SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
ENVIRONMENTAL SCIENCES
WATER MICROBIOLOGY

Received
10 March 2014

Accepted
21 May 2014

Published
10 June 2014

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The maximum specific hydrogen-producing activity of anaerobic mixed cultures: definition and determination

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Fermentative hydrogen production from wastes has many advantages compared to various chemical methods. Methodology for characterizing the hydrogen-producing activity of anaerobic mixed cultures is essential for monitoring reactor operation in fermentative hydrogen production, however there is lack of such kind of standardized methodologies. In the present study, a new index, i.e., the maximum specific hydrogen-producing activity (SHA_m) of anaerobic mixed cultures, was proposed, and consequently a reliable and simple method, named SHA_m test, was developed to determine it. Furthermore, the influences of various parameters on the SHA_m value determination of anaerobic mixed cultures were evaluated. Additionally, this SHA_m assay was tested for different types of substrates and bacterial inocula. Our results demonstrate that this novel SHA_m assay was a rapid, accurate and simple methodology for determining the hydrogen-producing activity of anaerobic mixed cultures. Thus, application of this approach is beneficial to establishing a stable anaerobic hydrogen-producing system.

Energy shortage is one of major global challenges, and thus there is an increasing interest in the search for renewable energy sources to meet the current and future energy requirements[1–3]. Hydrogen is an attractive potential alternative energy source due to its advantages including clean, efficient and non-polluting characteristics[4,5]. Compared with various chemical methods for hydrogen production, biological hydrogen production by fermentative process can be operated at ambient temperatures and normal pressures[6–8]. Furthermore, this process can reuse a large amount of various waste materials from industries and agriculture[9–11]. Therefore, biological hydrogen production facilitates both waste treatment and energy recovery[12].

During the start-up or steady-state operation of fermentative hydrogen-producing reactor, a sufficient quantity of active hydrogen-producing bacteria should be maintained within anaerobic reactors. Therefore, methodology for characterizing the hydrogen-producing bacteria and their activity is essential for monitoring reactors from the standpoint of the design and operation. Some techniques, such as microscopic counts, most probable number (MPN), adenosine triphosphate (ATP) and dehydrogenase activity, might be available to determine the level and activity of hydrogen-producing bacteria[13,14]. However, the use of the MPN test was found to be not practical due to long doubling times, requirements of strict anaerobic conditions, and the difficulty in cultivating some of the species involved. Both ATP and dehydrogenase activity assays cannot be used as reliable methods for determining the level of active hydrogen-producing bacteria[14]. In order to find out the optimum initial pH, substrate concentration, inocula, and enrichment procedure to start up a hydrogen-producing reactor, van Ginkelet al.[15] adopted a modified Gompertz equation to simulate a batch hydrogen production process and determine the hydrogen production rate of anaerobic mixed cultures. In another study, Zheng et al.[16] investigated the influence of illumination on the fermentative hydrogen production system, and also used a modified Gompertz equation to calculate the specific hydrogen production rate of anaerobic mixed cultures. However, three model parameters - lag time, H_2 production potential, and H_2 production rate, were adjusted to fit the experimental data in this empirical model. Even though this curve-fitting approach yields high correlation coefficients between the observed and fitted hydrogen evolution data, the three model parameters determined by curve-fitting were restricted to specific experimental conditions[17]. In a methane-producing reactor, the specific methanogenic activity (SMA) assay has been demonstrated to be an effective method and therefore has been standardized and widely used to determine the methanogenic ability of anaerobic mixed cultures under various conditions[18,19].

However, there is no standardized methodology for biological hydrogen-producing activity assay. Similar with the SMA assay, development of such a standardized methodology is essentially required and important for evaluating the hydrogen-producing ability of anaerobic mixed cultures under various conditions, especially for comparison purpose to arrive at conclusions.

Therefore, the present study aimed at developing and standardizing the biological assay for determining the ability of anaerobic mixed cultures to convert substrate to hydrogen. Based on the kinetic relationships between substrate, product and microorganism, a reliable and simple approach was proposed to determine the hydrogen-producing activity of anaerobic mixed cultures in this study. This method gave a direct measurement of the maximum rate of hydrogen production per unit of microbial biomass per unit time, e.g., the maximum specific hydrogen-producing activity (SHA_m) of anaerobic mixed cultures, thus, was named as the SHA_m test. Furthermore, the influences of various parameters, such as pH, substrate concentration and temperature, on the SHA_m determination were also evaluated. Additionally, this SHA_m assay was tested for different types of substrates and bacterial sources, and then verified with two case studies[20,21]. The use of such an SHA_m test could allow us to rapidly, accurately and simply determine the potential hydrogen-producing activity of anaerobic mixed cultures, which would be useful for establishing a stable anaerobic hydrogen-producing system.

Results

Definition of SHA_m. The relationship between microbial growth and product formation for the anaerobic hydrogen production by mixed anaerobic cultures was simulated by the Luedeking-Piret model[22]:

$$\frac{dP_i}{dt} = \alpha_i \frac{dX}{dt} + \beta_i X \tag{1}$$

where α_i is growth-associated formation coefficient of product *i*; β_i is non-growth-associated formation coefficient of product *i (Pi); X* is microorganism concentration (g-VSS/L); and VSS is volatile suspended solids.

Eq. (1) could be changed into:

$$\frac{dP_i}{Xdt} = \alpha_i \mu + \beta_i \tag{2}$$

where $dP_i/dt/X$ is specific formation rate of product *i*; and μ (1/d) is specific growth rate of microorganisms. A straight line could be obtained with an intercept of β_i and a slope of α_i, if plotting $dP_i/dt/X$ against μ.

In the anaerobic fermentative process, a typical fitted plot for hydrogen production by mixed anaerobic cultures was reported in Mu et al.[22] and the estimated β_{H2} value was nearly equal to zero, suggesting that the formation of hydrogen in such a process was mainly growth-associated. Although hydrogen can be produced during stationary phase(s) of bacterial cultures, several studies have also suggested that hydrogen was mainly produced during the period of biomass growth compared to the stationary phase in batch test for anaerobic mixed cultures[20,21,23–26], further suggesting that the formation of hydrogen was substantially (but not purely) growth-dependent in the anaerobic fermentative process by mixed cultures. As a consequence, the correlation between hydrogen and substrate could be expressed as Eq. (3)[27]:

$$\frac{dP_{H_2}}{dt} = -Y_{H_2} \times \frac{dS}{dt} \tag{3}$$

where *S* (mmol/L) is substrate concentration; and Y_{H2} (mL-H_2/mmol-substrate) is hydrogen yield coefficient.

In a batch fermentative hydrogen production process, Eq. (3) could be changed into:

$$\frac{dS}{dt} = -\frac{1}{Y_{H_2} \times V_R} \times \frac{dV_{H_2}}{dt} \times \frac{T_0}{T_1} \tag{4}$$

where V_{H2} (mL) is accumulated volume of hydrogen; V_R (L) is reactor volume; T_0 (K) is standard temperature, e.g., 273.15 K; and T_1 (K) is room temperature.

The degradation of substrate was expressed by Monod-type equation:

$$\frac{dS}{dt} = -\frac{U_{\max} \times S \times X}{K_s + S} \tag{5}$$

where U_{max} (1/d) is maximum specific substrate degradation rate; and K_s (mmol/L) is half-saturation constant.

The following Eq. (6) was obtained by combining Eqs. (4) and (5):

$$\frac{1}{Y_{H_2} \times V_R} \times \frac{dV_{H_2}}{dt} \times \frac{T_0}{T_1} = \frac{U_{\max} \times S \times X}{K_s + S}$$

$$\frac{1}{V_R} \times \frac{dV_{H_2}}{X \times dt} \times \frac{T_0}{T_1} = \frac{Y_{H_2} \times U_{\max} \times S}{K_s + S}$$

$$\frac{1}{V_R} \times \frac{dV_{H_2}}{X \times dt} \times \frac{T_0}{T_1} = Y_{H_2} \times U_{\max} \times \frac{S}{K_s + S} \tag{6}$$

At the beginning of fermentation, S≫Ks, thus Eq. (6) was simplified into:

$$\frac{1}{V_R} \times \frac{dV_{H_2}}{X \times dt} \times \frac{T_0}{T_1} = Y_{H_2} \times U_{\max} \tag{7}$$

Here, we defined the SHA_m of anaerobic mixed cultures (mL-H_2/g-VSS/d) as:

$$SHA_m = Y_{H_2} \times U_{\max} \tag{8}$$

Thus, Eq. (7) could be rewritten as:

$$\frac{1}{V_R} \times \frac{dV_{H_2}}{X \times dt} \times \frac{T_0}{T_1} = SHA_m \tag{9}$$

Eq. (9) indicates that the specific hydrogen production rate of anaerobic mixed cultures was a constant value at the beginning of fermentation, which was equal to the SHA_m.

The SHA_m determination of anaerobic mixed cultures could be useful in a fermentative hydrogen production process. At the beginning of the start-up period of a new reactor, the activities of the seeding anaerobic mixed cultures and its amount are of great importance. During the phases after start-up, a regular determination of SHA_m provides information about the change of the hydrogen-producing biomass, usually with a high level of inactive organic matter, until the consecution of biomass mostly composed by active bacteria. As a consequence, a simple method was developed to assess the SHA_m of anaerobic mixed cultures as follows.

Determination of SHA_m. A typical curve of the accumulated hydrogen with fermentative time is shown in Figure 1(A). After a lag time, the volume of accumulated hydrogen increased with the increasing fermentative time and a linearity of hydrogen production with time would be observed at the beginning of fermentation according to Eq. (9). Consequently, the value of SHA_m could be calculated from Eq. (10):

$$SHA_m = \frac{k \times 24}{V_R X} \times \frac{T_0}{T_1} \tag{10}$$

where *k* (mL-H_2/h) is slope of the linearity of hydrogen production.

The CH_4 formation was very limited for all of the SHA_m tests due to the significant elimination of methanogens in the inocula by

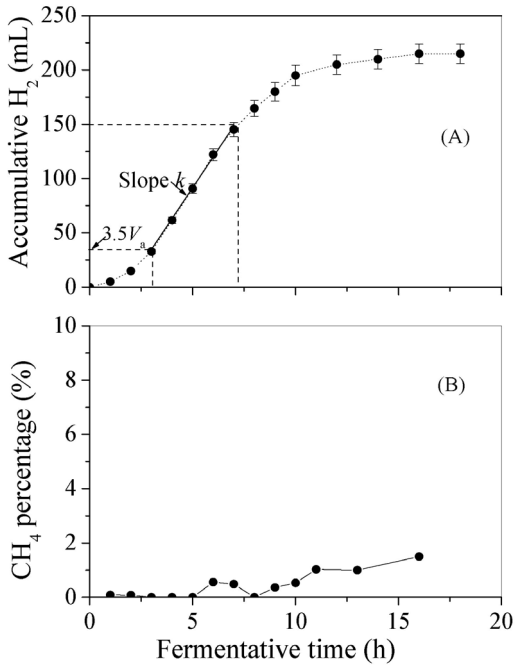

Figure 1 | A typical curve of (A) accumulated hydrogen and (B) CH_4 percentage in the gas phase with fermentative time (pH 5.0, sucrose concentration 3.4 g-COD/L, temperature 20.0°C, biomass concentration 3 g-VSS/L, and anaerobic sludge from a UASB reactor as inocula).

various pretreatments. As shown in Figure 1(B), the CH_4 percentage was lower than 3% in the gas phase for the heat-pretreated inocula. In addition, the produced H_2S could be completely removed in the washing flask with 5 mol/L NaOH solution. Therefore, it is expected that the formation of both CH_4 and H_2S should have no significant influence on the measurement of H_2 volume with the liquid displacement method.

It is noteworthy that a higher volume of gas would be measured at initial fermentation, due to nitrogen gas in the fermentor. The fraction of nitrogen gas in the gas collector could be expressed as follows[13]:

$$E = \exp(-V_g(t)/V_a) \tag{11}$$

where E is nitrogen percentage in the gas collector; V_g (mL) is volume of accumulated gas at fermentative time t (h); and V_a (mL) is gas-space volume of fermentative reactor.

A small E_a value of 0.03 is able to be obtained when the ratio of $V_g(t)$ and V_a is 3.5, implying that the nitrogen in the fermentor had been almost removed. Therefore, in order to eliminate the influence of nitrogen gas, the initial point for SHA_m calculation should be chosen when the accumulated gas is 3.5 times in volume of gas-space of fermentor, as indicated in Figure 1. The gas-space volume of the fermentative reactor was around 10 mL in this study.

Effects of various operational parameters on SHA_m. As shown in Figure 2, the SHA_m of the anaerobic mixed cultures increased from 419 ± 11 to 896 ± 8 mL-H_2/g-VSS/d as pH was increased from 4.0 to 5.5, then decreased to 496 ± 38 mL-H_2/g-VSS/d with a further increase to 7.0. A maximum SHA_m value was obtained at pH 5.5. The microbial activities may be controlled by the overall enzymatic activity, which is pH dependent, as shown in the follows[28]:

$$E^+ \leftrightarrow E + H^+$$

$$E \leftrightarrow E^- + H^+$$

where E represents the active enzyme, and E^+ and $E^-$are the less active forms of charge-carrying enzyme. Assuming K_H and K_{OH}

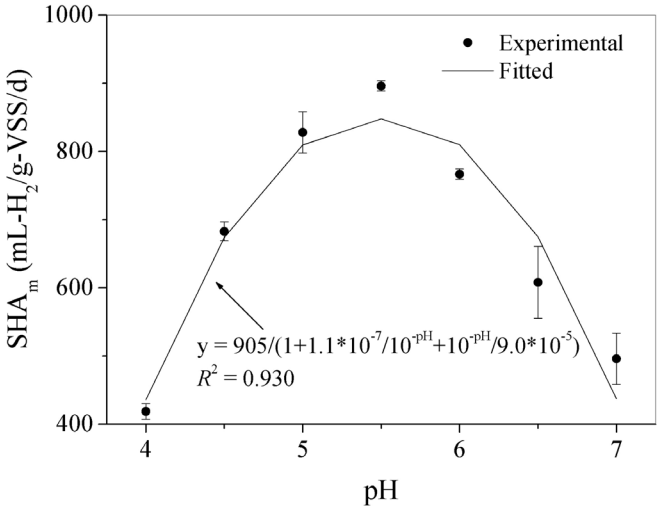

Figure 2 | **Effect of pH on the SHA_m (sucrose concentration 3.4 g-COD/L, temperature 20.0°C, biomass concentration 3 g-VSS/L, and anaerobic sludge from a UASB reactor as inocula).**

are the respective equilibrium constants of above two reactions, the SHA_m of anaerobic mixed cultures could be expressed as:

$$SHA_m = \frac{OSHA_m}{1 + K_{OH}/[H^+] + [H^+]/K_H} = \frac{OSHA_m}{1 + K_{OH}/10^{-pH} + 10^{-pH}/K_H} \tag{12}$$

where $OSHA_m$ is the optimum SHA_m (mL-H_2/g-VSS/d). As shown in Figure 2, the relationship between SHA_m and pH was well simulated by Eq. (12) with a high correlation coefficient of 0.930. The values of K_H, K_{OH}, and $OSHA_m$ were determined as 9.0 × 10^{-5} mol/L, 1.1 × 10^{-7} mol/L and 905 mL-H_2/g-VSS/d, respectively. The estimated maximum SHA_m of 905 mL-H_2/g-VSS/d was much close to the experimental maximum value of 896 ± 8 mL-H_2/g-VSS/d, whereas the predicted optimum pH [(pK_{OH}+ pK_H)/2] was equal to the experimental optimum pH of 5.5. Additionally, the final pH was in a range of 4.5–4.0 due to the production of volatile fatty acids in the fermentative hydrogen production process.

The effect of substrate concentration on the SHA_m of anaerobic mixed cultures is presented in Figure 3. The SHA_m increased from 555 ± 30 to 1400 ± 49 mL-H_2/g-VSS/d with the increasing sucrose concentration from 2.92 to 17.54 mmol/L, then decreased to 1049 ± 50 mL-H_2/g-VSS/d as the sucrose concentration was further increased to 20.47 mmol/L. The dependence of SHA_m on the initial substrate concentration could be described using the generalized Haldane equation[29]:

$$SHA_m = \frac{OSHA_m}{1 + \frac{K_s}{S} + (\frac{S}{K_i})^n} \tag{13}$$

and the non-competitive inhibition equation[29]:

$$SHA_m = \frac{OSHA_m \times S}{(K_s + S) \times (1 + \frac{I}{K_i})} \tag{14}$$

where K_i (mmol/L) is inhibition constant; I (mmol/L) is inhibitor concentration; and n is a constant (order of inhibition).

In parameter estimation, n was given values of 1 (referred to as Eq. (13a)) and 2 (referred to as Eq. (13b)). The fitted curves by Eqs. (13a), (13b) and (14) are shown in Figure 3 and the estimated values of various parameters are listed in Table 1. Although all of equations gave a well description about the relationship between SHA_m and substrate concentration, the K_s value, being higher than K_i estimated by both Eq. 13a and Eq. 13b, was not likely to be correct, on the basis of the values of K_s and K_i for methane production process found in

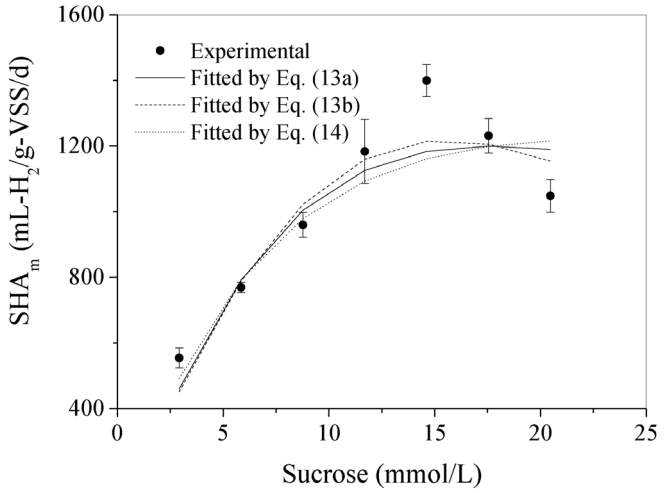

Figure 3 | Effect of substrate concentration on the SHA_m (pH 5.5, temperature 20.0°C, biomass concentration 3 g-VSS/L, and anaerobic sludge from a UASB reactor as inocula).

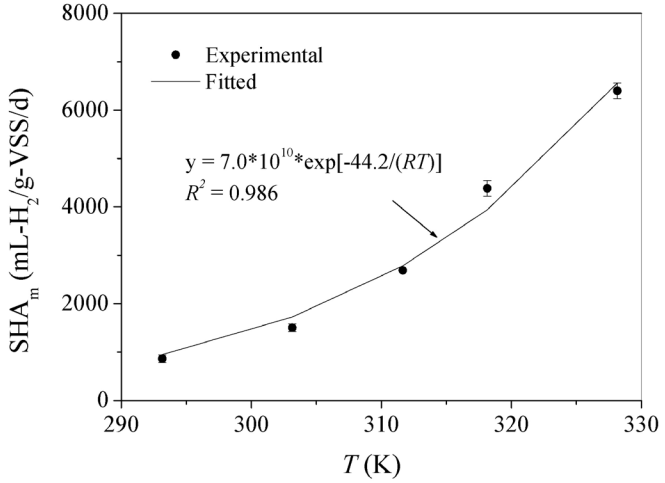

Figure 4 | Effect of temperature on the SHA_m (pH 5.5, sucrose concentration 3.4 g-COD/L, biomass concentration 3 g-VSS/L, and anaerobic sludge from a UASB reactor as inocula).

literatures[27,30]. Therefore, the non-competitive equation, i.e., Eq. (14), might be more suitable for describing the relationship and the following estimates for $OSHA_m$, K_s and K_i were selected as 4878 mL-H_2/g-VSS/d, 22.77 mmol/L and 22.77 mmol/L, respectively. In literatures very limited information about the values of K_s and K_i for fermentative hydrogen production processes is available. Although in general the value of K_i should be much higher than the K_s value, a previous study also reported that the value of K_i was close or even equal to the K_s value in biological methane production[29]. The reasons behind such an inconsistence need further investigations.

The effect of temperature on the SHA_m of anaerobic mixed cultures was investigated in a range of 20.0 to 55.0°C, as presented in Figure 4. An increase in SHA_m of anaerobic mixed cultures was observed from 864 ± 80 to 6400 ± 160 mL-H_2/g-VSS/d with the increase in temperature. The temperature dependence of anaerobic mixed cultures SHA_m was described by an Arrhenius type equation with a high correlation coefficient value of 0.986:

$$SHA_m = K \times e^{-E_a/(RT)} \quad (15)$$

where K (mL-H_2/g-VSS/d) is frequency factor; E_a (kJ/mol) is apparent activation energy; R (0.008314 kJ/mol/K) is gas constant; and T (K) is absolute temperature. As shown in Figure 4, the values of K and E_a were respectively estimated as 7.0×10^{10} mL-H_2/g-VSS/d and 44.2 kJ/mol.

Comparison of SHA_m for different substrates and bacterial sources. As shown in Table 2, the SHA_m values of anaerobic mixed culture were similar for different types of substrates and in a range of 900–1000 mL-H_2/g-VSS/d. This might be due to that all of substrates used were soluble and their hydrolysis was not a rate-limiting step for these substrates[31].

The SHA_m values had a notable difference for various bacterial sources, as summarized in Table 3. The anaerobic microflora from the upflow anaerobic sludge blanket (UASB) reactor had a highest SHA_m value of 1632 ± 288 mL-H_2/g-VSS/d, while a lowest value of 935 ± 12 mL-H_2/g-VSS/d for the anaerobically digested sludge from the municipal wastewater treatment plant. This result suggests that the method developed in this study could be applicable to assess the specific hydrogen-producing ability of different mixed bacterial inocula.

Application for two case studies. The methodology developed in this study was applied to describe the results reported in two cases[20,21]. Different factors were intensively examined for their effects on the H_2-producing activity of anaerobic mixed cultures in Case 1[20]. As shown in Figure 5(A), the relationship between the microbial growth and the product formation in their study was also well simulated by the Luedeking-Piret model with a regression coefficient higher than 0.8. Moreover, the estimated β_{H2} value was equal to zero, suggesting that the formation of hydrogen in such a process was mainly growth-dependent. Consequently, the SHA_m values of the anaerobic mixed culture were estimated using Eq. (10), and the results at various pH values are shown in Figure 5(B). A maximum SHA_m value was observed at pH 6.0 for the anaerobic mixed cultures in their study. The estimated β_{H2} value was also equal to zero in Case 2 (Figure 6A), where the activated sludge was adopted as the seed for hydrogen production[21], further suggesting that hydrogen production using mixed cultures was mainly growth-dependent. Additionally, the calculated SHA_m value was about 186 mL-H_2/g-VSS/d in Case 2 (Figure 6B).

Table 1 | Estimated kinetic constants for Eqs. (13a), (13b) and (14) at various substrate concentrations

Equation	$OSHA_m$	K_s	K_i	R^2
	(mL-H_2/g-VSS/d)	(mmol/L)	(mmol/L)	
13a Haldane (n=1)	5089	28.58	10.89	0.877
13b Haldane (n=2)	3598	20.28	19.28	0.899
14 non-competitive	4878	22.77	22.77	0.872

Table 2 | SHA_m values for various types of substrates

Substrate	SHA
	(mL-H_2/g-VSS/d)
Glucose	896 ± 8
Sucrose	896 ± 15
Starch	864 ± 53
Brewage wastewater	1036 ± 133

Note: pH 5.5, temperature 20.0°C, substrate concentration 3.4 g-COD/L, biomass concentration 3 g-VSS/L, and anaerobic sludge from a UASB reactor as inocula.

Table 3 | Comparison of SHA_m values for different bacterial sources

Bacterial sources	SHA_m (mL-H_2/g-VSS/d)
Anaerobic microflora from a UASB treating soybean-processing wastewater	1632 ± 288
Anaerobically digested sludge from the Zhuzhuanjing Municipal Wastewater Treatment Plant	935 ± 12
Anaerobic sludge from a CSTR treating chicken manure	990 ± 58
Cow dung compost from a farm	1051 ± 35
Sediments from Chaohu Lake	1081 ± 94

Note: pH 5.5, temperature 30.0°C, sucrose concentration 3.4 g-COD/L, and biomass concentration 3 g-VSS/L.

Discussion

Biological hydrogen fermentation is a microbial process, which requires careful design and control. In practice, the process design is usually based on sludge loading rate and VSS is usually used to characterize the concentration of hydrogen-producing sludge. However, the VSS is undefined as it includes active biomass, as well as dead cell and particulate organic matter. The SHA_m test proposed in this study is able to accurately quantify the hydrogen-producing activity of sludge in a simple and quick way under different conditions. Thus, the SHA_m of sludge is likely to be more useful than VSS as a parameter for the design and operation of anaerobic hydrogen-producing reactors.

Hydrogen partial pressure in the liquid phase is one of the key factors affecting hydrogen production[32]. Tanisho et al.[33] observed an increase in residual NADH by sparging with argon in the reactor, which expected to give an increased hydrogen production. Logan et al.[31] measured hydrogen production in two types of batch tests (Owen and respirometer) and found that the respirometric method resulted in the production of 43% more hydrogen from glucose than the Owen method. Therefore, a continuous gas release method (similar to respirometric one) was adopted in the SHA_m test, in order to reduce the influence of hydrogen partial pressure on hydrogen production in reactors.

Hydrogen production with anaerobic mixed cultures has been shown to be affected by many factors, such as pH, substrate concentration and temperature[34–36]. If we want to measure the real potential hydrogen-producing activity of anaerobic mixed cultures, the optimum conditions should be provided in the SHA_m test. Many studies have demonstrated that proper control of pH is crucial to the hydrogen production and the reported optimum pH value is in a wide range of pH 5.0 to pH 9.0 for different types of hydrogen-producing cultures[37–40]. This implies that the optimum pH for the SHA_m was likely different for various bacteria, although a peak value of SHA_m was observed at pH 5.5 for the anaerobic mixed cultures used in this study.

The substrate concentration is also critical in the microenvironment of the hydrogen-producing bacteria. A high substrate concentration could ensure a more rapid diffusion of substrate into microorganisms. However, as shown in Figure 3, when the substrate concentration was higher than 5.6 g-COD (chemical oxygen

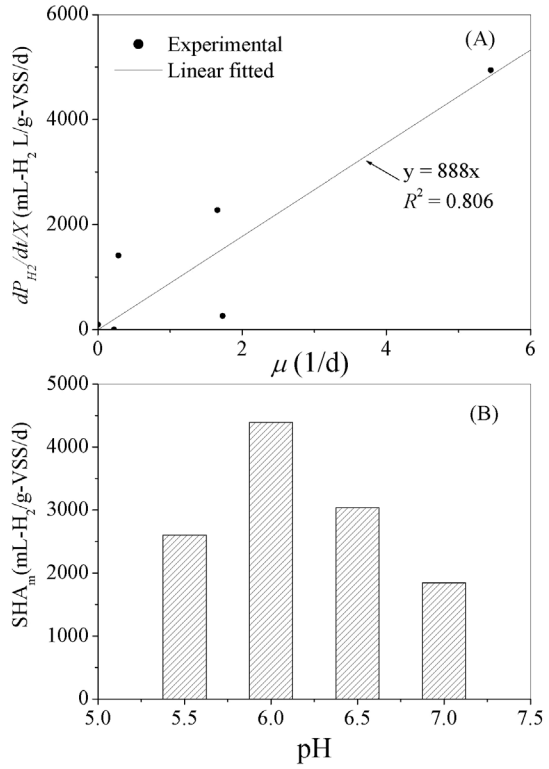

Figure 5 | (A) Luedeking-Piret model plot for the relationship between the hydrogen formation rate and the specific growth rate of microorganisms and (B) the estimated SHA_m values of the anaerobic mixed cultures at various pHs from Case 1[20].

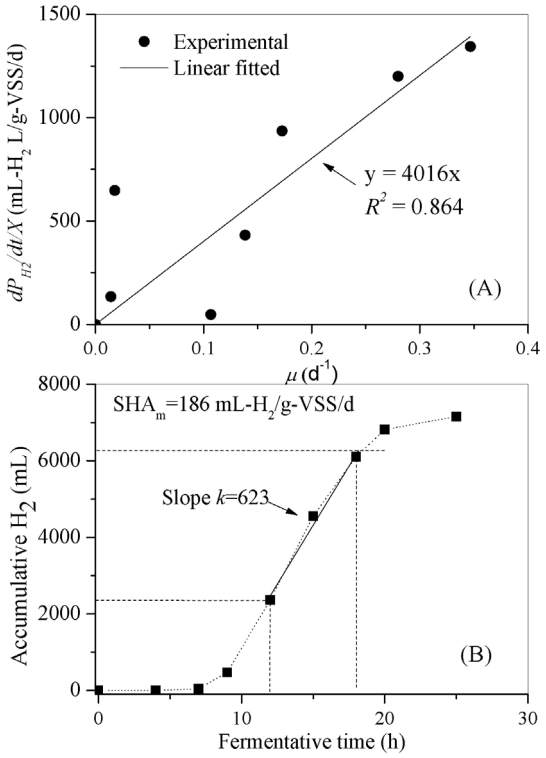

Figure 6 | (A) Luedeking-Piret model plot for the relationship between the hydrogenformation rate and the specific growth rate of microorganisms and (B) the estimated SHA_m value of the anaerobic mixed cultures from Case 2[21].

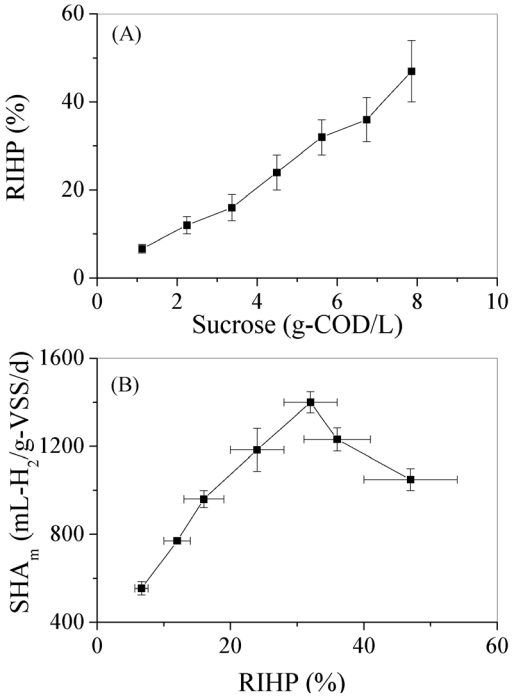

Figure 7 | (A)Relative increase in the hydrogen-producing biomass concentration (RIHP) at various substrate concentrations, and (B) the correlation between SHA_m and RIHP.

demand)/L, the SHA_m value of anaerobic mixed cultures in this study was reduced, which could be attributed to the possible inhibition caused by substrate and its products. On the other hand, the main purpose of this assay is to describe the hydrogen-producing activity of anaerobic mixed cultures. It is thus important to avoid the great changes of anaerobic mixed cultures in characteristics during the assay itself. Of particular importance is the possibility that the hydrogen-producing bacteria population significantly increases during the course of the assay. Figure 7(A) shows that the relative increase in the hydrogen-producing biomass concentration (RIHP) was increased from 6.7 ± 1.0% to 47 ± 7% with an increase in substrate concentration from 1.1 to 7.8 g-COD/L in the SHA_m tests. However, when the RIHP value was higher than 32% (i.e., substrate concentration of 5.6 g-COD/L), the SHA_m values started to decrease in the tests, as shown in Figure 7(B). Such a decrease could be attributed to the fact that the diffusion of substrate to bacteria might become a rate-limiting step if the biomass concentration was too high in the SHA_m tests. Therefore, the substrate concentration should be carefully selected based on two major reasons above and a much higher substrate concentration (e.g., more than 5.6 g-COD/L in this study) should not be applied in the SHA_m tests.

An increasing SHA_m was found with the increasing temperature, however, the temperature was a fixed condition for a given anaerobic hydrogen production system. In other words, it may not always be economical to heat or cool wastewater. It is obvious that if the tests were conducted at a lower or higher temperature than that of reactor system you are considering, the SHA_m measured would be either lower or higher than the real value.

It should be kept in mind that this SHA_m test is aimed at measuring the capacity of anaerobic mixed cultures to convert substrate to hydrogen only. In anaerobic methane production, the SMA test could be used to estimate the substrate-degrading ability of microorganisms because the end products are methane and carbon dioxide only[40,41]. However, in addition to hydrogen, volatile fatty acids and alcohols are also produced in fermentative hydrogen production. Therefore, this SHA_m assay cannot be adopted to determine the substrate-degrading ability of hydrogen-producing microorganisms. Additionally, soluble substrates are usually recommended to be used in the SHA_m assay as it is difficult to estimate biomass concentration when using complex heterogeneous substrate such as lignocellulosic biomass and starchy wastewater in experiment. However, if using insoluble matter as substrate in the test, the SHA_m method should be further improved and could couple with other methodologies of molecular biology to accurately determine the microbial biomass concentration.

In addition to for determining the hydrogen-producing activity of anaerobic mixed cultures, the SHA_m assay developed in this study could also be used for optimizing the operational conditions of hydrogen production reactors. Furthermore, this standardized methodology might be able to monitor any deterioration in the performance of reactors under variable operating conditions, such as toxicity of various heavy metals in wastewaters[42].

Methods

Hydrogen-producing mixed cultures and substrate. In this study five different bacterial sources were respectively taken as inocula, including (1) anaerobic microflora from a full-scale UASB reactor treating soybean-processing wastewater located in Benpu City, China; (2) anaerobically digested sludge from Zhuzhuanjing Municipal Wastewater Treatment Plant in Hefei City, China; (3) anaerobic sludge from a 600-m^3 CSTR (continuous stirred tank reactor) treating chicken manure located in Jieshou City, China; (4) cow dung compost from a farm in Benpu City, China; and (5) sediments from Chaohu Lake, China. Prior to use, these inocula were sieved to remove stone, sand and other coarse matters. Thereafter, the inocula were heated at 102°C for 90 min to inactivate the methanogens and to enrich the hydrogen-producing bacteria[31]. The initial concentration of biomass in the fermentor was about 3 g-VSS/L for all experiments.

On the other hand, various types of substrates, including glucose, sucrose, starch and brewage wastewater, were also compared in this study. Except for the brewage wastewater, all of other substrates were supplemented with buffering chemicals and balanced nutrients as follows (unit in mg/L): NH_4HCO_3 405; $K_2HPO_4{\cdot}3H_2O$ 155; $CaCl_2$ 50; $MgCl_2{\cdot}6H_2O$ 100; $FeCl_2$ 25; NaCl 10; $CoCl_2{\cdot}6H_2O$ 5; $MnCl_2{\cdot}4H_2O$ 5; $AlCl_3$ 2.5; $(NH_4)_6Mo_7O_{24}$ 15; H_3BO_4 5; $NiCl_2{\cdot}6H_2O$ 5; $CuCl_2{\cdot}5H_2O$ 5; $ZnCl_2$ 5. The characteristics of the brewage wastewater were similar to those reported by Yu et al[43]. The initial substrate concentration was about 3.4 g-COD/L for all experiments except for the tests with various substrate concentrations, where the amounts of all organic and inorganic constituents were adjusted *pro rata*.

Test procedures of SHA_m. Using a continuous gas release apparatus, the setup of the SHA_m test consisted of a 300-mL fermentor (serum vial), washing flask, gas collector, graduated flask, and vibrator with constant temperature. A required amount of anaerobic mixed cultures and substrate were respectively added into the serum vial, and the working volume was adjusted to 290 mL with distilled water. The required initial solution pH was adjusted using either 5 mol/L HCl or KOH. Then, the vials were flushed with nitrogen gas to remove oxygen from the solution, and were immediately capped with a rubber septum stopper and tied down with plastic fasteners. The vials were kept in incubator shaker at 180 rpm. The gas produced passed through carbon dioxide scrubber (5 mol/L NaOH solution) and was thus collected using water displacement method.

Analysis. Sucrose concentration was determined by using anthrone-sulfuric acid method[44], while COD and VSS were measured using the standard methods[45]. The percentage of H_2, N_2, and CH_4 in the gas was analyzed by using a GC (Model SP-6800A, Lunan Co., China) equipped with a thermal conductivity detector and a 3 m stainless column packed with 5 Å molecular sieve. The operational temperatures at the injection port, the column oven and detector were 70, 80 and 80°C, respectively.

In anaerobic fermentative hydrogen production process, the RIHP was defined as:

$$RIHP(\%) = \frac{X - X_0}{X_0} * 100\% \quad (16)$$

where X_0 (g-VSS/L) is initial hydrogen-producing biomass concentration; and X (g-VSS/L) is hydrogen-producing biomass concentration at fermentative time t (h).

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Acknowledgments

The authors wish to thank the Natural Science Foundation of China (51222812), the Recruitment Program of Global Experts, and the Program for Changjiang Scholars and Innovative Research Team in University of the Ministry of Education of China for the financial support of this study.

Author contributions

Y.M. carried out the experiments, analyzed the data, and wrote the paper; H.Y.Y., Y.Z.W., C.S.H. carried out the experiments; Q.B.Z. and Y.W. analyzed the data, and wrote the paper; H.Q.Y. designed the experiments, analyzed the data, and wrote the paper.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Mu, Y. *et al*. The Maximum Specific Hydrogen-producing Activity of Anaerobic Mixed Cultures: Definition and Determination. *Sci. Rep.* **4**, 5239; DOI:10.1038/srep05239 (2014).