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FERMENTATIVE HYDROGEN EVOLUTION BY *ENTEROBACTER AEROGENES* STRAIN E.82005

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Abstract—Fermentative hydrogen evolution by *Enterobacter aerogenes* strain E.82005 was studied with cultivations performed under both controlled and uncontrolled pH. Results show that the transient rate of hydrogen evolution on the glucose and peptone liquid culture exceeded 420 ml (g-dry cell·h)$^{-1}$ under uncontrolled cultivation and that about 1 mole of glucose was required to generate 1 mole of hydrogen by the bacterial fermentation. In addition, the pH of the culture was found to have important influences on the hydrogen evolving activities of the st. E.82005. The activity reached a maximum (520 ml (l-culture·h)$^{-1}$) at pH 6.0 asnd 40.5°C. However, the most vigorous growth of cell was found to occur at pH 7.0.

NOMENCLATURE

Y_{ATP} Growth yield defined as mass of cells formed from a mole of ATP; (g-dry cell) (mol-ATP)$^{-1}$

$Y_{A/S}$ ATP yield defined as moles of ATP formed from a mole of substrate; (mol-ATP) (mol-substrate)$^{-1}$

$Y_{H_2/S}$ H_2 yield defined as moles of H_2 evolved from a mole of substrate; (mol-H_2) (mol-substrate)$^{-1}$

INTRODUCTION

Biological hydrogen production has been investigated by many authors. In general, there are two ways to produce hydrogen with living organisms: one is hydrogen production by photosynthetic organisms and the other is fermentative hydrogen production. One well-known photosynthetic organism is *Oscillatoria* sp. Miami BG7 found by Mitsui *et al.* [1]. This organism which is a cyanobacterium living in the sea evolves hydrogen at a rate of 0.38 mmol (g-dry cell·h)$^{-1}$ under the light illuminated and nitrogen limited condition [2]. Another one of the well-known photosynthetic organisms is *Rhodopseudomonas capsulata* which is a phototrophic bacterium capable of evolving hydrogen at a rate of 5.3 mmol (g-dry cell·h)$^{-1}$ by using lactate as an electron donor [3]. For use as fermentative hydrogen producing organisms, Clostridia and *Escherichia coli* are widely studied. *Clostridium butyricum*, for example, has been reported to evolve hydrogen at a rate of 7.3 mmol (g-dry cell·h)$^{-1}$ under anaerobic cultivation of waste water from an alcohol factory (BOD = 10,000 p.p.m.) [4]. It seems that the rate of the fermentative hydrogen production is always faster than that of the photosynthetic hydrogen production. Other advantages of the fermentative hydrogen production are:

(1) It is possible to produce all day long without light.
(2) It is able to use photosynthetic products as substrates of the hydrogen evolution. This is an indirect use of solar energy.
(3) It is also able to use industrial and/or agricultural wastes as substrates.
(4) Metabolites except hydrogen can also be used.

Therefore the fermentation is most likely the technique to be adopted for industrial production.

The present authors found another species (*Enterobacter aerogenes* strain E.82005) also having a high hydrogen productivity in fermentation [5]. This species belongs to a family of Enterobacteriaceae and is a facultative anaerobe. Therefore, in contrast to *C. butyricum* which is a strict anaerobe, the utilization of the species is of great advantage as follows. Under aerobic conditions, this species grows vigorously because the growth yield of aerobic cultivation is far larger than that of anaerobic cultivation [6]. If anaerobically cultivated, it would evolve hydrogen in a metabolic pathway of glucose or certain energy substrates. An oxygen leakage is often harmful to hydrogenase in some hydrogen producing microbes, such as *C. butyricum*, but it would cause little problem for the species. This is because the species can rapidly consume oxygen and recover the activity of hydrogen evolution. Therefore, no special device is needed to degas the feed to a continuous hydrogen production tank containing this particular species.

In this article, we will report some important results on the rate of biological hydrogen production using *Enterobacter aerogenes* st. E.82005 under different conditions.

MATERIALS AND METHODS

Organism and culture conditions

Enterobacter aerogenes st. E.82005 was isolated from leaves of a plant as mentioned in a previous paper [5]. The species has been inoculated periodically in our

laboratory. A pre-culture was made by inoculating its colony grown on an agar plate on pre-culture liquid. The culture was then incubated aerobically, while under constant stirring for 20 h at 38°C. The culture medium consisted of 1.5% 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% $MgSO_4 \cdot 7H_2O$, all in weight percent.

250 μl or 10 ml of the incubated culture was inoculated respectively onto the 30 ml or 350 ml of glucose and peptone culture (GP culture). The GP culture contained 10 g of glucose and 50 g of peptone (Nissui Seiyaku Co. Ltd) in 1 l of ion exchanged water. During the incubation at 38°C the liquid was stirred with a magnetic stirrer.

The pH was controlled with an automatic pH controller (Tokyo Rikakikai Co. Ltd, FC-1) by the addition of 30% NaOH or 30% H_2SO_4 solution as required.

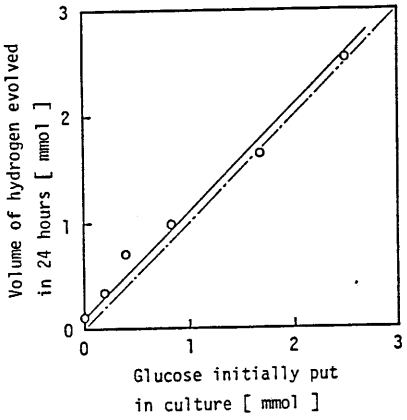

Fig. 1. Volume of hydrogen evolved on 5% peptone culture by *Enterobacter* st. E.82005.

H_2 production rate, dry cell weight and glucose concentration

The hydrogen evolution rate was measured by a liquid–gas exchange method using an upside-down measuring cylinder. The liquid was 30% NaOH solution. One ml of the gas exchanged was withdrawn from the cylinder periodically (30 min in case of rapid evolution and 1 h in slow evolution) and the component was analysed by gas chromatography using as packings the activated carbon for CO_2 and the molecular sieves 5A for H_2. However, because of the absorption of carbon dioxide by NaOH solution, the peak of carbon dioxide was not observed. Therefore, the gas evolution rate measured by the measuring cylinder was considered to be the hydrogen evolution rate.

Cells in a 30 ml culture liquid were separated by a centrifuge rotating at 10,000 rpm for 10 minutes and then washed by ion-exchanged water. After repeating the procedure twice, they were dried in a dryer and the amount of dried cells was weighted.

The glucose concentration was analysed enzymatically by using Diacolor-GC(Toyo Spinning Co. Ltd).

RESULTS

Cultivation without pH control

Enterobacter aerogenes st. E.82005 grows on the GP culture under the anaerobic condition. Figure 1 shows the total volume of hydrogen which was evolved for 24 h incubation at various amounts of glucose initially put in the culture with 5% of peptone. Hydrogen is evolved just a little amount in the culture consisted of peptone alone. However, the amount increases as the amount of glucose increases. Subtracting the amount of hydrogen which should be due to the peptone, the total volume of hydrogen evolved on the GP culture seems to be in the direct proportion with the initial amount of glucose as seen in the figure.

To get the transient information of growth and metabolism, 28 samples, each containing 1% glucose and 5% peptone in 30 ml culture, were prepared. These samples were inoculated at the same time and divided into 9 groups. From the range of 2.5 h to 6 h after the inoculation, the amount of hydrogen evolved, pH, glucose concentration and dry cell weight were measured by picking out a group successively at every 30-min interval. Data from the 8 groups were assumed to be the transient data of the 9th group measured 8 h after the inoculation. The results are shown in Fig. 2.

In Fig. 2(a) the time course of pH of the culture is shown. The pH decreases rapidly as the bacteria grows. However, below pH 5.8 the decrease becomes gentle and the pH is seen to approach a constant in spite of the vigorous evolution of hydrogen. This is presumably caused by the change of metabolic pathway from acids production to non-acids production. The change of metabolic pathway is a characteristic property of genus *Enterobacter*. Many genera belonging to the family Enterobacteriaceae (e.g. genus *Citrobacter*, genus *Escherichia* etc.) are widely known to produce hydrogen as the result of glucose fermentation. These genera, except the genus *Enterobacter*, are positive to Methyl Red (MR) tests and negative to Voges–Proskauer (VP tests, i.e. substantial amounts of acids are produced and the bacteria do not have the function to keep their residence at a suitable pH. On the other hand, the genus *Enterobacter* to which the st. E.82005 belongs is in general negative to MR test and positive to VP test, implying that not so much acids are produced and the bacteria should have the function to keep their residence at a suitable pH. Fig. 2(a) is evidence of this property.

The accumulated volume of hydrogen evolved by 9th group is shown in Fig. 2(b). As the cell number increases rapidly in the logarithmic stage, the rate of hydrogen evolution is accelerated about 3 h after inoculation and becomes nearly constant after about 4 h. The rate at the constant evolution is *ca* 270 ml (l-culture·h)$^{-1}$, i.e. *ca* 11

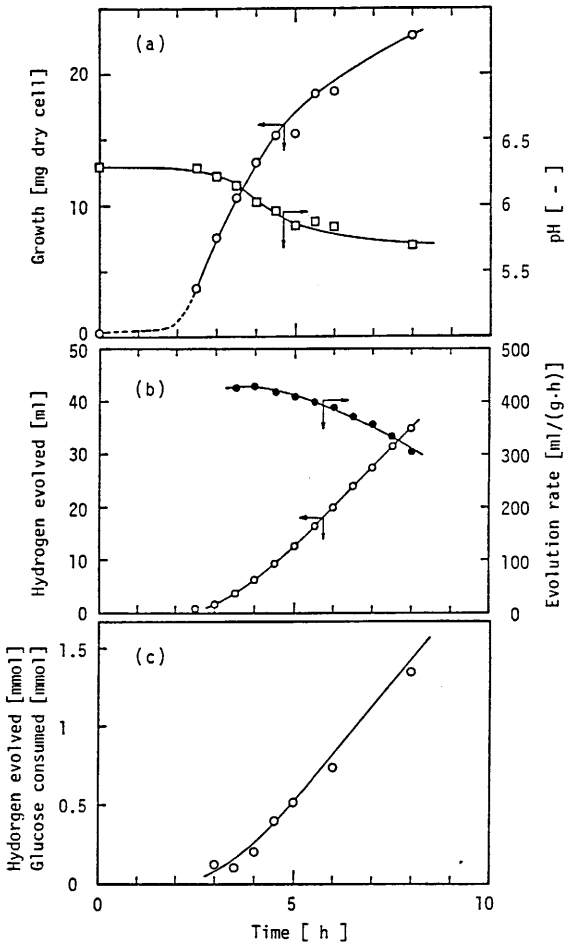

Fig. 2. Time course of growth, pH, glucose consumption and hydrogen evolution of *Enterobacter* st. E.82005.

mmol (l-culture·h)$^{-1}$. It is interesting that the pH of the culture also becomes nearly constant in concert with the constant evolution.

Transient rates of the hydrogen evolution per unit dry cell weight are also shown in the figure. The transient rate shows a maximum value when the culture pH is *ca* 6. The maximum rate exceeds 420 ml (g-dry cell·h)$^{-1}$, i.e. over 17 mmol (g-dry cell·h)$^{-1}$. This evolution rate per unit dry cell weight seems to be very sensitive to the culture pH.

In Fig. 2(c), the amount of glucose consumed is shown along with the amount of hydrogen evolved. The glucose consumption curve coincides very well with the hydrogen evolution curve. This means that the evolution of hydrogen runs parallel with the decomposition of glucose.

Cultivation under controlled pH

As seen in Fig. 2, the maximum hydrogen evolution may depend on pH of the culture. The dependency was studied by varying pH of the culture in a 300 ml fermenter. The mass of cells grown on 250 ml of GP culture was measured at the end of cultivations, which was taken to be the time when the evolution rate of hydrogen started to decrease drastically as indicated in Fig. 3. The cultures were cultivated at the liquid temperature of 37.5 ± 0.5°C The results are shown in Table 1.

The maximum production of cells was at *ca* pH 7. Cells of 468 mg were obtained after 5 h of cultivation at pH 7.0. However, at pH 5.0, the mass of cells was only 168 mg after 6 h cultivation and it decreased to 106 mg over a period of 23 h of cultivation, while the amount of hydrogen increased from 43 ml to 254 ml. The extraordinary increase of hydrogen volume was observed only at pH 5.0. Calculations of the mean production rate over the cultivation period also show a maximum at pH 7.0.

Table 1. Effect of pH on growth and hydrogen evolution

pH	Cultivation time	Cell production*	Production rate mg-cell	Growth yield $Y_{X/S}$ g-cell	ATP Yield† $Y_{A/S}$ mol-ATP	H_2 Yield $Y_{H_2/S}$ mol-H_2	H_2 Evolution rate ml-H_2	ml-H_2
	h	g-dry cell	h	mol-glucose	mol-glucose	mol-glucose	l-culture·h	g-dry cell·h
5.0	6.0	0.168	27.9	12.1	1.1	0.75	54	80
5.5	9.0	0.241	26.8	17.3	1.7	1.02	264	274
6.0	6.75	0.356	52.7	25.6	2.4	1.07	388	272
6.5	7.42	0.455	61.4	32.8	3.1	1.10	374	205
7.0	5.0	0.468	93.6	33.7	3.2	0.38	182	97
7.5	5.25	0.426	81.1	30.7	2.9	0.19	59	35
free‡	7.33	0.241	32.9	17.3	1.7	0.99	264	274

* Cell mass grown on 250 ml culture, which contained 1% glucose and 5% peptone.

† Calculated from the growth yield $Y_{X/S}$ on the assumption that Y_{ATP} is 10.5 g-cell (mol-ATP)$^{-1}$ in accordance with Hadjipetrou *et al.* [6].

‡ Cultivated without pH control. pH of the culture changed from 6.45 to 5.82 during the cultivation.

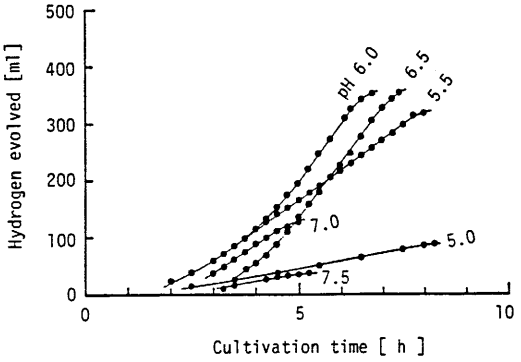

Fig. 3. Volume of hydrogen evolved under controlled pH.

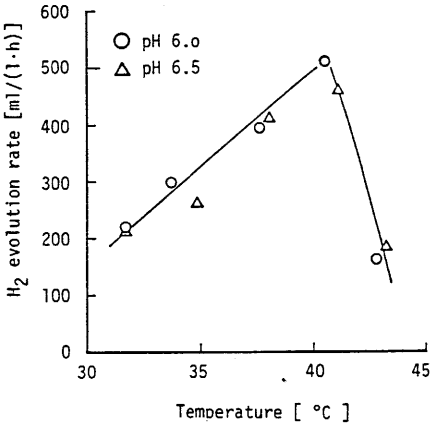

Fig. 5. Effect of temperature on a rate of hydrogen evolution.

Assuming that the growth yield for ATP (Y_{ATP}) is 10.5 g-dry cell per 1 mole ATP in accordance with the data of Hadjipetrou *et al.* [6], the ATP yield ($Y_{A/S}$) was found to be *ca* 3 when pH > 6.5. However, below pH 6.5 the yield decreases linearly with the pH. At pH 5.0 the yield is only one. These results, therefore, indicate that the optimum pH for growth is around pH 7.

Yields of hydrogen from glucose ($Y_{H_2/S}$) obtained in this study are shown in Table 1. The yield varies from 1.0 to 1.1 in the pH range from 5.5 to 6.5. The pH condition seems to be very good at 6.5 both for the evolution of hydrogen and for the growth of bacteria. At pH 5.0, where the growth condition for the bacteria is very severe, the yield $Y_{H_2/S}$ is small at 0.75. However, at pH 7.0 where the growth condition is very favourable, the yield becomes smaller (0.38). At pH 7.5, the yield is even smaller (0.19). This difference in behavior with respect to the suitable pH for the growth and the hydrogen evolution may be a very interesting fact for considering a mechanism of hydrogen evolution by fermentation.

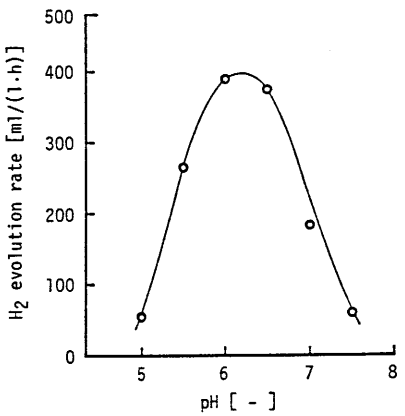

Fig. 4. Effect of pH on a rate of hydrogen evolution.

Accumulated volumes of hydrogen are shown in Fig. 3 as a function of time and the rates are given in Table 1. The rate of hydrogen evolution becomes nearly constant after about 4 hours of incubation, in common with case of cultivation without pH control. Figure 4 shows the variation of H_2 producing rate with pH. It is seen that the optimum pH at which the maximum evolution rate of hydrogen occurs is between 6.0 and 6.5. The rates at pHs 6.0 and 6.5 were *ca* 16 mmol (l-culture·h)$^{-1}$ and *ca* 15 mmol (l-culture·h)$^{-1}$, respectively. The optimum pH for evolution rate is the same as the optimum pH for the yield of hydrogen. However, the rate decreases significantly both below and above the optimum pH, e.g. the rate of pH 5.0 was only 20% of the rate of pH 6.0.

The evolution rates were measured at several temperatures while keeping pH constant. The results are shown in Fig. 5. The maximum rate of hydrogen evolution which occurred at 40.5°C was found to be *ca* 520 ml (l-culture·h)$^{-1}$ or *ca* 21 mmol (l-culture·h)$^{-1}$. The evolution rate increases linearly with increasing temperature when the temperature is below 40.5°C, but decreases rapidly at the ratio of 25%/°C when the temperature is greater than the optimum. A shift to higher temperatures from the optimum affects the bacterial life very drastically.

DISCUSSIONS

Comparison with other microorganisms

Many microorganisms can evolve molecular hydrogen; details of which may be found in the reviews [7–12]. Two categories of these microorganisms which show relatively high evolution rate of hydrogen are shown in Table 2. The first category is photohydrogen production which is subdivided into a double photosystem and a single photosystem, with the latter evolving molecular hydrogen faster than the former. The second

Table 2. Representative rates of hydrogen evolution

Category	mmol-H_2 / l-culture·h	mmol-H_2 / g-dry cell·h	pH control method	Reference
I. Photosynthetic bacteria				
Double photosystems				
Oscillatoria sp. Miami BG 7	0.4	0.4	Tris–HCl buffer	[2]
Anabaena cylindrica	1.2	1.3	$NaHCO_3$-CO_2 buffer	[13]
Single photosystem				
Rhodopseudomonas capsulata	5.3	5.3	phosphate buffer	[3]
Rhodosprillum rubrum	3.0	2.5	phosphate buffer	[14]
II. Fermentative bacteria				
Strict anaerobe				
Clostridium butyricum	—	7.3	—	[4]
	—	7.0	phosphate buffer	[15]
Facultative anaerobe				
Citrobacter intermedius	11	9.5	automatically controlled	[16]
Enterobacter st. E.82005	11	17*	uncontrolled	(this work)
	11	11	uncontrolled	(this work)
	21	11	automatically controlled	(this work)

* Dry cell weight of this data was measured at an appropriate instant of the cultivation. Dry cell weight in all other data was measured at the end of the cultivation.

category is fermentative hydrogen production. The category is also subdivided into strict anaerobes and facultative anaerobes based on the mode of ATP production. The rate of fermentative hydrogen production is generally faster than the rate of photohydrogen production, however, recently Miyake and Kawamura reported interesting results for the rate of photohydrogen evolution by *Rhodobacter sphaeroides* 8703, which evolved hydrogen at a very rapid rate such as 262 ml (g-dry cell·h)$^{-1}$, i.e. 10.4 mmol (g-dry cell·h)$^{-1}$, at 35°C under the illumination of 20,000 lux [17]. Therefore, more effective photohydrogen bacteria and/or suitable conditions for the hydrogen evolution may be found in the near future.

In case of industrial hydrogen production with bacteria from certain substrates, the hydrogen evolution rate is a very important factor and is required to be high. *Enterobacter* st. E.82005 is found to be capable of evolving hydrogen at a rate (11 mmol (g-dry cell·h)$^{-1}$) faster than other microorganisms without control of pH. Under controlled pH, the condition for hydrogen production becomes more suitable than free cultivation and the rate increases to 21 mmol (l-culture·h)$^{-1}$ at pH 6.0 and 40.5°C. This evolution rate appears to be the highest known to the authors. With this desirable property, *Enterobacter aerogenes* st. E.82005 will most likely find many useful applications in the fermentation industry.

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