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Oxygen Sensitivity of Carbon Monoxide-Dependent Hydrogen Production Activity in *Citrobacter* sp.

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Abstract A newly isolated *Citrobacter* sp. Y19 catalyzes the CO-dependent H_2 production (biological water-gas shift reaction) by the actions of CO dehydrogenase (CODH) and hydrogenase. Y19 requires O_2 for fast growth, but its H_2 production activity is significantly inhibited by O_2. In the present study, the effect of O_2 on the activities of CODH and hydrogenase was investigated quantitatively *in* both whole cells and broken cells, based on CO-dependent or methyl viologen (MV)-dependent H_2 production in addition to CO-dependent MV reduction. In crude cell extracts, CODH activity was mostly found in the soluble fraction. Inactivation of CODH and hydrogenase activities by O_2 followed the first-order decay kinetics, and the dependence of the rate constants on O_2 partial pressure could be expressed by the Michaelis-Menten equation. In whole cells, the maximum deactivation rate constants ($k_{d,max}$) of hydrogenase and CODH were quite similar: 0.07±0.03 min^{-1} and 0.10±0.04 min^{-1}, respectively. However, the first-order rate constant ($k_{d,max}/K_s$) of CODH (0.25 min^{-1} atm^{-1}) at low O_2 partial pressures was about 3-fold higher than that of the hydrogenase, since the half-saturation constant (K_s) of CODH was about half of that of hydrogenase. In broken cells, both enzymes became significantly more sensitive to O_2 compared to the unbroken cells, while $k_{d,max}/K_s$ increased 37-fold for hydrogenase and 6.7-fold for CODH. When whole cells were incubated under anaerobic conditions after being exposed to air for 1 h, hydrogenase activity was recovered more than 90% in 2 h suggesting that the deactivation of hydrogenase by O_2 was reversible. On the contrary, CODH activity was not recovered once deactivated by O_2, and the only way to recover the activity was to synthesize new CODH. This study indicates that O_2 sensitivity of H_2 production activity of *Citrobacter* sp. Y19 is an important drawback as in other H_2-producing bacteria.

Key words: *Citrobacter* sp. Y19, CODH, hydrogenase, oxygen sensitivity

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H_2 is a clean, recyclable, and an efficient energy carrier. It has a high potential as a major fuel for fuel-cell powered vehicle. Currently, H_2 is mainly produced from a steam reforming of hydrocarbons, however, it will be substituted by a more environment-friendly biological process in the future [16, 20]. The microorganisms producing H_2 obtain the reducing power for their H_2-producing enzymes from various external sources such as CO, sugars, or light. Among them, the CO-dependent H_2 production occurs by the following water-gas shift reaction [13, 14]:

$$CO+H_2O \rightarrow CO_2+H_2 \qquad \Delta G^{\circ}=-20.1\ kJ\ mol^{-1} \qquad (1)$$

The deactivation of hydrogenase by O_2 has been reported in many H_2-producing bacteria including photosynthetic algae, *Cyanobacteria* [11, 16]. The inactivation is caused by denaturation of the enzyme active site by oxygen free radicals or the loss of catalytic activity by the change in redox state [1, 16]. CO-dependent H_2-producing bacteria are mostly strict-anaerobic or photosynthetic, and they are also known to be O_2-sensitive. Two enzymes are involved in the CO-linked H_2 production: a CODH (carbon monoxide dehydrogenase) oxidizes CO to CO_2 with the production of reducing power, and a hydrogenase reduces protons to H_2 by using the electrons donated from CODH. It is known that, by O_2, not only the activities of these enzymes are inhibited, but their expression is also repressed at the gene level [4]. However, individual O_2 dependence of CODH and hydrogenase has not been studied from the engineering

point of view and, furthermore, no quantitative results have yet been reported.

Since the inactivation of hydrogenases by O_2 is a serious problem in development of H_2 production processes, many attempts have been made to avoid it. In a case of photoautotrophic process where O_2 is generated from water-splitting by photosystem II, various mechanical, chemical, and enzymatic methods have been suggested [11, 15]. Immobilization of *C. pasteurianum* has been reported to increase the stability of its hydrogenase to heat and O_2 [16]. However, these methods are economically not feasible at the present time due to high cost. Recently, Maness *et al.* [19] reported the isolation and characterization of an O_2-tolerant strain, *Rubrivivax gelatinosus*. The hydrogenase of this bacterium, linked to CO oxidation, had a long half-life of 21 h in air (21% O_2) and, furthermore, could function partially in the presence of O_2 [19].

Recently, a new chemoheterotrophic bacterium *Citrobacter* sp. Y19 was isolated [13, 14]. It can produce H_2 from fermentation of various sugars or from CO by water-gas shift reaction. With the CO-linked activity, Y19 differs from previously known strict anaerobes in that it grows fast under aerobic conditions and, on the other hand, produces H_2 under anaerobic conditions. Therefore, a two-stage cultivation that separates cell growth and H_2 production has been suggested as a desirable process. H_2 production activity of Y19 was also inhibited by O_2, but the individual sensitivity of CODH and hydrogenase has not been investigated.

The objective of this study was to quantitatively investigate the O_2 sensitivity of CODH and hydrogenase of Y19. Individual enzymatic activities were measured, based on CO-dependent or methyl viologen (MV)-dependent H_2 production along with CO-dependent MV reduction. Inactivation of hydrogenase and CODH was measured in unbroken whole cells and broken cells at various O_2 partial pressures, and the kinetic parameters were determined. Furthermore, reactivation or restoration of the enzymatic activities in O_2-inactivated whole cells and broken cells was studied.

MATERIALS AND METHODS

Cell Growth and Preparation of Crude Cell Extracts

Citrobacter sp. Y19 was grown in a serum bottle (total volume, 165 ml) on mineral salt medium [13] supplemented with 3 g yeast extract l^{-1} and 5 g sucrose l^{-1}. Y19 was cultivated for 12 h under air/CO (95:5, v/v), which was replenished once at 6 h. After the cell growth, the culture bottle was completely deprived of O_2 by Ar purging and added with 20% CO. The bottle was further incubated for 24 h for induction of H_2 production activity. Detailed methods for cell growth and activity induction were described previously [12, 14].

Active cells were washed twice with an ice-cold phosphate buffer (pH 7.0, 100 mM). Cells were disrupted by using a bead beater (Fastprep FP120, Qbiogene Inc., U.S.A.) at a speed of 6.0 for 20 s twice with 10 s intervals. The conditions for cell breakage were determined to give the maximum CODH activity in a broken-cell suspension (see below). All procedures except for centrifugation were conducted in an anaerobic chamber (Bactron 1.5, Sheldon Manufacturing Inc., U.S.A.). Broken cells were centrifuged at 10,000 ×g and 4°C for 10 min to remove unbroken cells and large cell debris, and the supernatant was further centrifuged at 38,000 ×g and 4°C for 1 h to separate the particulate fraction from the soluble fraction [7, 21]. Particulate fraction was resuspended in the same phosphate buffer. Protein contents were assayed by the Bradford method (Protein assay kit, Bio-Rad Laboratories, U.S.A.) [3]. The broken cells and crude enzyme extracts were stored in sample vials at 4°C before use.

Deactivation by O_2 and Reversibility Experiments

For studying quantitative deactivation kinetics, whole cells and broken cells in a phosphate buffer (pH 7.0, 100 mM) were incubated at 30°C under different O_2 partial pressures (0–60%, v/v). Every 10 min, samples (2 ml) were taken into separate vials (8 ml) where the enzymatic activities were measured. Before use, the vials were purged with Ar gas, and 5 µl of 1 M sodium dithionite (SDT) was added to minimize the effect of O_2 carried over with the sample solutions. It was confirmed beforehand that the trace amount of SDT did not affect the assays for hydrogenase or CODH activities.

The reversibility of the activities of O_2-exposed hydrogenase and CODH was investigated by incubating both inactivated whole cells and broken cells under anaerobic conditions. After being exposed to air (21% O_2, v/v) for 1 h, the cells were incubated under 100% Ar or Ar/CO mixture (80:20, v/v). Since mechanical shear can affect the enzymatic activity in broken cells, two different methods, purging the Ar gas directly into the liquid solution and indirectly into the headspace, were attempted and compared. The effect of chloramphenicol (50 µM), a transcriptional inhibitor, was also studied in order to elucidate whether the reactivation of whole cell was induced by the restoration of deactivated enzyme or the synthesis of new enzymes.

Activity Assay

CO-dependent H_2 production activity of whole cells were assayed as described previously [14]. CODH activity was determined by the reduction of CO-dependent MV as follows [5, 6]. The 990 µl of 3[*N*-morpholino]propanesulfonic acid (MOPS; pH 7.5, 100 mM) containing 15 mM of MV and 1 mM of EDTA were introduced into a 10 mm quartz cuvette with a stopper which was maintained at 30°C by constant-temperature chamber. The buffer solution was

bubbled with pure CO for 5 min, and 10 μl of cell suspension or prepared enzyme solution (0.6-0.8 mg protein ml^{-1}) was added to start the reaction. The reduction of MV (ε_{578}= 9.7 mM^{-1} cm^{-1}) was monitored at 578 nm for 20 min by a spectrophotometer (Lambda 20, Perkin-Elmer Inc., U.S.A.) [6, 9]. The absorbance increased linearly, and the activity was calculated from the slope of the absorbance change. Hydrogenase activity was determined by measuring the amount of H_2 produced with the reduced MV as an electron donor [5, 10]. Five-hundred μl of cell suspension or enzyme solution (0.6-0.8 mg protein ml^{-1}) containing 2.5 mM of MV and 200 mM of MOPS (pH 7.0) were introduced into an anaerobic serum vial (8 ml) and the head-space was replaced by purging Ar. The reaction was initiated by injecting an excess amount of 1 M SDT to become 50 mM. The total volume of liquid in the vial was 2 ml. The reaction mixture was incubated at 30°C in a reciprocally-shaking water bath (180 strokes min^{-1}) for 1 h, and the amount of H_2 produced was measured by a gas chromatograph (DS6200, Donam Instruments Inc., Korea) equipped with a thermal conductivity detector [5, 12, 23].

RESULTS AND DISCUSSION

Location and Activities of Hydrogenase and CODH

Table 1 shows the hydrogenase and CODH activities in whole cells and various fractions of crude cell extracts. Hydrogenase activity in a whole cell was 0.015 μmole H_2 min^{-1} mg $protein^{-1}$, while the CODH activity in a whole cell was 0.030 μmole CO min^{-1} mg $protein^{-1}$. Hydrogenase and CODH activities of the broken cells are significantly higher than those of the whole cells, indicating that the transfer of the reaction substrates through the intact cell wall could be an important barrier for the reactions. The higher CODH activity suggests that the hydrogenase activity might be the rate-limiting step for the CO-dependent H_2 production in Y19. This would be beneficial by enabling the cells to rapidly remove the intracellular CO, which is toxic to many cellular metabolisms. It should be noted that the specific CODH activity of Y19 broken cells (0.60 μmole CO min^{-1} mg $protein^{-1}$) was similar to that of unpurified Ni-deficient apo enzyme of *R. rubrum* [5, 8], and that the specific hydrogenase activity (0.090 μmole H_2 min^{-1} mg $protein^{-1}$) was about twice as high as that of *R. rubrum* (0.050) or *E. coli* (0.047) [2, 16]. This confirms that Y19 is a highly active biocatalyst for water-gas shift reaction [13].

Table 1 also shows the cellular localization of CODH and hydrogenase activities in Y19. After centrifugation at 38,000 ×g, most CODH activity was found in the soluble fraction, indicating that CODH is a cytoplasmic protein, as reported with *R. rubrum* [6]. The specific activity of CODH in the soluble fraction was approximately 2.80 μmole CO min^{-1} mg $protein^{-1}$, which was almost 5 times higher than that in broken cells. In the case of hydrogenase, no activity was found in the supernatant when the broken cell suspension was centrifuged at 10,000 ×g to remove unbroken cells and large cell debris. Consequently, no activity was found in either soluble fraction or particulate fraction following high-speed centrifugation. This observation, along with the fact that hydrogenase activity in broken cells was much higher than that of unbroken cells, suggests that most hydrogenase activity resides on cell debris in broken cells which are removed by the low-speed centrifugation. We also noticed that the cell breakage in this experiment was not complete, as evidenced by a large loss in protein content after low-speed centrifugation, and that hydrogenase enzyme molecules were not fully released from the subcellular compartment where they are located. Nevertheless, considering the appearance of high CODH activity in the 38,000 ×g fraction and a large increment of hydrogenase activity after cell breakage, some fractions of hydrogenase enzyme molecules must have been released during the cell breakage; however, their activity has been fully lost during the subsequent treatment, probably due to a high susceptibility of the hydrogenase to shear stress (see Fig. 3b). Hydrogenase activity in the cell-free system is important for its purification and characterization, but no further efforts were made in the present study.

Degradation of Hydrogenase and CODH Activities by Exposure to O_2

Figure 1 shows the deactivation of unbroken whole-cells and broken cells upon exposure to O_2. Both hydrogenase and CODH were very sensitive to O_2, and more than 50% of their activities were lost within 10 to 30 min when O_2 partial pressure was 17% (v/v). The deactivation rate increased with increasing O_2 partial pressure, as indicated by the increased slope. Compared to the unbroken cells, the enzymes of the broken cells were more sensitive to O_2.

Table 1. Distribution of CODH and hydrogenase activity in cell-free extract of *Citrobacter* sp. Y19.

	Whole cells	Broken cells	Supernatant at 10,000 ×g	Fractionation at 38,000 ×g	
				Soluble fraction	Particulate fraction
Protein content (μg ml^{-1})	620	600	210	50	160
Hydrogenase activity (μmole H_2 min^{-1} mg $protein^{-1}$)	0.015	0.090	0.0	0.0	0.0
CODH activity (μmole CO min^{-1} mg $protein^{-1}$)	0.030	0.60	0.71	2.80	0.10

[a] Prepared by centrifuging broken-cell suspension at 10,000 ×g to remove the unbroken cells and large cell debris.

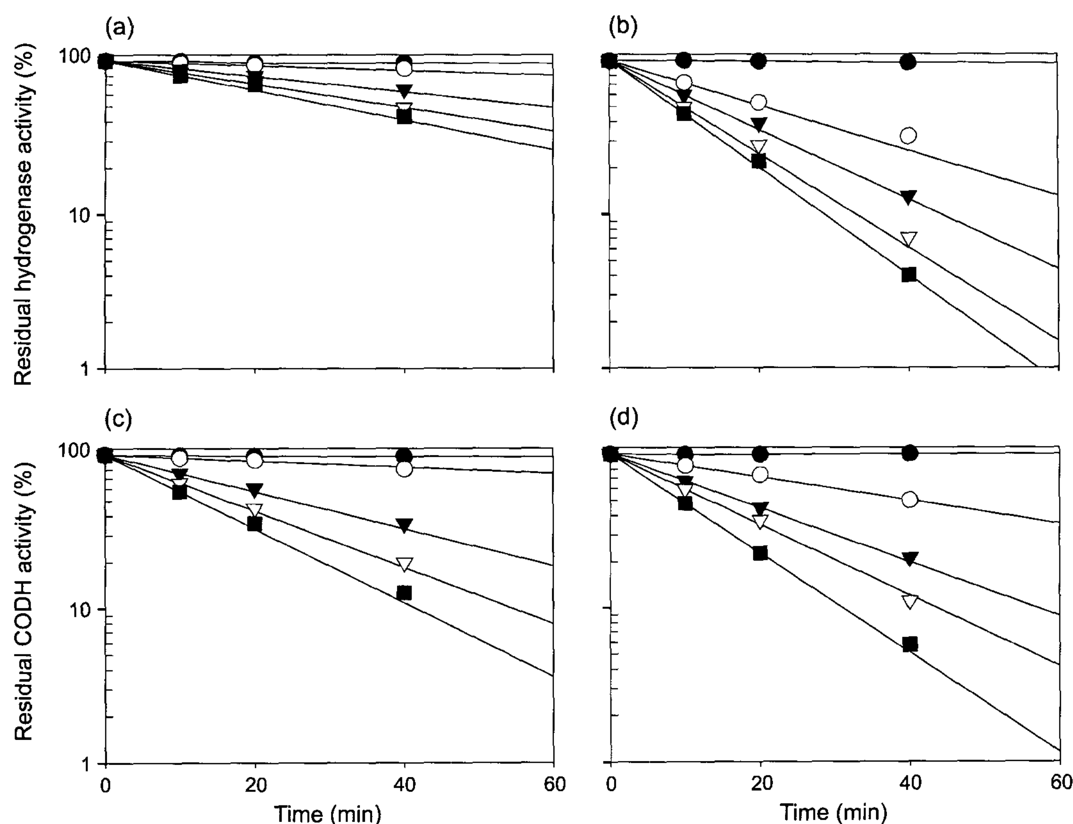

Fig. 1. Effect of O_2 partial pressure (%, v/v) on (a) hydrogenase of whole cells, (b) hydrogenase of broken cells, (c) CODH of whole cells, and (d) CODH of broken cells.
Symbols: -●-, 0%; -○-, 5.0 %; -▼-, 17.0%; -▽-, 27.0%; -■-, 60.0%.

The rate of activity loss followed the 1st order decay kinetics regardless of O_2 partial pressure.

$$-\frac{de_a}{dt}=r_d=k_d e_a \quad (2)$$

where e_a represents active enzyme concentration (μg ml^{-1}) or relative activity (%), and k_d represents deactivation rate constant (min^{-1}). The rate constant k_d was determined from the slope of Fig. 1 and followed the Monod-type kinetics.

$$k_d=\frac{k_{d,max}P_{O_2}}{K_S+P_{O_2}} \quad (3)$$

where $k_{d,max}$ represents maximum deactivation rate constant of the loss of activity upon exposure to O_2 (min^{-1}); K_s, half-saturation constant (atm), and P_{O_2}, oxygen partial pressure (atm).

Table 2 summarized the kinetic parameters related with O_2 deactivation. In the case of the whole cell, the maximum deactivation rate of CODH was about 50% higher than that of hydrogenase. However, the 1st order activity loss rate ($k_{d,max}/K_s$) of CODH at low O_2 partial pressures was about 3 times higher than that of hydrogenase, since K_s value of CODH was approximately half of that of hydrogenase. As comparison, kinetic parameters of O_2 deactivation of the CO-dependent H_2 production activity in a whole cell are also given. The $k_{d,max}/K_s$ of CO-dependent H_2 production activity was 0.17, which was higher than that of hydrogenase but lower than that of CODH. After the cell breakage, O_2

Table 2. Kinetic parameters of deactivation of hydrogenase and CODH by O_2[a].

Type of exposure to O_2	Enzyme	$k_{d,max}$ (min^{-1})	K_s (atm)	$k_{d,max}/K_s$
Whole cell	Hydrogenase	0.07±0.03	0.83±0.10	0.08
	CODH	0.10±0.04	0.41±0.08	0.25
Broken cell	Hydrogenase	0.23±0.06	0.08±0.02	3.01
	CODH	0.42±0.10	0.25±0.03	1.68
Whole cell	Overall CO-dependent H_2 production activity	0.03±0.01	0.16±0.03	0.17

[a]Assays were run in duplicate and averaged.

sensitivity of both hydrogenase and CODH greatly increased, as evidenced by 3-4 times higher $k_{d,max}$ and 2-10 times lower K_s. This result suggests that the presence of cell membrane contributes to stabilizing CODH as well as hydrogenase, and the stabilization is related to a slower O_2 transfer in intact cells. Alternatively, O_2 scavenger systems such as superoxide dismutase (SOD) and catalase could play some important roles. The oxygen scavenger system is known to exist in facultative anaerobes such as *Citrobacter* sp. [22], but not in strict anaerobes.

Reactivation of Hydrogenase and CODH Under Anaerobic Conditions

In order to investigate whether the inactivation of hydrogenase and CODH by O_2 was reversible or not, the cells were exposed to air for 1 h and then incubated under anaerobic condition. Figure 2 shows the results on the CO-dependent H_2 production activity in whole-cell suspension, while Fig. 3 shows the results on both hydrogenase and CODH activities in a whole-cell or broken-cell suspension. To prevent the cell growth during the experiment, 100 mM phosphate buffer at pH 7.0 was used as a suspending medium.

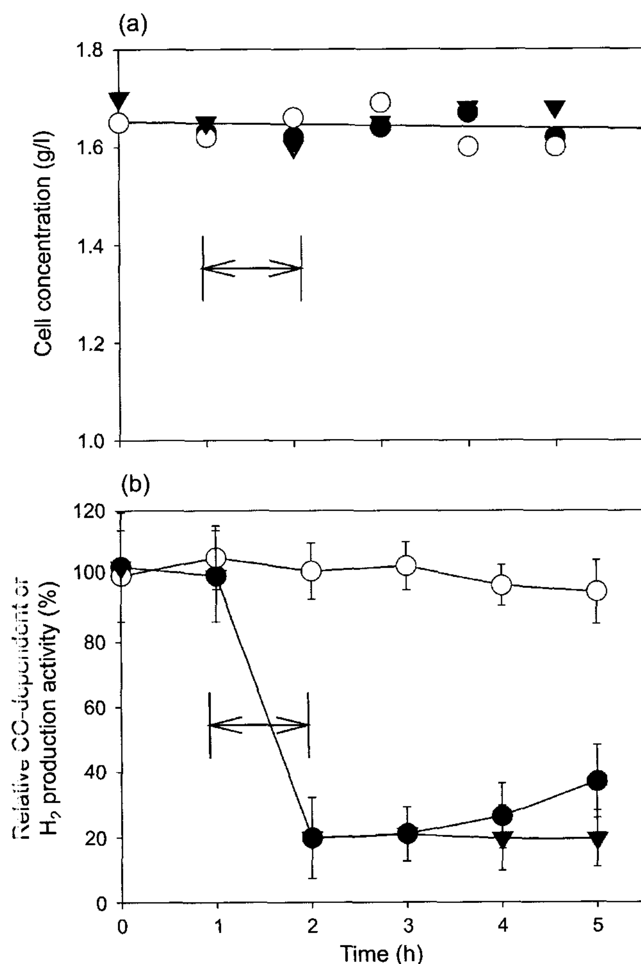

Fig. 2. Effect of transcription inhibitor (chloramphenicol) on (a) cell concentration, and (b) CO-dependent H_2 production activity of *Citrobacter* sp. Y19 upon exposure to air and reactivation by a subsequent anaerobic incubation.

Arrow represents the exposure period in full air. Symbols: -○-, No exposure (control); -●-, Exposure to air without chloramphenicol; -▼-, Exposure to air with chloramphenicol.

When the whole-cell suspension was exposed to air for 1 h, about 80% of the CO-dependent H_2 production activity was destroyed. When the inactivated cells were incubated under Ar/CO (80:20, v/v) mixture, the activity began to recover slowly after 1 h. However, the cells incubated with chloramphenicol, which is a transcriptional inhibitor, did not show any recovery. Cell density did not change for both cases. These results suggest that the reactivation during the anaerobic incubation is due to synthesis of new enzymes rather than reactivation of the enzymes present before the O_2 exposure.

Figure 3 shows the changes in hydrogenase and CODH activities during inactivation and subsequent reactivation processes. Forty % of hydrogenase activity in whole cells was lost and 80% in broken cells, when exposed to air for 1 h (Figs. 3a and 3b). When the inactivated cells were transferred to an anaerobic condition (100% Ar+ chloramphenicol), reactivation was observed for both cells. In the case of the whole cells, 90% of the initial activity was recovered in 2 h, whereas the recovery was hardly detectable in the case of the broken cells, when Ar was bubbled through the broken-cell suspension to develop the anaerobic condition. When Ar was purged through the headspace instead, about 25% of the original activity was recovered in 2 h. These results indicate that the inhibition of hydrogenase by O_2 is reversible, although the enzyme is highly sensitive to shear stress. Reversible characteristics of hydrogenases have been reported with other strains [16, 18]. Figure 3(c) and 3(d) show the change in CODH activity. After 1 h exposure to air, CODH activity of the whole cells and broken cells decreased by 90% and 80%, respectively. Incubation of the inactivated cell suspensions under anaerobic condition (100% Ar) in the presence of chloramphenicol did not yield any reactivation differently from the case of hydrogenase. These results indicate that inactivation of CODH by O_2 is irreversible.

Reactivation of CO-dependent H_2 production requires both CODH and hydrogenase activities. Since CO-dependent H_2 production activity in whole cells was recovered during the incubation in Ar/CO (80:20, v/v) mixture (Fig. 2) but CODH activity was not recovered even after incubation in 100% Ar in the presence of chloramphenicol (Figs. 3c and 3d), the effect of CO and chloramphenicol on reactivation of the enzymatic activities were further investigated (Table 3). CODH activity in whole cells was recovered only, when CO was present but chloramphenicol was not. If chloramphenicol was present or CO was absent, CODH

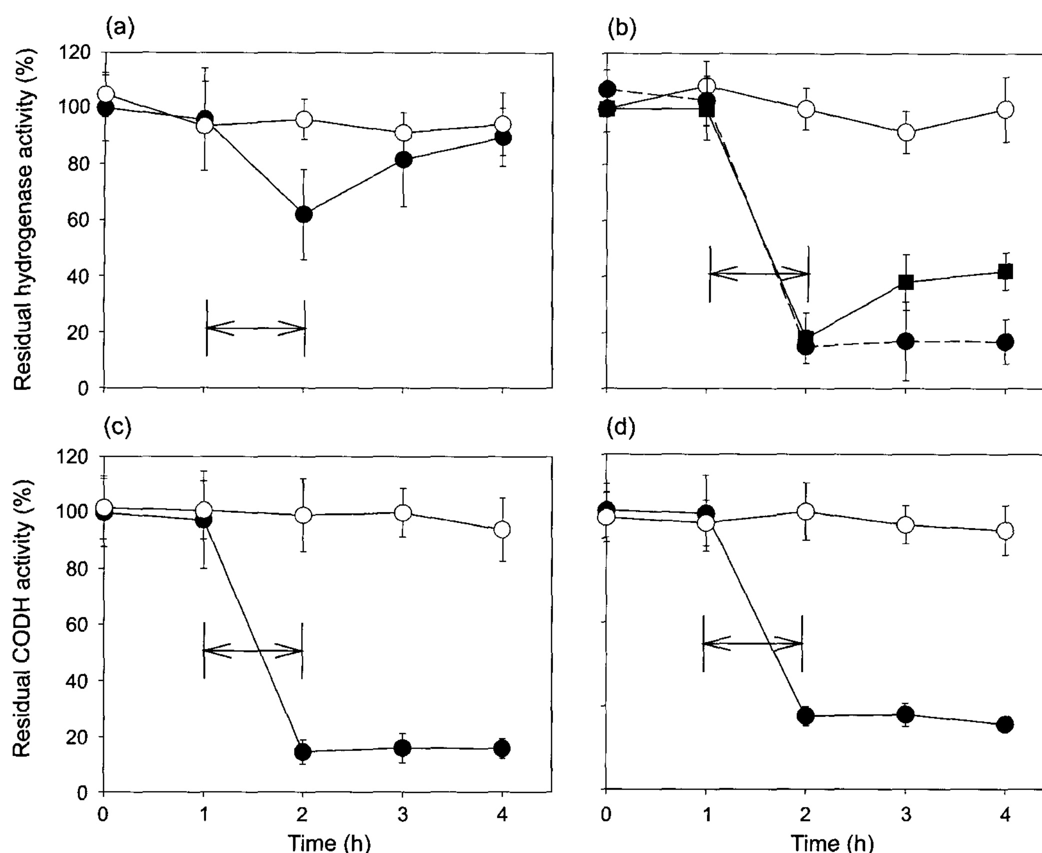

Fig. 3. Deactivation of H_2 production activity of Y19 upon exposure to air and reactivation by a subsequent anaerobic incubation.
Arrow represents the exposure period in full air. (a) Hydrogenase of whole cells, (b) hydrogenase of broken cells, (c) CODH of whole cells, and (d) CODH of broken cells. Symbols: -○-, No exposure (control); -●-, Exposure to air and a subsequent anaerobic incubation on 100% Ar. For anaerobic conditions O_2 was removed by bubbling Ar in reaction mixture except for the one case with (b) indicated by symbol -■- where Ar was supplied to the headspace of reaction vial.

activity was not recovered by the anaerobic incubation. On the contrary, hydrogenase activity was not affected by the presence of chloramphenicol or CO. These results indicate that, once Y19 is exposed to O_2 and its CO-dependent activity is lost, the synthesis of new CODH is essential to recover the whole-cell H_2 production activity. CO as an inducer seems to promote the expression of CODH at gene level. Furthermore, since the reactivation of CODH occurs in a buffer solution which does not support the cell growth, *de novo* pathway seems to contribute to the synthesis of new enzymes.

The results from Figs. 1-3 and Table 3 indicate that both hydrogenase and CODH activities of Y19 are greatly inhibited by O_2, but their inhibition modes are quite different. Because of irreversibility, O_2 inhibition of CODH might be more problematic than that of hydrogenase. However, it should be noted that Y19 has some advantages over strict anaerobes in recovering from O_2 inactivation. Y19 is a facultative anaerobe, and can aerobically grow very fast, when switched from an anaerobic condition [13]. This means that, although H_2 production

Table 3. Recovery of H_2 production activities in *Citrobacter* sp. Y19 after O_2 exposure[a].

Conditions	Hydrogenase[b]		CODH[b]		CO-dependent H_2 production in whole-cell suspension[b]
	Whole cell	Broken cell	Whole cell	Broken cell	
100% Ar+Chloramphenicol	+	+	-	-	-
100% Ar-Chloramphenicol	+	+	-	-	-
Ar/CO (80:20, v/v)+Chloramphenicol	ND[c]	ND[c]	-	-	-
Ar/CO (80:20, v/v)-Chloramphenicol	ND[c]	ND[c]	+	-	+

[a]Cells were incubated in 100 mM phosphate buffer (pH 7.0) that does not support cell growth.
[b](+) represents recovery of activity, while (-) represents no recovery.
[c]Not determined.

activity may be lost by accidentally incorporating O_2 into the H_2 production process, O_2 can be depleted quickly from the fermentation broth, and both CODH and CO-dependent H_2 production activity could be recovered at a high rate under anaerobic condition. In the present study, we focused our effort on reversibility of enzymatic activities and used 100 mM phosphate buffer. If nutrient broth is used, the reactivation process can be accelerated, owing to the rapid cell growth. Lee *et al.* [17] have reported some supportive results with the facultative bacterium *Rhodopseudomonas palustris* P4. The CO-dependent H_2 production activity in this strain which had been deactivated by O_2, could be successfully reactivated under oxygen-free condition by adding yeast extract and sucrose to the fermentation broth.

CONCLUSION

In *Citrobacter* sp. Y19, the enzyme activities related with CO-dependent H_2 production were severely inhibited by O_2. The rate of activity loss followed the 1st order decay kinetics, and its dependence on O_2 partial pressure could be expressed by the Michaelis-Menten kinetic equation. Hydrogenase was less sensitive to O_2 than CODH, and the inhibition was reversible. The inactivation of CODH by O_2 was irreversible and, once Y19 was exposed to O_2, the synthesis of new CODH was required for Y19 to recover the CO-dependent H_2 activity.

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NOMENCLATURE

Ar : argon
CO : carbon monoxide
MOPS: 3[*N*-morpholino]propanesulfonic acid
MV : methyl viologen
SDT : sodium dithionite
e : active enzyme concentration (μg ml^{-1}) or relative activity (%)
r_d : volumetric rate of deactivation (μg ml^{-1} min^{-1})
k_d : 1st order deactivation rate constant of the loss of activity upon exposure to O_2 (min^{-1})
$k_{d,max}$: maximum deactivation rate constant of the loss of activity upon exposure to O_2 (min^{-1})
K_s : half-saturation constant for deactivation by O_2 (atm)
P_{O_2} : O_2 partial pressure in gas phase (atm)

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