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# Comparison of Hydrogenases from *Clostridium butyricum* and *Thiocapsa roseopersicina*: Hydrogenases of *C. butyricum* and *T. roseopersicina*

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**Abstract** The properties related to the temperature and oxygen stability of the cytoplasmic hydrogenases from the fermentative strict anaerobic bacterium, *Clostridium butyricum* NCIB 9576 (*Cl. butyricum*), and purple sulfur phototrophic bacterium, *Thiocapsa roseopersicina* NCIB 8347 (*T. roseopersicina*), were compared. The optimum temperatures for the growth of *Cl. butyricum* and *T. roseopersicina* were 37°C and 25°C, respectively, whereas those for the $H_2$ evolution of the cytoplasmic hydrogenases prepared from *Cl. butyricum* (C-$H_2$ase) and *T. roseopersicina* (T-$H_2$ase) were 45°C and 65°C, respectively. The T-$H_2$ase was more thermostable than the C-$H_2$ase and retained its full activity for 5 h at 50°C under anaerobic conditions and 90% of its activity at 60°C, whereas the C-$H_2$ase lost its activity drastically at 50°C. The optimum pHs for $H_2$ oxidation of the C-$H_2$ase and T-$H_2$ase were 9.0 and 7.5, respectively. Both enzymes showed a maximum $H_2$ evolution activity at pH 7.0. Under aerobic conditions, 80% of the T-$H_2$ase activity was retained for 10 h at 30°C, and 50% of the activity remained after 6 days under the same experimental conditions. However, the C-$H_2$ase was labile to oxygen and lost its activity immediately on exposure to air. Therefore, these properties of the T-$H_2$ase are expected to be advantageous for application in *in vitro* biological $H_2$ production systems.

**Key words:** Hydrogenase, *Clostridium butyricum*, *Thiocapsa roseopersicina*, characterization

Hydrogenase ($H_2$ase) was first described by Stepheneson and Stickland in 1931 [1] as a bacterial enzyme that reversibly catalyzes the oxidation and production of hydrogen according to the following reaction: $2H^+ + 2e \leftrightarrow H_2$. $H_2$ase activity was subsequently found in a number of anaerobic and aerobic bacteria, various groups of algae, anaerobic protozoa, and archaea. The physiological function of $H_2$ases varies: they can serve as redox safety valves to dispose of excess reducing power; function as generators of chemical energy by taking up and oxidizing $H_2$; or work to maintain a reducing environment for reactions of crucial importance, such as the fixation of dinitrogen [2]. $H_2$ases (cytochrome c3, oxidoreductase, EC 1.18.99.1) are classified into two major families on the basis of the metal content of their respective dinuclear catalytic centers: *i.e.*, nickel-iron (NiFe)-$H_2$ases and iron only (Fe)-$H_2$ases [1]. Some NiFe $H_2$ases also contain selenium at their catalytic center in the form of selenocysteine. The two $H_2$ases families differ functionally from each other in that NiFe $H_2$ases tend to be involved in $H_2$ oxidation, whereas Fe $H_2$ases are involved in $H_2$ production. Moreover, NiFe $H_2$ases are approximately $10^{-1}$–$10^{-2}$ times less active and have $10^2$ times more affinity for $H_2$ than Fe $H_2$ases. It is also known that some NiFe $H_2$ases retain their activity during exposure to oxygen and carbon monoxide. $H_2$ases isolated from the phototrophic bacteria *Thiocapsa roseopersicina* and *Rhodobacter capsulatus* are NiFe-$H_2$ases, whereas those isolated from *Clostridium pasteuranium* and *Clostridium acetobutyricum* are Fe-$H_2$ases.

In recent years, interest in bio-$H_2$ production has resulted in a considerable amount of research into the use of whole cell or cellular components [3–5]. Such application-oriented studies have also increased basic knowledge on $H_2$ evolving microorganisms and the properties of various $H_2$ases [6]. Yet, although related research is being actively pursued with some encouraging preliminary results, major problems related to the utilization of $H_2$ases still remain. Specifically, $H_2$ases are generally unstable and sensitive to oxygen.

Accordingly, this study prepared fractions of cytoplasmic $H_2$ases from the fermentative strict anaerobic bacterium *Clostridium butyricum* NCIB 9576 (*Cl. butyricum*), and the purple sulfur phototrophic bacterium *Thiocapsa roseopersicina* NCIB 8347 (*T. roseopersicina*), and compared their

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properties in terms of their temperature, oxygen, and pH stabilities to determine the characteristics of the $H_2$ases for bio-$H_2$ production.

## MATERIALS AND METHODS

### Strains and Cultivation

The *Clostridium butyricum* NCIB 9576 was cultivated in a 5-l flask containing 3 l of a PYG medium supplemented with 1% glucose at 37°C [7]. After inoculation, the flask was flushed with argon gas for 20 min to develop anaerobic conditions. The inoculum was then cultivated in a 165-ml serum bottle (working volume of 50 ml) under the same conditions. The cells were harvested during the late exponential phase by centrifugation after 6 h of cultivation. Meanwhile, the *Thiocapsa roseopersicina* NCIB 8347 was photoautotrophically grown at 27°C in a 3.6-l flat vertical acryl reactor containing 3 l of Pfennig's medium containing 0.05% acetate [8]. The culture was continuously illuminated by 5 klux from a halogen lamp and sparged with nitrogen gas containing 1% carbon dioxide for agitation and to supply a carbon source at a flow rate of 200 ml/min. The cells were harvested by centrifugation during the late exponential phase after 18 h of cultivation.

### Preparation of Cytoplasmic Enzyme Extract

The cells were suspended in a 50 mM phosphate buffer (pH 7.0) and disrupted by sonication (Sonic dismembrator 550, Fisher) with continuous argon gas flushing at 4°C. The cell debris was removed by ultracentrifugation (80,000 ×*g*, 4°C, 1 h) and the resulting supernatant used as the cytoplasmic $H_2$ase [9].

### $H_2$ase Assay

The $H_2$ evolution activity was assayed by the evolution of $H_2$ from methyl viologen reduced by sodium dithionite [10]. In a 5-ml vacuum vial, 1.4 ml of a reaction mixture containing a 50 mM sodium phosphate buffer (pH 7.0), 2.5 mM methyl viologen, and the enzyme solution was prepared under a nitrogen atmosphere. The reaction was then started by adding 0.1 ml of 230 mM sodium dithionite to the reaction mixture after preincubation. The $H_2$ evolution was measured every 10 min using a gas chromatograph 14-B (Shimadzu, Kyoto, Japan) equipped with a molecular sieve 5A column (3 mm×2 m) and thermal conductivity detector. One unit of activity was defined as the amount of $H_2$ase evolving 1 μmol $H_2$ per minute.

The hydrogen-oxidation activity of the $H_2$ases was measured spectrophotometrically using methylene blue as an electron acceptor based on the method of Cammack *et al.* [11]. The reaction mixture (2.5 ml) was prepared with 0.5 mM methylene blue in a 50 mM sodium phosphate buffer (pH 7.0), and then placed in a rubber stoppered cuvette and saturated with $H_2$. The reaction was initiated by adding the enzyme preparation to the cuvette after 10 min of preincubation at the same temperature as the reaction. The reduction of methylene blue was monitored by measuring the absorbance at a wavelength of 570 nm. One unit of enzyme activity was defined as the amount of hydrogenase oxidizing 1 μmol methylene blue per min.

The protein concentration was determined by the Lowry method using a protein assay kit (Bio-Rad, U.S.A.). Bovine serum albumin was used as the standard.

### Oxygen Stability

The cytoplasmic preparation was exposed to an air or oxygen atmosphere for 10 h at 30°C, and then the residual enzyme activity for $H_2$ evolution was measured at 40°C for the C-$H_2$ase and 50°C for the T-$H_2$ase. In addition, the residual enzyme activity for $H_2$ evolution was also measured at 50°C after incubating the T-$H_2$ase cytoplasmic preparation in an air or oxygen atmosphere for 6 days at 4°C or 25°C.

### Temperature

The optimum temperatures for $H_2$ evolution and oxidation reactions by the cytoplasmic preparations were determined by measuring each activity over a temperature range of 30 to 80°C. Meanwhile, the thermostability was examined by incubating the enzyme preparations at various temperatures, and assessing the remaining activity by measuring the $H_2$ evolution activity at 40°C for the C-$H_2$ase and 50°C for the T-$H_2$ase.

### pH

The effect of pH on the $H_2$ase activity of the cytoplasmic preparations was investigated at various pH values. The $H_2$ evolution activity was measured in a 50 mM buffer over pH ranges of 6.0 to 7.5 (PIPES), 7.5–8.5 (EPPS), and 8.5–10 (CHES) at 50°C, whereas the $H_2$ oxidation activity was measured in a 50 mM sodium phosphate buffer (pH 6.0–7.5), 50 mM Tris-HCl buffer (pH 7.5–9.0), and 50 mM glycine-NaOH buffer (pH 9.0–10.5) at 50°C.

## RESULTS AND DISCUSSION

### Growth and $H_2$ Production

The $H_2$ productivity, cell growth, and $H_2$ase activity of the mesophilic strict anaerobic bacterium *Cl. butyricum* NCIB 9576 were measured under the experimental conditions described in Materials and Methods. The logarithmic growth phase continued for 4 h in a synthetic medium containing 1% glucose, and the cell generation time for this growth phase was 20 min. The cell concentration reached 0.9 g-DCW/l-culture after 6 h of cultivation, and the pH gradually decreased to 4.5, resulting in a cessation

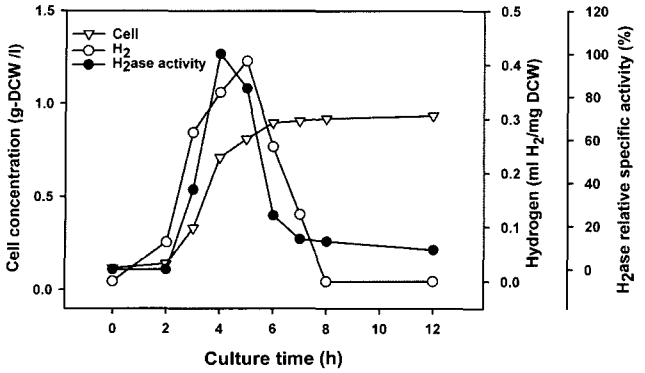


**Fig. 1.** Cell growth and hydrogenase activity of *Cl. butyricum* NCIB 9576.

▽, Cell concentration; ○, $H_2$ produced; ●, $H_2$ evolution activity. *Cl. butyricum* NCIB 9576 was cultivated in PYG medium containing 1% glucose. $H_2$ production was determined by gas chromatography. $H_2$ evolution activity was measured at 40°C using reduced methyl viologen.

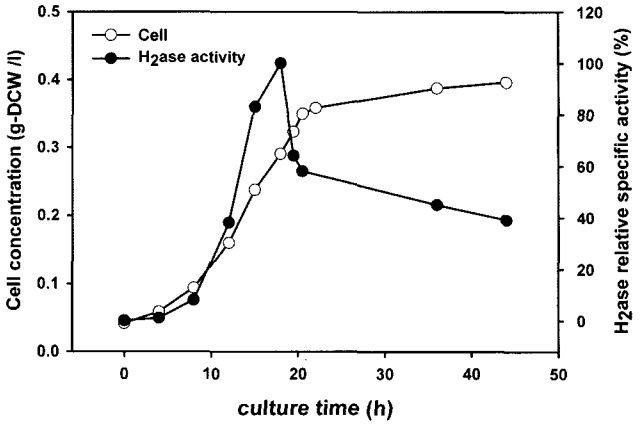


**Fig. 2.** Cell growth and hydrogenase activity of *T. roseopersicina* NCIB 8347.

○, Cell concentration; ●, $H_2$ evolution activity. *T. roseopersicina* NCIB 8347 was cultivated under photoautotrophic conditions in Pfennig's medium. $H_2$ evolution activity was measured at 50°C using reduced methyl viologen.

of cell growth. The $H_2$ase activities were influenced by the cell growth during the logarithmic growth phase (Fig. 1).

*Cl. butyricum* produced $H_2$ at 1.17 l-$H_2$/g-DCW for 12 h using 1% glucose as the carbon source. During the logarithmic growth phase, the $H_2$ase activity significantly increased when the cell concentration increased, yet it drastically decreased during the stationary growth phase, resulting in a decrease in the $H_2$ production rate. Changes in the rate of $H_2$ production during the growth phase have also been observed by other researchers and may be explained as follows: *Clostridium* sp. showed a biphasic fermentation pattern on a glucose medium under anaerobic conditions. As such, after producing acetate and butyrate from glucose, *i.e.*, the acidogenic phase, the organism switches to the solventogenic phase, where acetone, butanol, and ethanol are formed, shortly before entering the stationary phase. This change in the carbon flow from acids to solvents appears to be associated with a modification in the electron flow. In the acidogenic phase, ferredoxin, an electron mediator, is reduced to oxidize the excess NADH produced during glycolysis. The oxidized ferredoxin is regenerated by hydrogenase, using the protons as electron acceptors, whereas in the solventogenic phase, the solvent-producing pathways require more NAD(P)H than can be produced during glycolysis. The reoxidation of the reduced ferredoxin to produce NAD(P)H then competes with the oxidation of ferredoxin by hydrogenase. As a consequence, the rate of hydrogen production is decreased [12]. The $H_2$ partial pressure of the culture indicated that it had an effect on $H_2$ production and $H_2$ase regulation. Thus, the activity of the NAD(P)H-dependent Fe-$H_2$ase in *Clostridum* sp. decreased as the $H_2$ partial pressure increased in the culture.

The purple sulfur bacterium *T. roseopersicina* NCIB 8347 was grown photoautotrophically in a modified Pfennig medium that contained 0.05% acetate, plus bubbling nitrogen

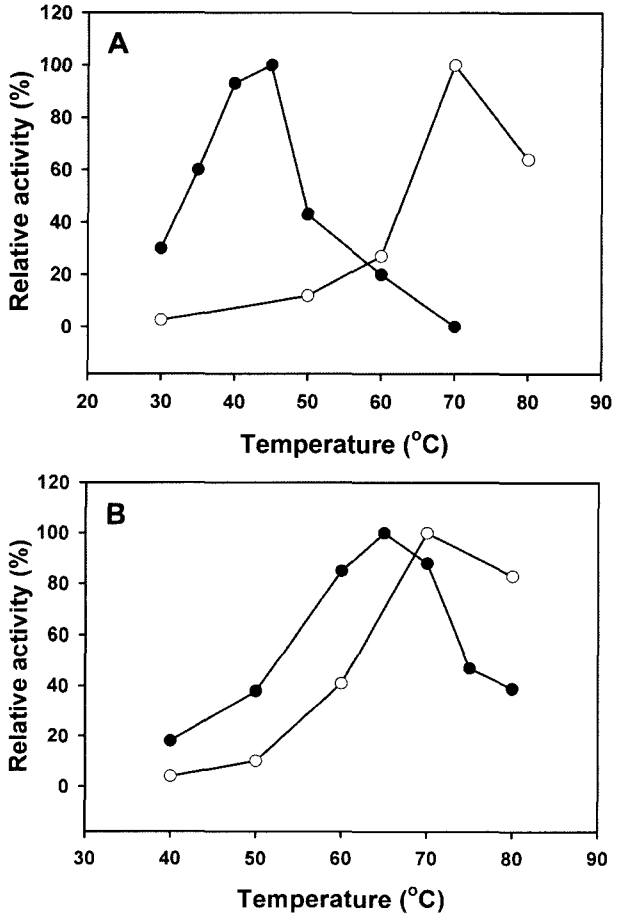


**Fig. 3.** Temperature profile for C-$H_2$ase (**A**) and T-$H_2$ase (**B**).

Activities of C-$H_2$ase were measured in 50 mM PIPES buffer (pH 7.0) for $H_2$ evolution (●) and in 50 mM Glycine-NaOH buffer (pH 9.0) for $H_2$ oxidation (○). Activities of T-$H_2$ase were measured in 50 mM PIPES buffer (pH 7.0) for $H_2$ evolution and in 50 mM Tris-HCl buffer (pH 7.5) for $H_2$ oxidation.

containing 1% $CO_2$. The cell concentration increased to 0.29 g-DCW/l-culture after 18 h of cultivation (Fig. 2). The $H_2$ase-specific activity increased as the cell concentration increased during the logarithmic growth phase, and then decreased drastically during the stationary phase. *T. roseopersicina* NCIB 8347 showed a different pattern of $H_2$ase activity compared with *T. roseopersicina* grown under photoheterotrophical culture conditions using lactate and glutamate as the carbon and nitrogen source, respectively, as the $H_2$ production started from the late logarithmic growth phase and continued to the stationary phase until the cell growth leveled out.

The cytoplasmic fraction prepared from *Cl. butyricum* showed both evolution and oxidation $H_2$ase activities, yet the membrane fraction showed neither. Adams *et al.* [6] already reported on two soluble $H_2$ases from *Cl. pastertianum* involved in $H_2$ evolution and oxidation. However, under the present experimental conditions, the cytoplasmic fraction of *T. roseopersicina* only exhibited evolution $H_2$ase activity, whereas the membrane fraction showed both activities, with mainly oxidation activity. This appears to correspond with a previous report by Kovacs *et al.* [2] wherein two membrane-bound $H_2$ases were identified in *T. roseopersicina*, a soluble $H_2$ase and a sensor $H_2$ase.

**Table 1.** Determination of D-value and z-value for C-$H_2$ase and T-$H_2$ase.

| | Temp (°C) | D-value (h)[a] | z-value (°C)[b] |
|---|---|---|---|
| C-$H_2$ase | 30 | 393.3 | 10.63 |
| | 40 | 33.79 | |
| | 45 | 13.67 | |
| | 50 | 5.210 | |
| T-$H_2$ase | 50 | 121.6 | 19.61 |
| | 60 | 65.50 | |
| | 65 | 35.00 | |
| | 70 | 10.27 | |

[a]The D-value was determined by the time in hours required for a 90% reduction in the enzyme activity.
[b]The z-value is the number of degrees of temperature change necessary to change the D-value by a factor of 10.

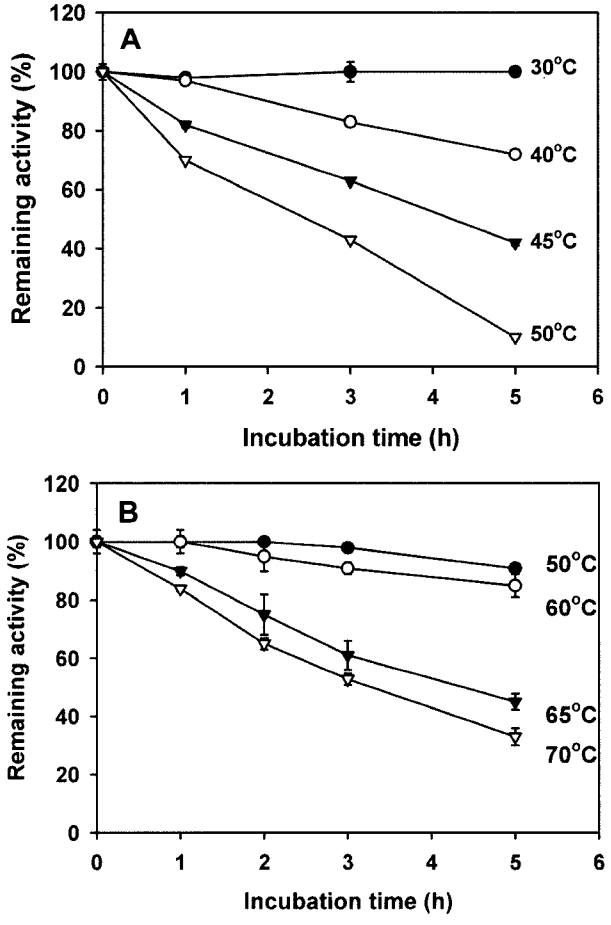


**Fig. 4.** Thermostability of C-$H_2$ase (**A**) and T-$H_2$ase (**B**) for $H_2$ evolution.
Enzymes were incubated at various temperatures under argon, and the remaining $H_2$ evolution activity was measured at 40°C for C-$H_2$ase and 50°C for T-$H_2$ase, respectively.

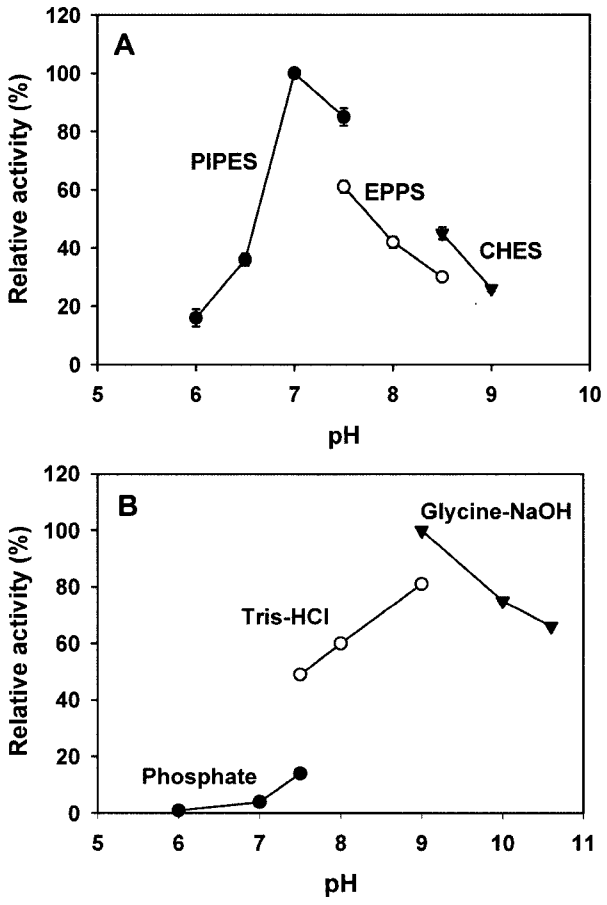


**Fig. 5.** pH profile for *Cl. butyricum* NCIB 9576 $H_2$ase activity.
$H_2$ evolution (**A**) and $H_2$ oxidation (**B**) activities of C-$H_2$ase were measured in buffers with various pH values at 40°C.

**Temperature**

The optimum temperatures for the $H_2$ evolution of the C-$H_2$ase and T-$H_2$ase were 45°C and 65°C, respectively, whereas the optimum growth temperatures for *Cl. butyricum* and *T. roseopersicina* were 37°C and 25°C, respectively (Fig. 3). The temperature profiles for the $H_2$ oxidation reactions of the C-$H_2$ase and T-$H_2$ase were similar, and both $H_2$ases showed a maximum activity at 70°C. The C-$H_2$ase retained its full activity at 30°C for 5 h, and retained 75% and 45% of its activity after heating at 40°C and 45°C for 5 h, respectively (Fig. 4A). The T-$H_2$ase was more thermostable than the C-$H_2$ase, retaining its full activity after heating at 50°C for 1 h at 50°C, and losing only 10% of its activity after 5 h (Fig. 4B). It also maintained 80% of its activity after incubation at 70°C for 1 h.

The D-value and z-value, indicating the index for the enzyme thermostability, were calculated and are compared in Table 1. The D-value for the C-$H_2$ase at 50°C was 5.2 h, whereas that for the T-$H_2$ase was 121 h. The z-value for the C-$H_2$ase was 10.6°C, whereas that for the T-$H_2$ase was 19.6°C. Therefore, the T-$H_2$ase was more thermostable than the C-$H_2$ase. Nishihara *et al.* [13] previously reported a thermophilic and oxygen-stable membrane-bound $H_2$ase from the marine hydrogen oxidizing bacterium *Hydrogenovibrio marinus*, which retained 90% of its activity after heating at 70°C for 50 min. When compared with their data, the present T-$H_2$ase showed a similar thermostability with *H. marinus*, although originated from a mesophile.

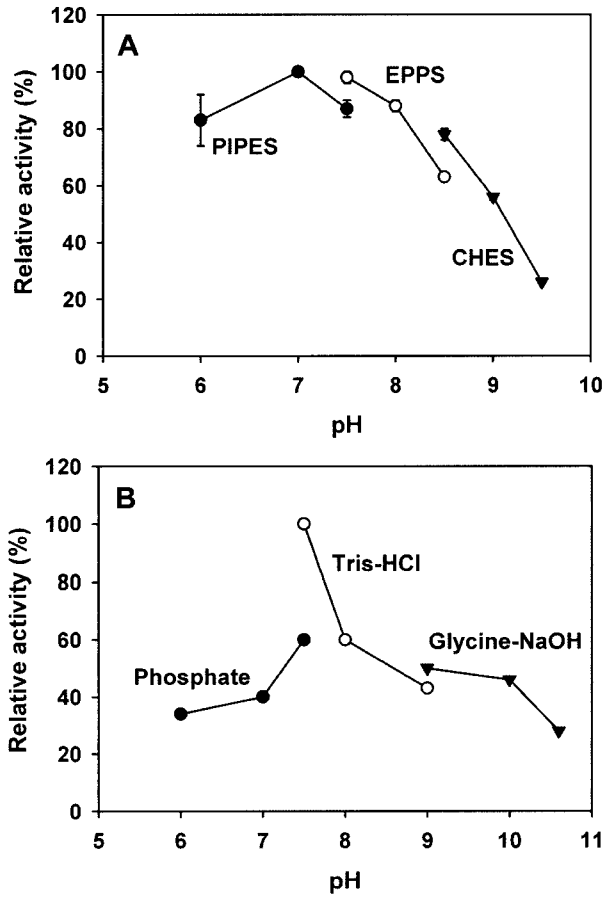


**Fig. 6.** pH profile for *T. roseopersicina* NCIB8347 $H_2$ase activity.

$H_2$ evolution (**A**) and $H_2$ oxidation (**B**) activities of T-$H_2$ase were measured in buffers of various pH values at 50°C.

**pH**

A wide pH range from 6 to 10 was investigated using different types of buffer to determine the optimum pH for $H_2$ase activity. The $H_2$ase activities were influenced according to the pH range and type of buffer. As shown in Fig. 5, the optimum pH values for $H_2$ evolution and $H_2$ oxidation for the C-$H_2$ase were 7.0 (50 mM PIPES) and 9.0 (50 mM Glycine-NaOH), respectively, indicating that the chemical equilibrium of the $H_2$ase reaction was controlled by the pH value. A neutral pH was preferable for $H_2$ production, and an alkaline pH for $H_2$ oxidation. As shown in Fig. 6, the T-$H_2$ase showed a high $H_2$ evolution activity within a broad range of pH values from 6.0 to 8.0, with the optimum pH value for $H_2$ evolution at 7.0 (50 mM PIPES). The optimum pH value for $H_2$ oxidation by the T-$H_2$ase was pH 7.5 (50 mM Tris-HCl), with minimal activity at pH 6.0.

**Oxygen**

As expected from the strict anaerobe *Cl. butyricum*, the C-$H_2$ase was more sensitive under aerobic conditions than the T-$H_2$ase, originating from the obligate anaerobic phototroph *T. roseopersicina* (Fig. 7). The T-$H_2$ase showed a relatively high oxygen stability; specifically, it retained 80% of its original activity at 30°C under exposure to air for 10 h, whereas the C-$H_2$ase lost 50% of its original activity after only 3 min. To further investigate the prolonged

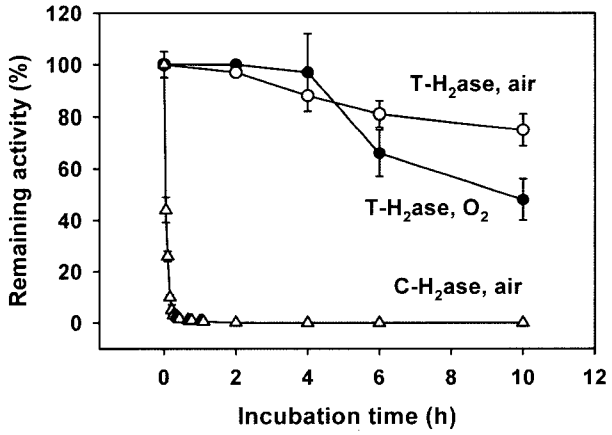


**Fig. 7.** Oxygen stability of C-$H_2$ase and T-$H_2$ase for $H_2$ evolution.

C-$H_2$ase and T-$H_2$ase were incubated in stoppered glass vials in air atmosphere (△, ○) and T-$H_2$ase was incubated in oxygen atmosphere (●) at 4°C. The remaining $H_2$ evolution activity was measured at 40°C for C-$H_2$ase and 50°C for T-$H_2$ase, respectively.

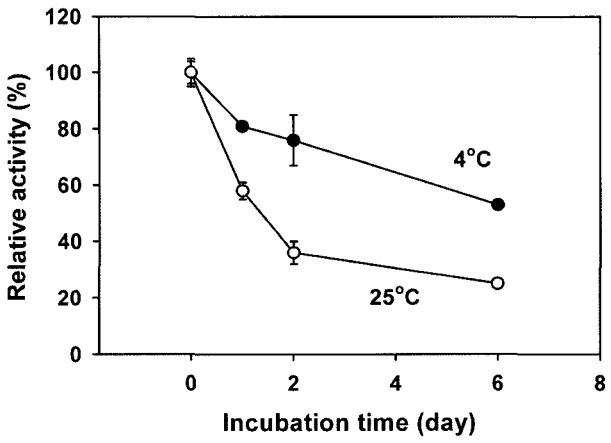


**Fig. 8.** Prolonged oxygen stability of *T. roseopersicina* hydrogenase for $H_2$ evolution.
T-$H_2$ases were incubated in stoppered glass vials in an air atmosphere at 4°C (●) or 25°C (○) for 6 days. The remaining $H_2$ evolution activity was measured at 50°C.

oxygen stability of the T-$H_2$ase, the enzyme was stored at 4 and 25°C for 6 days and the residual activities determined (Fig. 8). At 4°C, the T-$H_2$ase retained 80% of its original activity after 24 h of storage and 50% after 6 days of storage. At an ambient temperature, the enzyme retained 60% of its activity after 24 h of storage and 30% after 6 days of storage. Several thermophilic $H_2$ases have already been reported from hyperthermophilic archaea, *Pyrodictium brockii* and *Pyrococcus furiosus*, which grow optimally at 105°C and 100°C, respectively [14, 15]. The $H_2$ oxidizing activity in the crude extract from *Pd. brockii* increases up to 90°C, and retains 80% of its activity at 90°C for 15 min under anaerobic conditions. However, the enzyme is very sensitive to oxygen and cannot produce $H_2$ from reduced methyl viologen. The optimal temperature for the soluble $H_2$ase from *Pc. furiosus* for both $H_2$ oxidation and evolution activities is above 95°C, plus it is remarkably thermostable under anaerobic conditions. However, it loses half of its activity after 6 h of exposure to air [13].

In conclusion, the T-$H_2$ase originating from the mesophile *Thiocapsa roseopersicina* showed similar characteristics to the $H_2$ases from thermophiles in terms of its thermostability and thermophilicity, and was vastly superior to any other $H_2$ase in terms of its oxygen stability. Therefore, these properties are expected to be advantageous for its application in *in vitro* biological $H_2$ production systems.

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