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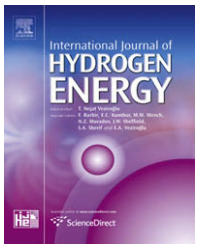

Isolation of anoxygenic photosynthetic bacteria from Songkhla Lake for use in a two-staged biohydrogen production process from palm oil mill effluent

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ABSTRACT

We are developing a process to produce biohydrogen from palm oil mill effluent. Part of this process will involve photohydrogen production from volatile fatty acids under low light conditions. We sought to isolate suitable bacteria for this purpose from Songkhla Lake in Southern Thailand. Enrichment for phototrophic bacteria from 34 samples was conducted providing acetate as a major carbon source and applying culturing conditions of anaerobic-low light (3000 lux) at 30 °C. Among the independent isolates from these enrichments 19 evolved hydrogen with productivities between 4 and 326 ml l^{-1} d^{-1}. Isolate TN1 was the most efficient producer at a rate of 1.85 mol H_2 mol $acetate^{-1}$ with a light conversion efficiency of 1.07%. The maximum hydrogen production rate for TN1 was determined to be 43 ml l^{-1} h^{-1}. Environmentally desirable features of photohydrogen production by TN1 included the absence of pH change in the cultures and no detectable residual CO_2.

1. Introduction

As oil prices rise and concerns regarding the environmental problems accompanying the use of fossil fuels escalate, there is an increasing demand for reliable and effective energy alternatives [1–3]. Hydrogen is regarded as a clean fuel since its complete combustion only emits water (H_2O). Furthermore, the technology is already available that makes it useable as a fossil fuel alternative [4–9]. However, using hydrogen as a fuel source will only be an acceptable alternative provided its production is environmentally sound [10,11].

A major industry in southern Thailand is the extraction of palm oil. The waste effluent from this process, POME (Palm Oil Mill Effluent), is rich in organic material and could therefore potentially support the bioproduction of hydrogen. The main biological hydrogen production systems already in use are based on dark-fermentation and phototrophic metabolisms [12–17]. A thermophilic hydrogen-producing anaerobic bacterium that was isolated from the high temperature POME (70–80 °C) has been shown to be well-suited for dark-fermentation hydrogen production [18–21]. But the products of its fermentative growth are VFAs (Volatile Fatty Acids) and CO_2, which means that (i) hydrogen bioconversion of the organic material is incomplete and (ii) the process is generating an environmentally adverse greenhouse gas. A particularly promising addition to the dark-fermentation process is a second stage in which anoxygenic photosynthetic bacteria (PSB) would further metabolize the VFAs [4,5,22–25],

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producing additional hydrogen, while also consuming CO_2 evolved during both stages [26,27].

We have previously isolated and characterized a variety of PSB [28]. However, none of these are useful in the second stage of biohydrogen production from POME; they grow poorly in the presence of VFAs, they have low hydrogen production, or they are inefficient at hydrogen production in the low transparency of the effluent (unpublished results). Towards engineering a 2-staged system, we identified and characterized a new natural isolate of PSB from Songkhla Lake that has a high-hydrogen production capacity in low light using acetate. While Songkhla Lake is the largest lake in Thailand and the second largest in Southeast Asia [29], to our knowledge, there are no previous descriptions of PSB isolated from this lake.

2. Materials and methods

2.1. Isolation of non-sulfur photosynthetic bacteria (PSB) from Songkhla Lake

A total of 34 water and lake bed samples were collected from Songkhla Lake in the Phatthalung and Songkhla Provinces of Thailand. Non-sulfur photosynthetic bacteria were enriched for by inoculating with the samples 50 ml serum vials that had been completely filled with GA medium, which is basal medium [23] modified by the addition of 5 mM glutamate as a major nitrogen source and 20 mM acetate as a major carbon source. Anaerobic conditions were established by flushing the sealed vials with argon gas. The vials were incubated at room temperature (30 ± 2 °C) in low light (3000 lux). After 10 days of incubation, the cultures varied in color, and included dark red, pink, brown and yellow. Bacteria from each culture were streak-purified on GA agar plates that were then incubated in anaerobic jars with a CO_2 generator (AnaerocultA system, Merck, Germany) placed in front of a tungsten lamp. One isolated colony from each plate was cultured in GA agar and permanent stocks were prepared from samples of these cultures.

2.2. Culturing of natural isolates of non-sulfur photosynthetic bacteria

Each permanent stock was used to inoculate three 50 ml serum vials completely filled with GA and closed with a silicon stopper. The vials were incubated under anaerobic-low light conditions at 30 °C for 2 days in a water bath to cut off infrared light from the lamp. Because nitrogenase, a hydrogen-producing enzyme, is inhibited by ammonium [30,31], the GA medium was modified by substituting Na_2MoO_4 for $(NH_4)_6Mo_7O_{24}$ at a concentration of 0.75 mg l^{-1}.

2.3. Analytical methods

Cell densities were monitored by measuring light scattering at 660 nm with a Zenyth 200 microplate reader (Anthos Labtec., UK). Dry weights (DCW) were calculated from a standard curve that was generated for each of the different isolates by the method of Sasaki et al. [32].

The biogas production in each culture vial was measured by a syringe technique described by Owen et al. [21]. Hydrogen content of the biogas was determined using an Oldham MX 2100 gas detector (Cambridge Sensotec Ltd., England).

Acetate utilization was determined by measuring free fatty acid concentrations in samples of the cultures using an HP 6850 Gas Chromatogram equipped with a frame ionization detector (Hewlett Packard, USA) and a 30 m × 0.25 mm × 0.25 μm capillary packed with nitroterephthalic acid-modified polyethleneglycol (DB-FFAP, Agilent J&W GC columns, USA). The samples were first centrifuged at 10,502 *g* for 5 min, acidified by using 0.2 N oxalic acid, and then passed through a 0.2 μm nylon membrane. The operational temperatures at the injection port and detector were 230 and 250 °C, respectively. The oven was programmed as follows: (i) hold at 70 °C for 3 min, (ii) ramp for 5 min at 20 °C min^{-1} to a final temperature of 180 °C, (iii) hold at 180 °C for 3 min. Helium was used as carrier gas with a flow rate of 1.2 ml min^{-1}.

2.4. Calculation of hydrogen yield and production efficiency

Hydrogen yields (Y) were expressed as the total hydrogen gas produced (p) per gram of dry cells (x).

The efficiency of hydrogen production from acetate was defined as indicated in Eq. (1) [25]:

$$H_2(\%) = \frac{\text{Amount of } H_2 \text{ produced (mol)}/\text{Amount of acetate consumed (mol)}}{\text{Theoretical amount of } H_2 \text{ produced (mol)}/\text{Amount of acetate consumed (mol)}} \times 100 \quad (1)$$

2.5. Kinetic analysis

A modified Gompertz equation (Eq. (2)) [33] was used to fit the cumulative hydrogen production data for TN1 cultures to obtain P, R_{max}, and λ:

$$H = P \exp\left\{-\exp\left[\frac{R_{max}e}{p}(\lambda - t) + 1\right]\right\} \quad (2)$$

H: cumulative H_2 production (ml l^{-1}), P: maximum cumulative H_2 production (ml l^{-1}), R_{max}: maximum H_2 production rate (ml l^{-1} h^{-1}), λ: lag time (h), t: culture time (h), and e: irrational constant (2.718).

The kinetic parameters were determined by best-fitting the hydrogen production data for Eq. (2) using Sigma Plot version 9.0.

2.6. Light conversion efficiency

Light conversion efficiency (η) is defined as the efficiency by which the light energy can be transformed into H_2 [34]. The η value was calculated using the following equations:

$$\eta(\%) = \frac{H_2 \text{ energy content} \times H_2 \text{ output}}{\text{light energy input}} \times 100 \text{ [34]} \quad (3)$$

$$= \frac{241.83\left(\text{kJ mol}^{-1}\right)2\left(g_{H_2}\text{mol}^{-1}\right)\rho\left(\text{gl}^{-1}\right)V(\text{l})}{I(\text{Js}^{-1}\,\text{m}^{-2})t(\text{h})3600\left(\text{sh}^{-1}\right)A(\text{m}^2)} \times 100$$

$$= \frac{33.6\rho_{H_2}V_{H_2}}{ItA} \times 100 \quad (4)$$

where ρ_{H_2} is the density of H_2 production ($g\,l^{-1}$), V_{H_2} is the volume of H_2 production (l), I is the light intensity in $W\,m^{-2}$ ($1\text{ lux} = 0.0161028\,W\,m^{-2}$), t is the duration time of H_2 production (h), A is the irradiated area (m^2). $A = \pi \times H \times D$, where the H is the height of culture broth and D is the diameter of the serum vial. The enthalpy of water formation in the gas phase is 241.83 kJ mol^{-1} [35].

3. Results and discussion

3.1. Isolation of, and hydrogen production by newly isolated photosynthetic non-sulfur bacteria

It has been reported that photosynthetic non-sulfur bacteria can produce hydrogen from metabolizing volatile fatty acids such as acetate that are generated from fermentations by anaerobic bacteria [12,14,35]. Using modified GA medium that contains 20 mM sodium acetate as a major carbon source, photosynthetic non-sulfur bacteria were isolated from 34 individual samples of Songkhla Lake waters and lake bed sediments. Among the independent PSB isolates obtained from these samples, 19 could produce hydrogen with a range of 4–326 ml $l^{-1}\,d^{-1}$ (Table 1). The top six hydrogen-producing isolates, designated SL2, SL3, SL8, SL15, SL24, and TN1 had production rates of 326, 305, 308, 157 and 301 ml $l^{-1}\,d^{-1}$, respectively, with hydrogen yields of 543, 564, 424, 209, and 456 ml g DCW^{-1}, respectively. Cultures of isolate SL8 were not consistent with respect to hydrogen production, and so this isolate was not examined further. The remaining five were chosen for further studies.

3.2. Growth, acetate consumption, and hydrogen production of high-hydrogen-producing natural isolates of PSB

To be useful in our two-staged biohydrogen production process, the PSB should grow well in low light and in media with acetate, and hydrogen production should be dependent upon acetate availability. The top five hydrogen producers were evaluated with respect to these parameters (Fig. 1 and Table 2). Measurements were performed on triplicate cultures of each isolate over the entire duration of logarithmic growth, a period of 72 h.

The specific growth rates of the five high-hydrogen producers were variable. The lowest rate was 0.0269 h^{-1} for isolate SL15 and the highest rate was 0.0475 h^{-1} for isolate SL24. For all of the high-hydrogen producers, acetate was depleted within 48 h (Fig. 1) with a concomitant accumulation of hydrogen. The efficiency of hydrogen production from acetate was determined by comparing the theoretical yield to the measured amount of hydrogen produced by these isolates (Table 2). Isolate TN1 had a production efficiency of 46.31%, which was the highest of the five isolates. The corresponding light conversion efficiencies calculated from the amount of hydrogen produced during 72 h ranged from <0.01 to 1.07% (Table 2). Thus, we found that while SL24 had the highest specific growth rate, it had the lowest hydrogen production efficiency from acetate. By contrast, TN1 had a high specific growth rate and also the highest production efficiency.

Table 1 – Hydrogen production by new PSB isolates in 20 mM sodium acetate under anaerobic-low light conditions (3000 lux) at 30 °C.

Strain	Color appearance in anaerobic-light	Dry cell weight ($g\,l^{-1}$)	Total biogas ($ml\,l^{-1}\,d^{-1}$)	Total H_2 ($ml\,l^{-1}\,d^{-1}$)	% H_2	Y p/x (ml g DCW^{-1})
SL1	Red–pink	0.78	24	22	91.67	14.10
SL2	Red–pink	0.30	350	326	93.14	543.33
SL3	Pink	0.27	337	305	90.50	564.81
SL6	Pink	0.30	22	20	90.91	33.33
SL7	Pink	0.28	22	19	86.36	33.33
SL8	Orange–pink	0.28	183	167	91.26	303.64
SL10	Red–pink	0.24	131	96	73.28	200.00
SL14	Orange–pink	0.67	38	28	73.68	20.90
SL15	Pink	0.24	215	208	96.74	424.49
SL19	Orange–pink	0.52	104	92	88.46	88.46
SL21	Red–pink	0.78	22	4	18.18	2.56
SL24	Orange–pink	0.27	190	157	82.63	290.74
TN1	Orange–pink	0.33	341	301	90.27	456.06
TN3	Orange–pink	0.81	38	36	94.74	22.22
TN4	Orange–pink	0.74	30	20	66.67	13.51
TN5	Red	0.57	131	96	73.28	84.21
TN6	Red	0.82	22	20	90.91	12.20
TN7	Brown	0.74	36	34	94.44	22.97
TN8	Brown	0.87	23	20	86.96	11.49

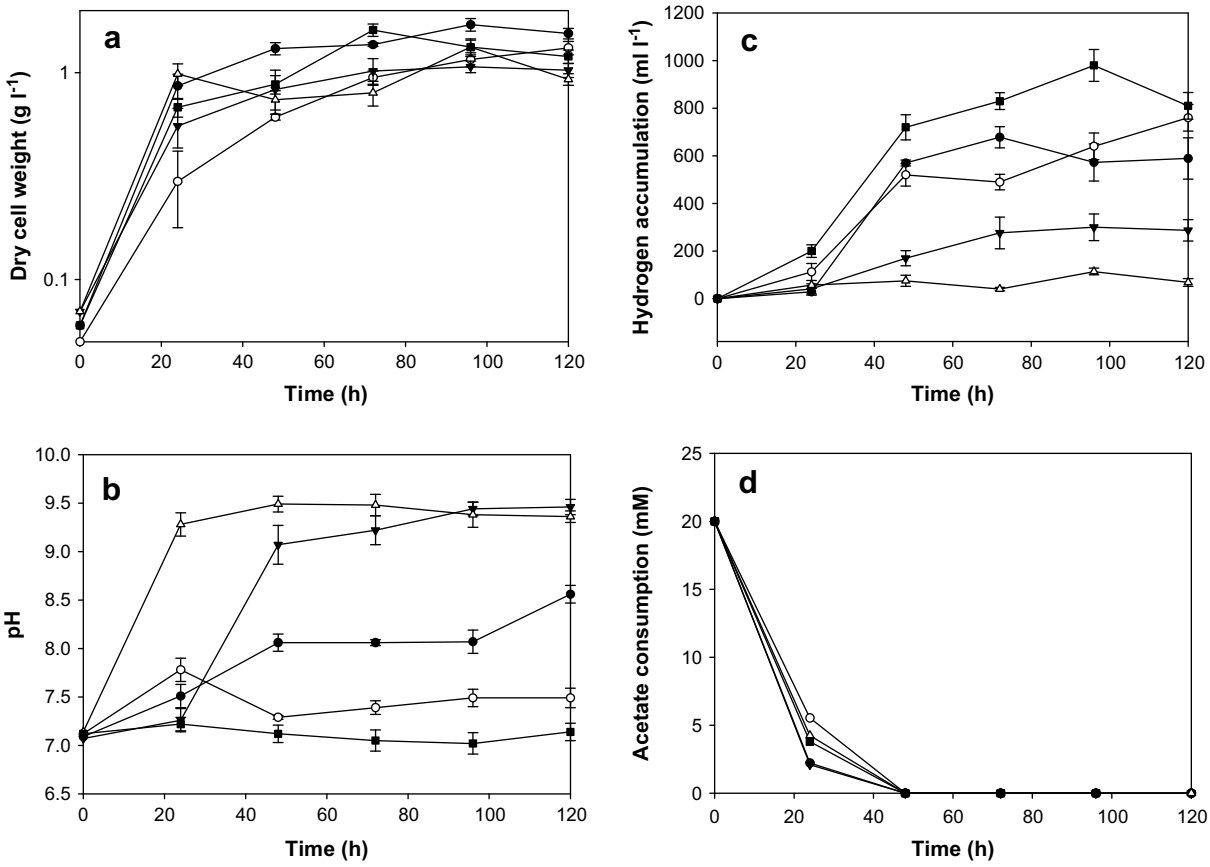

Fig. 1 – Time course of photohydrogen production by new natural isolates under anaerobic-low light conditions (3000 lux) at 30 °C; cell growth (a), pH change (b), hydrogen accumulation (c), and acetate utilization (d). In each panel, symbols are ● for SL2, ○ for SL3, ▼ for SL15, △ for SL24, and ■ for TN1.

3.3. *pH of the cultures*

A negative impact of elevated pH on hydrogen production has been reported for various PSB [31,36]. It may be that these high pHs are suboptimal for hydrogen-producing nitrogenase, for uptake hydrogenase, or for both. We measured the change in pH of the culture with time for the five high-hydrogen-producing isolates (Fig. 1) and found that there was a close correspondence between pH changes of the culture (Fig. 1) and hydrogen productivity (Table 2). Thus, the greatest pH change occurred in cultures of SL15 and SL24, and these also had the lowest efficiencies of hydrogen production, while the pH changed by less than 0.1 unit in the cultures of TN1, which had the highest efficiencies of hydrogen production. Perhaps the lack of pH change observed for the TN1 cultures reflects a particularly high capacity of this isolate to minimize pH change through the use of alternate electron sinks for reductants, in particular, poly-3-hydroxybutyrate (PHB) [37,38].

3.4. *Kinetic analysis of hydrogen production by TN1*

Among the five high-hydrogen producers, isolate TN1 generated the most hydrogen per mol of acetate, and so had

Table 2 – Characteristics of the top five H_2-producing PSB new natural isolates.

Strains	Specific growth rate (h^{-1})	H_2 production (mol H_2 mol acetate^{-1})[a]	Efficiency of H_2 production (%)[a]	Light conversion efficiency (%)[a]	Final pH[a]
SL2	0.0300	1.37	34.17	0.63	8.06
SL3	0.0298	1.09	27.34	0.33	7.39
SL15	0.0269	0.56	13.91	0.10	9.22
SL24	0.0475	0.09	2.23	<0.01	9.48
TN1	0.0328	1.85	46.31	1.07	7.05

a After 72 h cultivation.

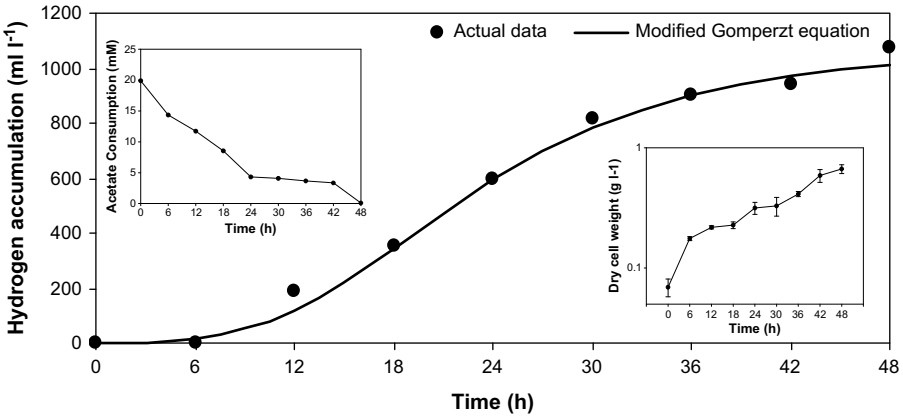

Fig. 2 – Hydrogen accumulation curve fitted by the modified Gompertz equation for triplicate cultures of isolate TN1 cultivated for 48 h.

the highest light conversion efficiency. It was therefore regarded as the best candidate for our two-staged biohydrogen production system from POME. In order to further investigate its suitability, we examined its kinetics of hydrogen production using a modified Gompertz equation [33,39,40], as described in the Materials and methods. TN1 was again cultured in triplicate under anaerobic-low light (3000 lux) conditions in modified GA medium and measurements of growth, acetate utilization, hydrogen production, and pH were taken every six hours over the course of two days. The inverse correlation between the amount of acetate available and the amount of hydrogen produced was observed again, as was a lack of change in pH of the cultures, which remained at pH 7.0 throughout. As shown in Fig. 2 by the solid line, there was a good fit of the hydrogen production data to the modified Gompertz equation, with an R^2 of 0.995. The R_{max} defined by the equation was 43 ml l^{-1} h^{-1}. It will be important to perform similar kinetic analyses when the bacteria are cultured in media that more closely approximates the VFA composition of the effluent after the anaerobic-dark-fermentation stage.

3.5. *Performance assessment of photohydrogen production from acetate by isolate TN1*

From our analyses, TN1 possesses the following attributes with respect to photohydrogen production from acetate:

- Isolate TN1 produces high amounts of hydrogen from acetate at an efficiency of 46.31%. A recent survey of the ability to use cultures to generate hydrogen for electricity found the best rate for photo-fermentation among PSB was 0.16 mmol l^{-1} h^{-1} [6]. TN1 compares favorably with a rate of 1.73 mmol l^{-1} h^{-1} (converted from the R_{max} value using a factor of 24.8 ml $mmol^{-1}$ at 30 °C and 1 atm pressure [7]).
- The pH of TN1 cultures remains near neutral. Not only does this seem to be important in terms of hydrogen production, since we found that the pH change in cultures of our isolates correlated with a lower hydrogen production, but this is also desirable in terms of environmental impact or other downstream applications.
- When cultured in modified GA medium (20 mM acetate), there was no detectable CO_2 in the biogas when all of the acetate had been utilized (results not shown). Therefore the hydrogen content of the biogas is very high. This could simplify the downstream purification step to obtain hydrogen suitable for fuel cell use in generating electricity [9,41].

4. Conclusions

Songkhla Lake harbors PSB that have a high capacity to use acetate in photohydrogen production with rates of 157–326 ml of H_2 l^{-1} d^{-1}. While species identification was not part of this investigation, we found that the color of cultures of the top five high-hydrogen producers we isolated differed. We also determined that these isolates differed with respect to their growth rates, rates of hydrogen production, relative light conversion efficiencies, and the change in culture pH *versus* time. Collectively these observations and measurements argue that the isolates may represent considerable genetic diversity.

Among our new natural isolates, we were able to identify a good candidate for our two-staged biohydrogen production process from POME. TN1 grows well in acetate in low light conditions and produces hydrogen with the high efficiency. The calculated light conversion efficiency for TN1 was 1.07%. We have attempted to compare the light conversion efficiency for TN1 to reports in the literature for other PSB. However, the experimental conditions are not equivalent, and so the values could not be compared.

Since there was no measurable CO_2 when all acetate had been consumed in cultures of TN1, the capacity of this bacterium to fix carbon had apparently not been exceeded. This promising result suggests the PSB could also reduce or perhaps even eliminate CO_2 produced from the anaerobic-dark-fermentation of POME. Optimization of the two-staged process will involve examining TN1 biohydrogen production from VFAs other than acetate, as well as the actual mixed products of the first stage of the process.

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