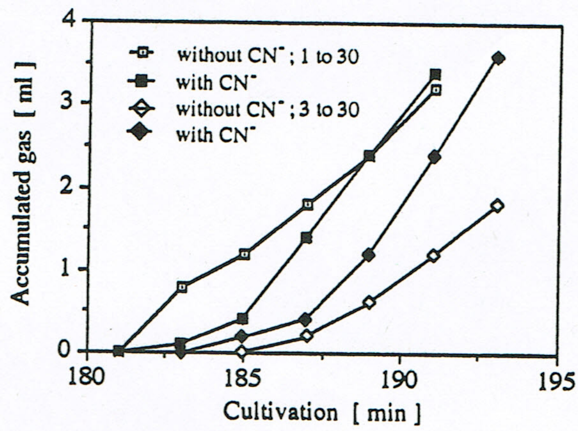

Fig. 7 Accumulated Volume of Gas Evolved just after Periodic Aeration

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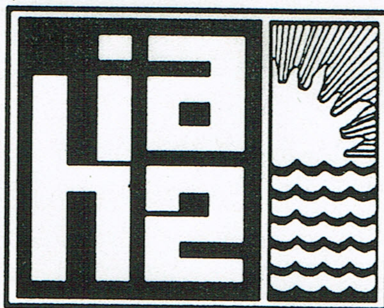

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