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Hydrogen production characteristics of photoheterotrophic *Rubrivivax gelatinosus* L31

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ABSTRACT

A new strain of *Rubrivivax gelatinosus* (designated as L31) was isolated from the sediment of a local reservoir. Testing against 10 organic substrates, this strain could produce hydrogen from carbohydrates, including glucose, sucrose and starch, as well as from lactate and malate. Even though it could use acetate, propionate, butyrate, succinate and glutamate as substrate, it could not produce hydrogen from them. Based on the determined kinetic parameters derived from experimental data, lactate produced the highest amount (225.4 ml) of hydrogen with a hydrogen conversion efficiency of 50.5%, whereas starch exhibited the highest production rate of 829 ml/g/h after an extensive lag phase of 870 h. The increase of nitrogenase activity, which ranged from 9.0 to 36.3 μl-C_2H_4/h/mg-VSS, generally resulted in higher substrate degradation and hydrogen conversion efficiency. Although both formations of hydrogen and intracellular poly-β-hydroxybutyrate consumed electrons, there was no noticeable quantitative correlation between them.

1. Introduction

Hydrogen is an ideal fuel which does not produce greenhouse gases upon combustion. It is commercially produced by electrochemical and thermochemical processes. However, hydrogen may also be produced biologically via either non-phototrophic (often referred as dark) or phototrophic fermentation. It may be harvested during biological treatment of waste and/or wastewater, the organic pollutants in which serve as carbon and energy sources for the microbes. The non-phototrophic process, which has been studied extensively in the past decade [1], converts no more than 40% of chemical energy in the organic pollutants into hydrogen, leaving the majority in the form of fatty acids and alcohols. Only the phototrophic process is able to convert these residual fatty acids and alcohols completely into hydrogen.

Most of phototrophic biohydrogen studies were conducted for pure cultures of four purple non-sulfur bacteria (PNS), including *Rhodobacter sphaeroides* [2], *Rhodobacter capsulatus* [3], *Rhodopseudomonas palustris* [4] and *Rhodospirillum rubrum* [5], using organic substrate as carbon source. Another PNS, *Rubrivivax gelatinosus* can also produce hydrogen but mainly using carbon monoxide as carbon source.

This research was conducted to study the hydrogen production characteristics of a new strain of *R. gelatinosus*, which was isolated from a local reservoir sediment, using various organic substrates. These characteristics were then correlated with the activity of its nitrogenase, which is responsible to photoheterotrophic hydrogen production [6], and the accumulation of PHB (poly-β-hydroxybutyrate), an intracellular polymer which may compete with hydrogen for electrons [7].

2. Materials and methods

2.1. Culture enrichment and isolation

A mixed phototrophic sludge culture was firstly enriched from the sediment of a local reservoir. The enrichment was carried

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out in a 125 ml serum bottle at 30 °C under 200 W/m^2 of illumination by a tungsten lamp using the common phototrophic Medium 27 (DSMZ, Germany). After eight enrichment cycles, the diluted culture liquids were spread onto the nutrient agar plates, containing sodium acetate and sodium glutamate as respective carbon and nitrogen sources, and incubated under illumination at 200 W/m^2. After 7 days of incubation, a phototrophic bacterium, which was subsequently identified as a new strain (designated as L31) of *R. gelatinosus*, was isolated.

2.2. PCR, sequencing and identification

The 16S rDNA fragment of this bacterium was amplified by whole-cell polymerase chain reaction (PCR) using the *Bacteria*-specific primer EUB8F (5′-AGAGTTTGATCMTGGCTCAG) and universal primer 1492R (5′-GGTTACCTTGTTACGACTT). The DNA sequence of the amplified fragment was then compared with those available in the GenBank by BLAST search for preliminary identification. They were then aligned and checked manually using BioEdit. A phylogenetic tree was subsequently constructed using the neighbor-joining method with MEGA 3.1 [8].

2.3. Media

A basal medium was used to prepare for tests of bacteria growth and hydrogen production conducted in this study. The basal medium was added (for each liter of ultra-pure water) with 0.75 g K_2HPO_4, 0.85 g KH_2PO_4, 2.8 mg H_3BO_3, 0.75 mg $Na_2MoO_4 \cdot 2H_2O$, 0.24 mg $ZnSO_4 \cdot 7H_2O$, 2.1 mg $MnSO_4 \cdot 4H_2O$, 0.04 mg $Cu(NO_3)_2 \cdot 3H_2O$, 0.75 mg $CaCl_2 \cdot 2H_2O$, 2.0 mg EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 11.8 mg $FeSO_4 \cdot 7H_2O$, 3.78 mg vitamin B1, 3.57 mg biotin, 5.25 mg p-aminobenzoic acid, and 6.48 mg nicotinamide [9]. The aSy medium, which was used for the cultivation of bacteria, was then prepared from the basal medium by adding in extra 1.25 g/l $(NH_4)_2SO_4$, 9.8 g/l sodium succinate and 1 g/l yeast extract.

Ten additional media with individual organic substrates for hydrogen production tests were also prepared from the basal medium by, respectively, adding 30 mM each of sodium lactate, sodium acetate, sodium propionate, sodium butyrate, sodium malate, sodium succinate, sodium glutamate, and glucose, or 15 mM of sucrose and 5.4 g/l of starch. Glutamate were also added as nitrogen source to each medium to keep the C/N ratio at 25/1, except in one medium where glutamate served as the sole substrate, the C/N ratio of which was 4.3/1.

The aSy medium and the six media dosed with lactate, acetate, propionate, butyrate, malate, and succinate were autoclaved at 120 °C for 20 min for sterilization. The remaining four media dosed with glutamate, glucose, sucrose, and starch were sterilized by 0.20 μm membranes. The initial pH in all media was adjusted to 7.1–7.2 by $NaHCO_3$.

2.4. Cultivation of the isolate and hydrogen production conditions

The bacteria were cultured in the aSy medium at 30 °C under 200 W/m^2 of illumination by a tungsten lamp. After 48 h of incubation, bacterial cells were harvested by centrifugation at 4000 rpm for 20 min for the subsequent phototrophic biohydrogen production experiments conducted in batches. All of these experiments were conducted in duplicate in 125 ml serum bottles filled with 100 ml media. The initial cell concentration, expressed as volatile suspended solids (VSS), in each bottle was 13 ± 2 mg/l. The bottles were submerged in circle in a 30 °C water bath. A tungsten lamp was installed above the center of the circle, providing a uniform light intensity of 105 W/m^2.

2.5. Chemical analysis

The volume of biogas produced from each serum bottle was measured using a glass syringe. Hydrogen content was analyzed by a gas chromatograph (Hewlett Packard 5890 II, USA) equipped with a thermal conductivity detector and a 30 m × 0.53 mm PLOT (Porous-Layer Open-Tubular) silica capillary column (Supelco Carboxen 1010) [10].

As reported by Yetis et al. [11], bacterial concentrations in a series of standard solutions ranged 0–0.78 g/l were found in this study increased linearly ($R^2 = 0.97$) with the optical density at 660 nm as measured by a UV spectrophotometer (Shimadzu UV-160, Kyoto, Japan). Consequently, biomass concentrations in this study were estimated from the optical density measurements at 660 nm, a unit of which was found equivalent to a cell density of 0.573 g/l.

Concentrations of low molecular weight volatile fatty acids (VFA), including lactate, acetate, propionate, butyrate, malate, and succinate, were measured by a high performance liquid chromatograph (SCL-10 AVP, Shimadzu, Kyoto, Japan) equipped with a 0.3 m × 6.5 mm column (OA-1000, Alltech, USA), and a SPD-10 (Shimadzu, Kyoto, Japan) UV–visible detector (210 nm), using 0.01 N sulfuric acid as the mobile phase with a flow rate of 0.80 ml/min. Other VFA and alcohols, including i-butyrate, valerate, i-valerate, caproate, methanol, ethanol, propanol and butanol, were analyzed by another gas chromatograph (6890N, Agilent Technologies, USA) equipped with a hydrogen flame ionization detector and a 10 m × 0.53 mm fused-silica capillary column (HP-FFAP). Concentration of glutamate, which could not be satisfactorily measured by these two chromatographs, was analyzed by its chemical oxygen demand (COD) when it was used as the sole carbon source. Carbohydrates were analyzed by the anthrone method [12].

The PHB content was determined by a gas chromatographic method [13] using pure poly-β-hydroxybutyrate (Sigma-Aldrich Co.) as the standard. About 10–20 mg of lyophilized biomass was mixed with 2 ml acidified methanol (20% sulfuric acid) and 2 ml chloroform, and incubated for 4 h at 105 °C. After cooling to room temperature, 0.5 ml of 25% aqueous ammonia solution was added. The mixed solutions were shaken vigorously, and then centrifuged at 3000 rpm for 10 min. The methylated ester product from PHB was extracted into the dense chloroform phase and was analyzed by a gas chromatograph (6890N, Agilent Technologies, USA) equipped with a hydrogen flame ionization detector and a Neutrabond-1 capillary column (GL Science; internal diameter 0.25 mm, film thickness 0.25 μm, and length 30 m).

2.6. Nitrogenase activity

The nitrogenase activity of each biomass sample was measured based on its ability of reducing acetylene to ethylene [14]. Bottles (11 ml) were autoclaved at 120 °C for 20 min and added

with 1 ml of culture solution with individual substrates. The bottles were then purged with argon, followed by acetylene. After 20 h of incubation, the concentrations of acetylene and ethylene (i.e. the product of nitrogenase reduction) in the headspace were analyzed with a gas chromatograph (6890N, Agilent Technologies, USA) equipped with a hydrogen flame ionization detector at 250 °C and a 50 m × 0.535 mm column (AT-Alumina PLOT GC, Alltech, USA) at 100 °C. Helium was the carrier gas at a flow rate of 6.7 ml/min. Nitrogenase activity was expressed by the average ethylene formation rate for each mg of biomass in 20 h.

2.7. Kinetic modeling

The cumulative hydrogen volume in batch experiments followed the modified Gompertz equation [15]:

$$H = P \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\}, \quad (1)$$

where H represents the cumulative hydrogen production (ml), λ the lag phase time (h), P the hydrogen production potential (ml), and R_m the maximum hydrogen production rate (ml/h). The values of P, R_m and λ for each batch were determined by best fitting the hydrogen production data for Eq. (1) using Microsoft's software Excel 2000.

2.8. Accession number

The nucleotide sequence of the isolate found in this study, which was subsequently identified as a new strain (designated as L31) of *R. gelatinosus*, has been assigned an accession number of EF094990 by the GenBank, EMBL and DDBJ databases.

3. Results and discussion

3.1. Microbial identification and phylogenetic analysis

Fig. 1 illustrates the phylogenetic relationship of this isolate with commonly studied phototrophic hydrogen-producing bacteria, including fresh water species, such as *R. sphaeroides* [16], *R. capsulatus* [17], *R. palustris* [18], *R. rubrum* [19], and *R. gelatinosus*, as well as marine species, such as *Rhodovulum* sp. [20], and *Rhodovulum sulfidophilum* [21]. Based on BLAST analysis of the 16S rDNA sequence (1394 bp), this new isolate is most closely related to *R. gelatinosus* (99.6% similarity to strains OK303 and L144, and 99.5% similarity to TUT3906). This implies that this isolate is a new strain (designated as strain L31 in this study) of *R. gelatinosus*.

3.2. Hydrogen production

Results of batch tests using individual organic substrates showed that *R. gelatinosus* L31 was unable to produce hydrogen from acetate, propionate, butyrate, succinate and glutamate. Fig. 2 illustrates the cumulative hydrogen production data using the other five substrates, i.e. lactate, malate, glucose, sucrose and starch, as well as the respective best-fit curves using Eq. (1). Table 1 summarizes the hydrogen production potential (ml) and maximum volumetric production rate (ml/l/h) estimated from the best-fit curves of Eq. (1). It also lists the estimated specific production rate (ml/g-VSS/h), conversion efficiency of hydrogen (%), hydrogen yield (mol-H_2/mol-substrate), biomass increase (g-VSS/l) and yield (g-VSS/g-C), substrate removal efficiency (%), final pH of mixed liquor, nitrogenase activity (μl-C_2H_4/h/mg-VSS) and PHB content (g-PHB/g-VSS). The hydrogen conversion efficiency is defined as the ratio between the actual hydrogen production and the stoichiometric value according to Eq. (2) [6]:

$$C_aH_bO_c + (2a - c)H_2O \rightarrow aCO_2 + (2a - c + 0.5b)H_2. \quad (2)$$

Fig. 2 illustrates that lactate and malate had a shorter lag phase and produced more hydrogen than three carbohydrates (i.e. glucose, sucrose and starch). Results in Table 1 show that lactate had a lag phase of 158 h and produced a maximum amount of 225.4 ml of hydrogen, as compared to the corresponding values of 870 h and 73.9 ml for starch. On the other hand, the carbohydrates produced hydrogen, after a

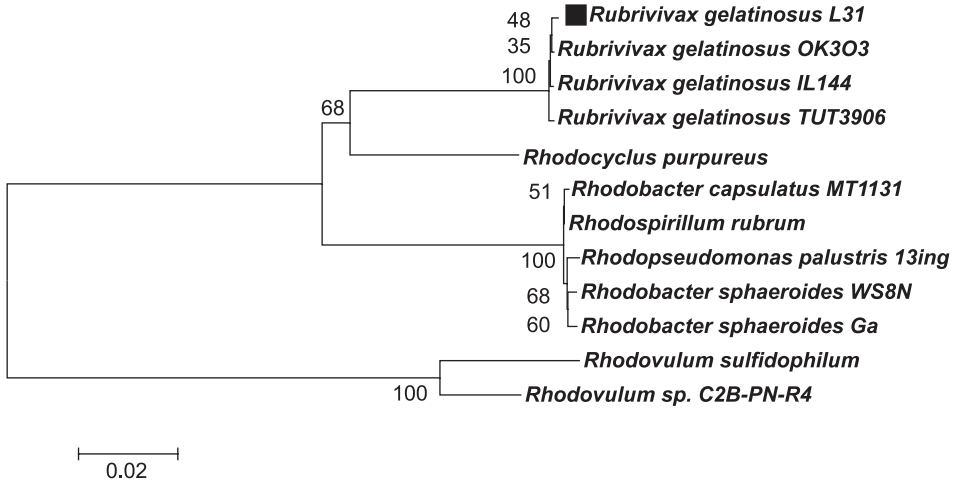

Fig. 1 – Phylogenetic relationship between L31 and other phototrophic purple non-sulfur bacteria commonly used for hydrogen production.

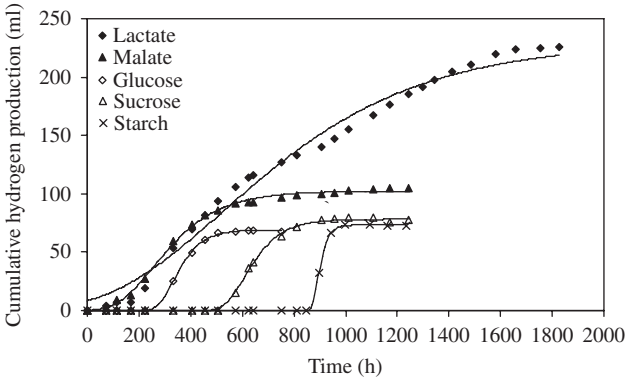

Fig. 2 – Cumulative hydrogen production from various carbon sources.

long lag phase, at higher rates. The maximum specific hydrogen rates were 829 ml/g/h for starch, as compared to 193 ml/g/h for malate. Lactate had the highest conversion efficiency of 50.5%, as compared to 7.4–8.8% for the three carbohydrates.

Table 2 compares the maximum volumetric and specific hydrogen production rates, as well as conversion efficiencies, of the tested organic substrates and the corresponding values in literature. Results in Table 2 show that the maximum specific hydrogen production rates of *R. gelatinosus* L31 (193–829 ml/g/h) are much higher than those of other phototrophs using the same substrate, except the reported value of 670 ml/g/h by *R. palustris* P4 using glucose [22]. The conversion efficiency of 50.5% for lactate in this study is higher than most reported data in literature (12.4–26.1%) and comparable to the 52.7% by *R. capsulatus* JP91 [23]. The conversion efficiencies of malate, glucose, sucrose, and starch are all comparable to those reported in literature.

Starch has been rarely used for phototrophic hydrogen production. Ike et al. [24] has found that although *R. marinum* A-501 could produce hydrogen from glucose and sucrose (Table 2), but could not produce from starch. For *R. gelatinosus*, the hydrogen was produced from starch at a maximum rate of 12.1 ml/l/h in this study, which is higher than the 7.8–11.3 ml/l/h produced from starch sources such as cassava, rice, and corn [25].

Table 1 shows that the conversion efficiencies of *R. gelatinosus* L31 were 50.5% for lactate and 24.6% for malate, both of which were substantially higher than the 7.4–8.8% for the three carbohydrates. Table 1 also shows that *R. gelatinosus* L31 could not produce hydrogen from acetate, propionate, butyrate, and succinate, even though these organic acids could produce hydrogen by other species such as *R. sphaeroides*, *R. capsulatus*, *Rhodopseudomonas* sp., and *R. palustris* R1, as shown in Table 2. There is no study so far reporting any phototroph capable of producing hydrogen from glutamate.

3.3. *Bacteria growth in various substrates*

Although it could not produce hydrogen from glutamate, acetate, propionate and butyrate, *R. gelatinosus* L31 may still use these substrates for growth. The biomass concentration increases of *R. gelatinosus* L31 were 1.06 g/l for glutamate and

Table 1 – Hydrogen production related parameters from various carbon sources

Substrate	H_2 (ml)	λ (h)	Max. H_2 rate (ml/l/h)	Max. H_2 rate (ml/g/h)	Biomass increase (g/l)	Biomass yield (g/g-C)	Conversion efficiency (%)	Hydrogen yield (mol-H_2/mol-substrate)	Substrate removal (%)	Final pH	Nitrogenase activity (μl-C_2H_4/h/mg)	PHB (g/g-VSS)
Glucose	68.7	272	4.1	241	0.79	0.37	7.4	0.9[a]	69.2	5.3	16.2	0.14
Sucrose	79.5	529	3.6	327	0.81	0.38	8.6	1.0[a]	21.1	7.0	9.0	ND[b]
Starch	73.9	870	12.1	829	0.76	0.35	8.8	1.1[a]	41.6	5.4	10.6	ND
Lactate	225.4	158	2.9	264	0.40	0.37	50.5	3.0	93.4	7.4	36.3	0.25
Malate	104.7	116	2.7	193	0.48	0.33	24.6	1.5	56.1	7.7	15.7	0.22
Acetate	0.7	–	–	–	0.75	1.04	0.2	0.01	53.4	10.5	0	0.65
Propionate	7.5	–	–	–	0.85	0.78	1.4	0.1	38.6	10.0	0	0.18
Butyrate	1.8	–	–	–	0.70	0.48	0.2	0.02	29.6	10.0	0	0.42
Succinate	4.1	–	–	–	0.38	0.26	0.8	0.06	26.6	9.9	0	ND
Glutamate	0.0	–	–	–	1.06	0.59	0.0	0.0	63.0[c]	9.6	0	ND

[a] Mol-H_2/mol-hexose.
[b] ND, not detectable.
[c] Removal based on COD.

Table 2 – Comparison of Hydrogen production by *R. gelatinosus* L31 with reported data in literature

Substrate	Bacteria	Max. H_2 rate (ml/l/h)	Max. H_2 rate (ml/g/h)	Conversion efficiency (%)	Reference
Lactate	*R. gelatinosus* L31	2.9	264	50.5	This study
Lactate	*R. sphaeroides* RV	30.0	65	26.1	[28]
Lactate	*R. capsulatus* JP91	38.5	42	52.7	[23]
Lactate	*R. palustris* R1	9.1	83	12.6	[4]
Lactate	*Rhodobium marinum* A-501	9.1	–	12.4	[24]
Malate	*R. gelatinosus* L31	2.7	193	24.6	This study
Malate	*R. sphaeroides* O.U.001	8.0	10	36.0	[29]
Malate	*R. palustris* R1	5.8	53	36.0	[4]
Malate	*Rhodopseudomonas* sp.	1.1	2	6.6	[4]
Malate	*Rhodobium marinum* A-501	5.7	–	7.8	[24]
Glucose	*R. gelatinosus* L31	4.1	241	7.4	This study
Glucose	*R. sphaeroides* VM81	2.2	4	3.5	[26]
Glucose	*Rhodobium marinum* A-501	5.3	–	7.2	[24]
Glucose	*R. palustris* P4	–	670	5.5–23.0	[22]
Sucrose	*R. gelatinosus* L31	3.6	327	8.6	This study
Sucrose	*R. capsulatus* Z-1	–	60	6.0	[3]
Sucrose	*Rhodobium marinum* A-501	3.0	–	4.1	[24]
Starch	*R. gelatinosus* L31	12.1	829	8.8	This study
Starch	*R. gelatinosus* SB24	7.8–11.3	7–17	–	[25]
Acetate	*R. sphaeroides* KD131	4.9	–	8.0	[30]
Acetate	*R. capsulatus* B100	26.2	–	53.0	[17]
Acetate	*R. palustris* R1	2.2	20	14.8	[4]
Butyrate	*Rhodopseudomonas* sp.	7.6	17	8.4	[4]
Butyrate	*R. palustris* R1	Nil	Nil	Nil	[4]
Propionate	*R. capsulatus* Z-1	–	40	–	[3]
Succinate	*R. capsulatus* Z-1	–	100	72.0	[3]

All studies were by batch experiments.

0.70–0.85 g/l for the three acids. These are much higher than the reported 0.12–0.60 g/l for *R. palustris* R1 and *Rhodopseudomonas* sp., using acetate [4]. For those substrates from which hydrogen was produced, the increase of *R. gelatinosus* L31 concentration was 0.38–0.48 g/l for lactate, malate, and succinate, and 0.76–0.81 g/l for the three carbohydrates. However, the biomass yields of 0.35–0.38 g/g-C from carbohydrates were much lower than the 0.78–1.04 g/g-C using acetate and propionate. For comparison, the biomass growth of 0.11 g/l by *R. sphaeroides* VM 81 using glucose [26] was much lower than 0.79 g/l by *R. gelatinosus* L31 in this study.

3.4. *Substrates degradation*

Table 1 shows that *R. gelatinosus* L31 degraded 93.4% of lactate; such a substrate degradation efficiency is much higher than the reported 58.8–63.0% by *R. sphaeroides* [27]. *R. gelatinosus* L31 was also able to degrade 69.2% of glucose, 56.1% of malate, 53.4% of acetate, and 63.0% of glutamate. Substrate degradation efficiencies were much lower for the other substrates.

Degradation of carbohydrates produced mostly acetate (60–420 mg/l), propionate (300–700 mg/l) and ethanol (400 mg/l), plus traced amount of i-valerate (3–26 mg/l). Lactate (up to 380 mg/l) was found in the mixed liquor during the batch reaction, but was completely degraded at the end. Degradation of lactate, propionate, butyrate and succinate produced 0–92 mg/l of ethanol, 46–68 mg/l of acetate, and 0–35 mg/l of i-butyrate. On the other hand, there was no detectable VFA and alcohols in batches degrading acetate, malate and glutamate.

3.5. *Nitrogenase activity*

Table 1 shows that the nitrogenase activities were 9.0–36.3 μl-C_2H_4/h/mg-VSS for media containing *R. gelatinosus* L31 and hydrogen-producing substrates, including lactate, malate, glucose, sucrose, and starch. These values are comparable to the nitrogenase activity of 43.4 μl-C_2H_4/h/mg-VSS for *R. capsulatus* [17], and much higher than 6.4 μl-C_2H_4/h/mg-VSS for *R. palustris* [14]. There was no measurable nitrogenase activity in media which did not produce hydrogen. Fig. 3 illustrates that substrate degradation and hydrogen conversion efficiency increase in general with the increase of nitrogenase activity.

Literature data on the correlation between nitrogenase activity and hydrogen production rate were limited and conflicting. Results of this study showed that there was no noticeable correlation between the nitrogenase activity and the hydrogen production rate, as reported by Watanabe et al. [31] for *Rhodopseudomonas gelatinosa*. However, Kim et al. [14]

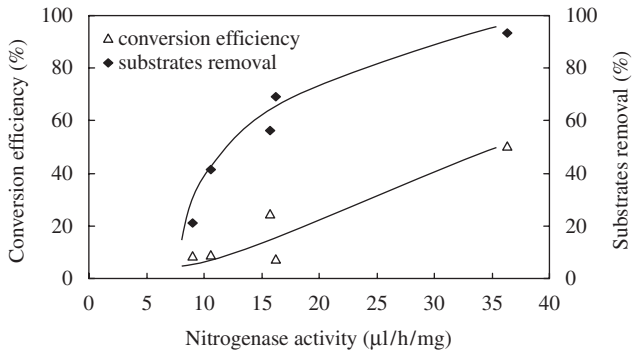

Fig. 3 – Relationship between nitrogenase activity and hydrogen conversion efficiency and substrate removal efficiency.

reported that both hydrogen yield and production rate increased with nitrogenase activity for *R. palustris*.

3.6. *Final pH and PHB production*

Although the initial pH was 7.1–7.2 in all tests, the final pH changed only slightly to 7.4–7.7 for lactate and malate, but decreased to pH 5.3–5.4 for glucose and starch due to the formation of VFA. On the other hand, the final pH increased to pH 9.6–10.5 for those substrates that did not produce hydrogen.

Photosynthetic bacteria may use the available electron to produce intracellular PHB, instead of hydrogen [7]. Table 1 shows that *R. gelatinosus* L31 could produce PHB using glucose, lactate, malate, acetate, propionate and butyrate. It, however, could not produce PHB using sucrose, starch, succinate and glutamate. Calculations based on data in Table 1 further show that there is no quantitative correlation between the productions of PHB and hydrogen.

4. Conclusions

A new strain of *Rubrivivax gelatinosus* (designated as L31) was isolated from the sediment of a local reservoir. Testing against 10 organic substrates, this strain could produce hydrogen from carbohydrates, including glucose, sucrose and starch, as well as from lactate and malate. Even though it could use acetate, propionate, butyrate, succinate and glutamate as substrate, it could not produce hydrogen from them. Based on the determined kinetic parameters derived from experimental data, lactate produced the highest amount (225.4 ml) of hydrogen with a hydrogen conversion efficiency of 50.5%, whereas starch exhibited the highest production rate of 829 ml/g/h after an extensive lag phase of 870 h. The increase of nitrogenase activity, which ranged from 9.0 to 36.3 μl-C_2H_4/h/mg-VSS, generally resulted in higher substrate degradation and hydrogen conversion efficiency. However, there was no correlation between nitrogenase activity and hydrogen production rate or biomass yield. Although formations of hydrogen and intracellular PHB consumed electrons, there was no noticeable quantitative correlation between them.

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