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Biohydrogen Production from Carbon Monoxide and Water by *Rhodopseudomonas palustris* P4

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Abstract A reactor-scale hydrogen (H_2) production *via* the water-gas shift reaction of carbon monoxide (CO) and water was studied using the purple nonsulfur bacterium, *Rhodopseudomonas palustris* P4. The experiment was conducted in a two-step process: an aerobic/chemoheterotrophic cell growth step and a subsequent anaerobic H_2 production step. Important parameters investigated included the agitation speed, inlet CO concentration and gas retention time. P4 showed a stable H_2 production capability with a maximum activity of 41 mmol H_2 g cell^{-1}h^{-1} during the continuous reactor operation of 400 h. The maximal volumetric H_2 production rate was estimated to be 41 mmol H_2 L^{-1}h^{-1}, which was about nine-fold and fifteen-fold higher than the rates reported for the photosynthetic bacteria *Rhodospirillum rubrum* and *Rubrivivax gelatinosus,* respectively. This is mainly attributed to the ability of P4 to grow to a high cell density with a high specific H_2 production activity. This study indicates that P4 has an outstanding potential for a continuous H_2 production *via* the water-gas shift reaction once a proper bioreactor system that provides a high rate of gas-liquid mass transfer is developed.

Keywords: hydrogen production, carbon monoxide, water-gas shift reaction, *Rhodopseudomonas palustris*, two-step process

Carbon monoxide (CO) is a common constituent in many industrial gas emissions [1]. It is generated from mobile and stationary sources such as automobiles, electric power plants and iron furnaces. It can also be produced by steam reforming of methane or pyrolysis of various biomasses [2]. CO is toxic to humans in extremely small doses; nevertheless, it is a valuable resource since it can be used for the biological production of hydrogen (H_2), acetate or ethanol [3,4]. H_2 production, through the well-known water-gas shift reaction as seen below, has been studied most extensively.

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

There are many bacterial strains known to catalyze the water-gas shift reaction. A partial list includes several photosynthetic bacteria, such as *Rhodobacter* sp., *Rubrivivax gelatinosus* and *Rhodospirillum rubrum*, and a strictly anaerobic bacterium such as *Methanosarcina barkeri* [5-8]. Among them, the photosynthetic purple nonsulfur bacteria, *Rh. rubrum* and *Ru. gelatinosus* have drawn much attention due to their high specific CO uptake rates and high conversion yields close to the theoretical value of 1.0. Many bioreactor-level continuous experiments have been carried out with microorganisms [5,8-10]. Several bioreactor systems such as the continuously stirred tank reactor (CSTR), bubble-column reactor (BCR), and trickle-bed reactor (TBR) have been examined for improving the mass transfer rate of CO (a sparingly soluble gaseous substrate) to liquid media. However, in most bioreactor systems, very low volumetric CO conversion rates (especially below 5 mmol CO L^{-1}h^{-1}) and low volumetric H_2 production rates are obtained. Furthermore, CO conversion efficiencies are also low, especially when the CO loading rates are high.

We have been studying two new H_2-producing bacteria, *Rhodopseudomonas palustris* P4 [4,11-13] and *Citrobacter* sp. Y19 [14-17]. These bacteria were originally isolated for CO-dependent H_2 production through the water-gas shift reaction, but we discovered they also have the ability to ferment various sugars. In our previous studies, these bacteria rapidly grew to high densities under aerobic conditions, but H_2 production occurred under anaerobic conditions only. Since the optimal conditions for cell growth and H_2 production were different from each other, a two-step process, which separates the growth phase from the H_2 production phase, has been suggested for maximizing H_2 production.

The present study aims at elucidating the H_2 production potential of *Rps. palustris* P4 *via* the water-gas shift reaction in reactor-scale experiments. Continuous experiments were conducted for 450 h to evaluate the long-term sta-

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bility of P4 during H_2 production. The relationship between volumetric H_2 productivity and the CO mass transfer rate was examined by varying the agitation speed, inlet CO concentration, and gas retention time. Finally, the results were compared to that of other CO-catalyzing microorganisms.

Rps. palustris P4 isolated from a sludge digester was used in this study [4]. Cells were cultivated in a medium containing (per liter): sucrose, 10 g; yeast extract, 6 g; KH_2PO_4, 1.5 g; NaH_2PO_4, 1.5 g; $MgCl_2$, 1.5 g; $ZnSO_4 \cdot 7H_2O$, 0.0006 g; $MnCl_2 \cdot 4H_2O$, 0.0006 g; $NiCl_2 \cdot 6H_2O$, 0.00012 g; and, $FeSO_4 \cdot 7H_2O$, 0.012 g. Experiments were carried out in a two-step manner: an aerobic/chemoheterotrophic cell growth step and a subsequent anaerobic H_2 production step. The 7-L bioreactor (KF-series, Korea Fermenter Co., Korea; working volume of 5 L) was inoculated with 500 mL of seed culture, which was aerobically grown to a late exponential phase, in 1.0-L flasks (working volume of 150 mL). During aerobic cell growth in the bioreactor, air was bubbled at 500 mL/min. The agitation speed was adjusted to the range of 500 to 700 rpm to maintain dissolved oxygen (DO) levels above 10% of saturation. The temperature was maintained at 30°C, and pH at 7.0 ± 0.1 by the automatic addition of 2.5 N NaOH and 30% HCl. To achieve a high cell density, 100 mL of 255 g/L sucrose solution was supplemented once at approximately 6 h into aerobic cell growth at which time the carbon source was depleted. When cell growth reached a stationary phase at 24 h, the culture was switched into an anaerobic condition. A N_2/CO mixture (9:1, v/v) was fed at 500 mL/min to induce H_2 production activity while agitating the bioreactor at 700 rpm.

Specific H_2 production activity (mmol H_2 g dry cell^{-1}h^{-1}) was determined for the cells taken from the bioreactor during the continuous operation. The cells were harvested by centrifugation, washed once with an ice-cold buffer solution (pH 7.0, 100 mM MOPS), and re-suspended in the same buffer to 0.5~0.7 mg/mL. Then, 1.0 mL aliquots were placed in 8.0 mL serum vials with 12 mm-thick butyl rubber septa and aluminum caps. All the procedures except for centrifugation were conducted in an anaerobic chamber (Bactron 1.5, Sheldon Manufacturing Inc., Oregon, USA; 100% Ar gas atmosphere). The vials were flushed with Ar gas (99.999%) for 5 min and then the headspaces were charged with a CO-Ar gas mixture (1:4, v/v). H_2 production was monitored for 1 h while shaking the vials at 160 strokes/min in a 30°C water bath [15].

Cell concentration was measured by a spectrophotometer (Lambda 20, Perkin-Elmer, CT, USA). Sucrose was quantified by the phenol sulfuric acid method [18]. Gas composition was determined by a gas chromatograph equipped with a thermal conductivity detector and a stainless steel column (1.8 m × 1/8") packed with Molecular Sieve 5A (Alltech, Deerfield, IL, USA). Detailed procedures and conditions were followed as previously described [11,15,19-21].

Rps. palustris P4 was isolated according to its ability to grow on CO under light [4]; however, it showed better growth under an aerobic/chemoheterotrophic condition than an anaerobic/photosynthetic or an anaerobic/chemoheterotrophic condition in serum bottle- and flask-cultures [11]. H_2 production, however, was observed under anaerobic conditions only. In order to obtain a high cell density, the cells were initially cultivated under an aerobic/chemoheterotrophic condition (data not shown). A high specific growth rate of 0.62 h^{-1} was observed and the cell density reached 6 g/L at about 6 h. Since the carbon source was depleted at 6 h, a 40% sucrose solution was supplemented into the bioreactor to 5 g sucrose/L and cells were further cultivated. At 18 h, the final cell density of 10 g/L was achieved (data not shown) and the reactor was switched into an anaerobic mode. H_2 production was examined at two different inlet CO concentrations: 10% (v/v) (phase I) and 20% (v/v) (phase II) (Fig. 1). When 10% CO was added under an anaerobic condition, the volumetric H_2 production rate gradually increased and, at about 150 h, reached 11 mmol H_2 L^{-1}h^{-1} with the CO conversion efficiency of 49% (phase I in Fig. 1). There was a long lag of 50 h before an active H_2 production began. This is in accordance to our previous report that stated the presence of CO is necessary for the induction of H_2 production activity in P4 [11]. Two enzymes are involved in the CO-linked H_2 production: a carbon monoxide dehydrogenase (CODH) which oxidizes CO to CO_2 with the production of reducing equivalent, and a CODH-dependent hydrogenase which reduces protons to H_2 by using the electrons donated from CODH [17]. It is known that, in *Rh. rubrum*, these two enzymes are expressed when CO is available only [22,23]. At 240 h, the inlet CO concentration was increased to 20% (v/v), concurrently, the agitation speed was increased from 500 rpm to 900 rpm (phase II). The volumetric H_2 production rate and CO conversion efficiency increased up to 28 mmol H_2 L^{-1}h^{-1} and 68%, respectively. These high values were maintained over 100 h and decreased after 400 h. Although there were some variations, the yield of H_2 on CO was in the range of 0.80 and 0.94 mmol/mmol, which was considerably lower than the theoretical value of 1.0 mmol/mmol (Eq. 1). This suggests that some H_2 are consumed by uptake hydrogenase [24] and used for maintaining cellular functions of P4. However, cell growth was not observed during the H_2 production period.

Fig. 1C shows the variations of specific H_2 production activity. The measurements were conducted in serum vials with the cells taken from the bioreactor. The specific activity gradually increased up to the maximum value of 41 mmol H_2 g cell^{-1}h^{-1} and then decreased. The volumetric production rates of H_2 during phase I and II were estimated to be in the range of 11~28 mmol H_2 L^{-1}h^{-1}, which corresponds to the specific H_2 production activity of 1.1~2.8 mmol H_2 g cell^{-1}h^{-1} at the cell density of 10 g/L. Since the apparent specific activity was much lower than the actual specific activity during the majority of the operation, we concluded that the overall H_2 production rate in the present CSTR system was not limited by the biological CO conversion activity of P4. Instead, the CO mass transfer rate was almost certainly responsible in determining the overall reaction rate (see below). Another

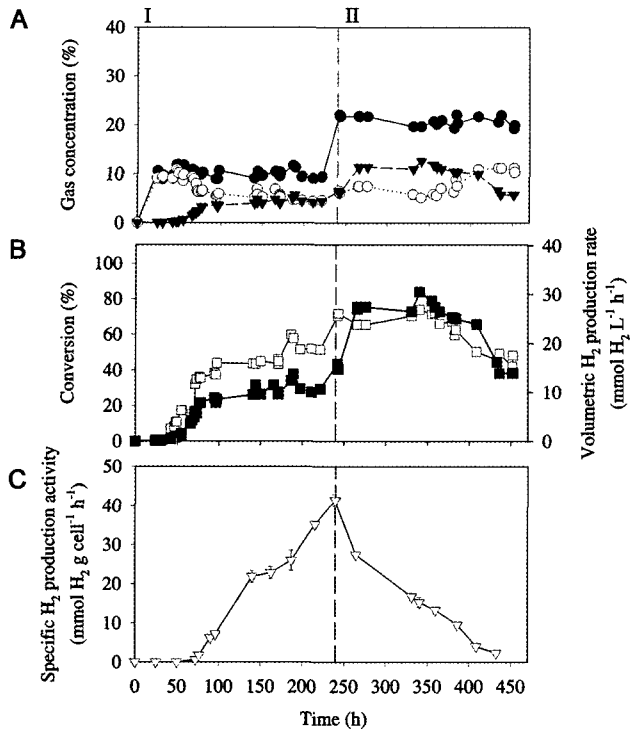

Fig. 1. H_2 production in a bioreactor culture. P4 was grown under aerobic/chemoheterotrophic conditions and shifted to an anaerobic condition at t=0. Symbols: Inlet CO (●), outlet CO (○), outlet H_2 (▼), CO conversion efficiency (□), volumetric H_2 production rate (■), and specific H_2 production activity (▽).

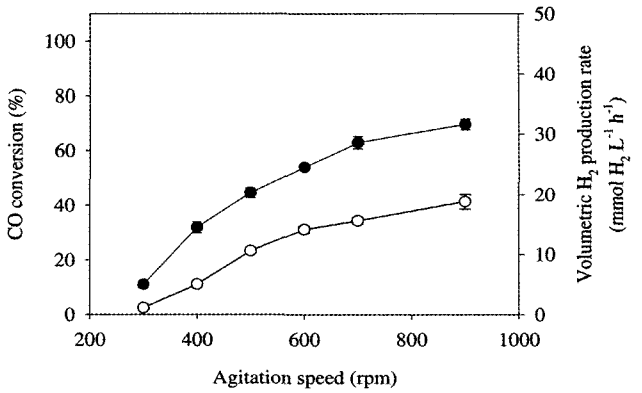

Fig. 2. Effect of agitation speed on CO conversion (●) and the volumetric H_2 production rate (○). The inlet CO concentration and gas retention time were fixed at 10% (v/v) and 10 min, respectively.

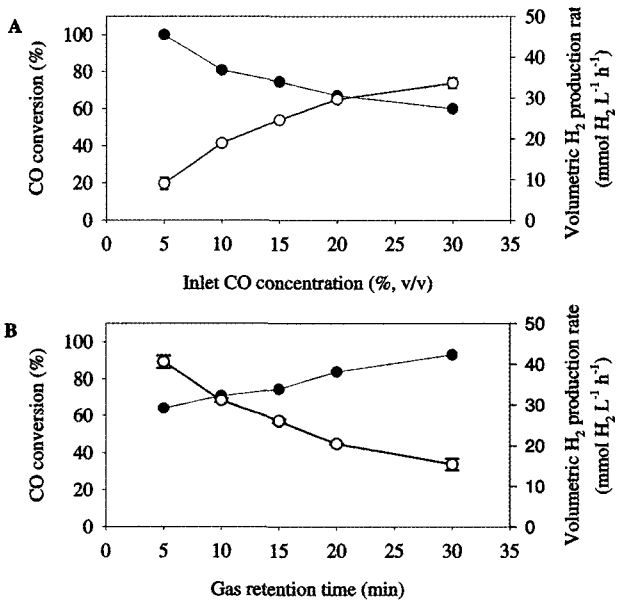

Fig. 3. Effect of the CO loading rate on CO conversion (●) and the volumetric H_2 production rate (○). The inlet CO concentration was varied at a constant gas retention time of 10 min (A) or the gas retention time was varied at a constant inlet CO concentration of 20% (v/v) (B).

important feature illustrated in Fig. 1 is that P4 exhibited a relatively stable H_2-production activity during the continuous H_2 production. Although continuously changing, the activity remained above 10 mmol H_2 g $cell^{-1}h^{-1}$ for about 300 h without any appreciable cell growth. This long stability has not been reported for other strains, and should be considered an important advantage of P4.

The effects of various operating conditions on H_2 production were studied at the end of phase I, where the specific H_2 production activity was approximately 40 mmol H_2 g $cell^{-1}h^{-1}$. The gas-liquid mass transfer rate is dependent on many parameters such as the agitation speed, gas flow rate, and inlet gas concentration. The effect of agitation speed was studied in the range of 300 and 900 rpm (Fig. 2). The inlet CO concentration and gas retention time were fixed at 10% (v/v) and 10 min, respectively. Upon increasing the agitation speed, the volumetric H_2 production rate and the CO conversion efficiency gradually increased. This indicates that the overall H_2 production performance is under a CO mass-transfer limitation. A similar study with *Rh. rubrum* also showed the existence of a mass transfer limitation in a stirred-tank reactor operated at 300~700 rpm [25]. The volumetric H_2 production rate obtained was estimated to be 19 mmol H_2 $L^{-1}h^{-1}$ even at the high agitation speed of 900 rpm, which corresponded to the apparent H_2 production activity as low as 1.9 mmol g $cell^{-1}h^{-1}$.

The effect of the CO loading rate was studied by changing the inlet CO concentration or gas retention time at an agitation speed of 900 rpm. Fig. 3A shows the effect of the inlet CO concentration at the gas retention time of 10 min. As the inlet CO concentration increased from 5% (v/v) to 30% (v/v), the CO conversion decreased gradually from 100% to 61% while the volumetric H_2 production rate increased from 10 mmol H_2 $L^{-1}h^{-1}$ to 33 mmol H_2 $L^{-1}h^{-1}$. Fig. 3B shows the effect of the gas retention time in the range of 5 and 30 min at the fixed inlet CO concentration of 20% (v/v). Upon decreasing

Table 1. Comparison of P4 with selected CO-dependent H_2 producers for continuous bioreactor operations

Microorganism	Reactor type (Working vol., L)	Culture condition	Cell density during H_2 production (g cell/L)	Operation time (h)	Specific H_2 production activity (mmol H_2 g cell^{-1}h^{-1})	Maximum volumetric H_2 productivity (mmol H_2 L^{-1}h^{-1})	CO Conversion (%)	Reference
Rhodopseudomonas palustris P4	CSTR (5)	Two-step aerobic-chemoheterotrophic cell growth/anaerobic H_2 production	10	450	~ 41[a]	41	61~100	Present study
	CSTR (5)	Two-step anaerobic-phototrophic cell growth/anaerobic H_2 production	1	50	~ 10[a]	5	< 35	Present study
Citrobacter sp. Y19	CSTR (3)	Two-step aerobic-chemoheterotrophic cell growth/anaerobic H_2 production	1.5	< 70	~ 20[a]	5.7	< 30	[15]
Rhodospirillum rubrum	CSTR (1.25)	Single-step anaerobic/phototrophic condition	< 0.9	< 100	~ 10[b]	4.7[c]	38~58	[25]
Rubrivivax gelatinosus CBS-2	TBR (0.95~1.1)	Two-step anaerobic-phototrophic cell growth/anaerobic H_2 production	1.35~1.65	Nr	13~33[b]	2.7[d]	< 75	[8]

Nr, not reported.
[a]The activities were measured in serum vials for the cells taken from the bioreactor during the continuous operation.
[b]Apparent specific H_2 production activity.
[c]The volumetric CO uptake rate (mmolL^{-1}h^{-1}) was converted using the H_2 yield of 0.88 mmol/mmol.
[d]The value was estimated from experimental data of the reference based on the empty bed volume of the bioreactor.

the gas retention time, the volumetric H_2 production rate gradually increased but the CO conversion efficiency decreased. The maximum H_2 production rate of 41 mmol H_2 L^{-1}h^{-1} was obtained at the short gas retention time of 5 min. Fig. 3 indicates that H_2 production rate and CO conversion efficiency upon the variation of CO loading rate by changing inlet CO concentration or gas retention time change always to the opposite ways. Increase of CO loading rate enhanced the H_2 production because of improved CO mass transfer rate, but resulted in low CO conversion yield due to the high CO concentration in the gas phase leaving the reactor. In order to improve CO conversion yields by *Rps. palustris* P4 under high CO loading rates, efficient gas mass transfer methods should be developed. The gas recycling, increased pressure and microbubble dispersions with surfactant might be attempted [26].

Table 1 summarizes the performances of some selected microorganisms during continuous bioreactor-scale H_2 production. Compared to serum bottle or flask-scale experiments [4,6,7,11,14,17,23,27], the results from bioreactor-scale experiments are limited [8,9,15,25]. The reactor performance depends on the characteristics of biocatalysts (such as specific H_2 production activity, stability, and cell growth rate) as well as the operating conditions (such as CO loading rate, agitation speed, recirculation rate, pH, and temperature). Table 1 indicates that even when considering the differences in experimental conditions, the present results are superior to most others. Especially, we notice that P4 grows to a much higher cell density of 10 g/L than the photosynthetic bacteria such as *Rh. rubrum* [25] and *Ru. gelatinosus* CBS-2 [8]. P4 does not require light for growth and its doubling time during aerobic growth is about 1 h. Most photosynthetic bacteria cannot be grown to a high density due to the limitation of light supply and consequently grow much slower. Since an efficient light supply becomes more difficult as the scale of the reactor increases, the use of light-dependent biocatalysts becomes less attractive for large-scale applications. The advantage of non-photosynthetic growth was confirmed by a separate experiment where P4 was grown under a photosynthetic condition (data not shown). Although the same 7-L bioreactor was used, P4 grew to the low cell concentration of 1 g/L under a constant light intensity of 2,000 lux, and the volumetric H_2 production rate was about 5 mmol H_2 L^{-1}h^{-1} (Table 1).

In summary, the present study showed the outstanding potential of *Rps. palustris* P4 in the reactor-scale water-gas shift reaction. This bacterium can grow to a high cell density and maintain the H_2 production activity stably for a long period of time. However, due to a serious gas-liquid mass transfer limitation of the CSTR used in this study, the CO conversion efficiency was low and a substantial amount of unreacted CO was discarded as the outlet gas stream. For an economical industrial application, the conversion yield can be enhanced so that the outlet CO content is maintained low regardless of the inlet CO concentration. This indicates that the development of a proper bioreactor system that provides a high gas-liquid mass transfer rate may ensure a large-scale biological water-gas shift reaction by P4.

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