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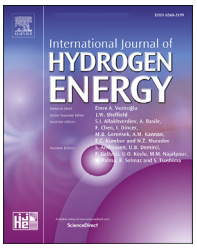

Biohydrogen production with lipid-extracted *Dunaliella* biomass and a new strain of hyperthermophilic archaeon *Thermococcus eurythermalis* A501

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HIGHLIGHTS

- *T. eurythermalis* A501 with *Dunaliella* biomass is an efficient H_2 production mode.
- Lipid-extracted *Dunaliella* algal residue can greatly improve the H_2 production.
- Algal concentration at 2.5 g/L and gas-liquid ratio at 2:1 reached optimal H_2 yield.
- 192.35 mL/g VS H_2 yield was obtained by *T. eurythermalis* A501 without pretreatment.

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ABSTRACT

Economic feasibility is important for the development of microalgae bioenergy industry. Dark fermentation of microalgal residue in a biorefinery context can improve the energy conversion efficiency of biomass and reduce the cost of microalgae industry. The present study proposes a promising dark fermentation model that combines thermophilic hydrogen-producing bacteria with algal residue substrates. Lipid-extracted *Dunaliella* residue can greatly improve hydrogen production by *Thermococcus eurythermalis* A501, the yields of which are more than four times higher than with algal cells as substrates. Under the optimal conditions of 2.5 g/L algal residue concentration and a 2:1 initial volume ratio of gas to liquid, the highest hydrogen yields of 192.35 and 183.02 mL/g volatile solid (VS) with algal residue of *Dunaliella primolecta* and *D. tertiolecta* are obtained, respectively, in less than 19 h without any pretreatment. This work may provide a biorefinery approach for comprehensive utilization of microalgae resources.

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Introduction

Increasing climate change and environmental pollution have prompted the search for clean energy [1]. Hydrogen has attracted more attention than other alternative energy sources, because of its high energy output and sole by-product of water [2]. However, most of the global hydrogen currently comes from steam reforming of non-renewable hydrocarbon in the petrochemical industry, which is not sustainable because of its dependence on fossil fuels [3]. In contrast, biohydrogen production from renewable source including biophotolysis, photo and dark fermentation has been increasingly emphasized [4]. Especially, dark fermentation can be served as a core bioprocess in the biorefinery concept, combined with sustainable biomass technology to produce hydrogen and other high-value byproducts, while utilizing organic residues from the upper biological processes [5]. In this context, dark fermentation, which combines hydrogen-producing bacteria with biomass, is attractive for further development.

Microalgae have recently been investigated as a source of biofuel production including biodiesel, bioethanol and biohydrogen [6]. Indeed, compare to terrestrial plants, microalgae offer a broad prospect for the biofuel production due to their high growth rate, lack of lignin and no need for arable land [7]. At present, the application of microalgae in biodiesel is heavily studied in the field of microalgae bioenergy. Nevertheless, microalgal biodiesel still cannot compete with conventional diesel because of the relatively high cost [8]. Comprehensive utilization of microalgal resources is a feasible way to reduce the production cost of biodiesel. *Dunaliella* is a unicellular species of microalgae that is promising for industrial applications in biodiesel production and carotenoids extraction [9,10]. *Dunaliella* residue after lipid extraction still contains high levels of sugar and protein, which are good substrates for dark fermentation [11,12]. Moreover, due to its characteristic of lacking a cell wall [13], it is easier to use *Dunaliella* as a substrate than other algae, due to lower pretreatment cost [14]. Therefore, the efficiency of microalgal biomass based production of energy can be improved by using *Dunaliella* algal residue as a substrate for dark fermentation, thus increasing the feasibility of microalgae bioenergy industrialization.

Various microorganisms function as biocatalysts in the dark fermentation process. The growth temperature categorizes these hydrogen-producing bacteria into ambient (15–30 °C), mesophilic (30–39 °C), thermophilic (50–64 °C) and hyper-thermophilic (>65 °C) [15]. When considering the effect of temperature on dark fermentation reaction, higher temperatures (≥60 °C) are thermodynamically more suitable for hydrogen production, which enables thermophiles to produce more hydrogen than mesophiles. Moreover, thermophilic and hyper-thermophilic hydrogen-producing bacteria usually have higher substrate conversion efficiency and hydrogen tolerance than mesophilic species [16]. Also, strictly thermophilic conditions can reduce the risk of contamination and inhibit activities of hydrogen consumers [17]. Currently, exploiting extreme environmental resources such as deep seas and hot springs has attracted increasing attention. Due to the advantages of thermophilic and hyper-thermophilic species, it is necessary to study the dark fermentation using hydrogen-producing microorganisms newly obtained from extreme environments.

Thermococcus eurythermalis A501 is a hyper-thermophilic archaeon with a wide range of growth conditions that was recently isolated from an oil immersed chimney of a deep-sea hydrothermal vent in the Guaymas Basin [18]. Based on a genome sequence analysis, *T. eurythermalis* A501 encodes a series of hydrogenase complexes and can thus be used to study the hydrogen production capacity of dark fermentation [19]. Therefore, in the present study, dark fermentation by a hyper-thermophilic archaeon *T. eurythermalis* A501 using *Dunaliella* biomass as a substrate was investigated. To compare this method of hydrogen production with previous studies, the kinetic parameters of dark fermentation were analyzed and hydrogen yields without any pretreatment were determined.

Materials and methods

Microalgal cultivation

The microalgae used were *Dunaliella primolecta* and *D. tertiolecta* acquired from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, China.

The microalgal species were cultivated in Dunaliella medium containing (g/L) NaCl, 87.69; $NaNO_3$, 0.42; $CaCl_2 \cdot 2H_2O$, 0.044; $NaHCO_3$, 0.84; $NaH_2PO_4 \cdot 2H_2O$, 0.0156; KCl, 0.074; $MgSO_4 \cdot 7H_2O$, 1.23; ferric citrate, 0.005 and 1 mL of microelement reagent containing (g/L) $MnCl_2 \cdot 4H_2O$, 1.81; $CuSO_4 \cdot 5H_2O$, 0.079; $ZnSO_4 \cdot 7H_2O$, 0.222; H_3BO_3, 2.86; $NaMoO_4 \cdot 5H_2O$, 0.39; $Co(NO_3)_2 \cdot 6H_2O$, 0.0494. In batch culture, *D. primolecta* and *D. tertiolecta* were cultivated in a 1 L Erlenmeyer flask and agitated by bubbling air, under 25 ± 2 °C and a light intensity of 4000 Lux (measured by a light meter).

Microalgae were collected by centrifugation at 6500 rpm for 5 min and washed twice with distilled water. The pellets were treated with a freeze dryer to obtain dried algal biomass and for further lipid extraction.

Microalgal biomass composition analysis

The method of Zhu et al. [20] was used to extract the total lipids from microalgae with some modifications. Algae powder (0.2 g) was added into a 5 mL organic reagent mixture of chloroform: methanol (2:1, v/v). After 20 min of shaking extraction, the samples were centrifuged at 8000 rpm for 10 min. This process was repeated until all lipids in the algal cells were extracted. Finally, all extracts were collected, steam dried at 60 °C in a water bath and weighed using an analytical balance.

The protein content was estimated by the Kjeldahl method and the total nitrogen content was converted to crude protein by a conversion factor of 6.25.

The carbohydrate content of microalgal biomass was measured using the 3.5-dinitrosalicylic acid colorimetric method (DNS method) [21]. The sample was pretreated as follows: algae powder (0.2 g) was added into 10 mL of 6 M HCl

and 15 mL distilled water, mixed and then boiled. Iodine solution was used as a reaction indicator. When the mixture boiled to the point where the iodine solution could not turn blue (30 min), the total carbohydrate was considered to have been hydrolyzed to monosaccharide.

Volatile solid (VS) content of microalgal biomass was measured by placing dry algal powder in a muffle furnace at 600 °C for 4 h. The volatile solid content is the percentage of the weight reduced after treatment to the total weight of dry samples.

Microorganism fermentation

T. eurythermalis A501 was used for fermentation experiments. The pre-inoculum was cultured in Thermococcales Rich Medium (TRM) [22], which contains (per liter of distilled water) yeast extract, 1 g; tryptone, 4 g; NaCl, 23 g; $MgCl{\cdot}6H_2O$, 5 g; $(NH_4)_2SO_4$, 0.5 g; KCl, 0.7 g; PIPES, 3.3 g; NaBr, 0.05 g; $SrCl_2$, 0.01 g; $CaCl_2{\cdot}2H_2O$ (2%), 1 mL; $NaWO_4$ (10 mM), 1 mL; $FeCl_3{\cdot}6H_2O$ (25 mM), 1 mL; KH_2PO_4 (6%), 1 mL; K_2HPO_4 (6%), 1 mL.

Dark fermentation

Batch experiments were conducted in 150 mL fermentation bottles that were sealed with butyl rubber stoppers and aluminum seals. In order to eliminate oxygen, nitrogen was filled as the gas phase. The pressure in the fermentation bottle was indicated by the barometer on the air pumping device. After replacing the air in the bottle with nitrogen, the pressure in the fermentation bottle was further adjusted to normal pressure with a syringe. All tests were performed at 85 °C for 18 h and included inoculations with late exponential-phase *T. eurythermalis* A501 (10^6 cells/mL). For these assays, the fermentation medium used was basal TRM (without yeast extract or tryptone) with microalgal biomass as a unique carbon source.

To analyze the potential of *Dunaliella* biomass, dry and wet and lipid-extracted microalgal biomass were used as substrates in fermentation experiments under three conditions. The different biomass concentrations and conditions used are shown in Table 1. The dry weight (g/L) of wet algal cells was calculated using the method proposed by Chiu et al. [23].

To optimize substrate concentration, the initial concentrations of lipid-extracted algal residues ranging from 0.625 to 10 g/L were investigated.

To analyze the effect of initial volume ratio of gas to liquid on hydrogen production, specific volumes of liquid medium were added to fermentation bottles to keep the ratios as 1:5, 1:2, 1:1, 2:1 and 5:1.

All the experiments and analysis were conducted in duplicate, and the results are shown as mean ± SD.

Analytical procedure for hydrogen

A 50 mL gas-tight syringe was used to measure the total volume of biogas accumulated during dark fermentation [24]. Hydrogen was detected by a gas chromatograph (GC-14B, SHIMADZU, Japan) equipped with a thermal conductivity detector (TCD) and a 1 m column (TDX-02, SHIMADZU, Japan). Argon was the carrier gas used in gas chromatography. The detector temperature was 120 °C, and the injector and column temperatures were both 100 °C.

The specific hydrogen yield (SHY; mL/g VS) was defined as the ratio of final volume of hydrogen produced (mL) to the original algal biomass weight (g VS). For the best operational conditions, the SHY was monitored using a modified Gompertz equation (Eq. (1)) [25]:

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

In this equation, H is the SHY (mL/g VS), λ is the lag time of hydrogen production (h), H_m is the maximum potential yield of hydrogen (mL/g VS), R_m is the peak rate of hydrogen production (mL/g VS/h), t is the cultivation time (h), and e is the exp (1) = 2.718.

Results and discussion

Microalgal biomass characterization

Carbohydrates and proteins are the main substrates used by microorganisms in the dark fermentation process. Several

Table 1 – Different added concentrations of dry and wet and lipid-extracted microalgal biomass under the conditions of same initial biomass concentration, same initial carbohydrate concentration and same initial protein concentration.

Algal biomass		Substrate concentration (in dry weight, g/L)		
		For same initial biomass concentration	For same initial carbohydrate concentration[c]	For same initial protein concentration[d]
Dunaliella primolecta	Dry algal cells	5	5	5
	Wet algal cells[a]	5	5	5
	Lipid-extracted algal residue	5	3.96	4.2
Dunaliella tertiolecta	Dry algal cells	5	5	5
	Wet algal cells[b]	5	5	5
	Lipid-extracted algal residue	5	2.75	4.85

[a] Dry weight of wet algal cells of *D. primolecta* was determined with the equation: Dry weight (g/L) = 0.4909 × OD_{680}+0.0955 (R^2 = 0.9903).
[b] Dry weight of wet algal cells of *D. tertiolecta* was determined with the equation: Dry weight (g/L) = 0.3573 × OD_{680}+0.1340 (R^2 = 0.9954).
[c] The initial carbohydrate concentrations of different algal biomass of *D. primolecta* and *D. tertiolecta* remained at 1.05 g/L and 1.02 g/L, respectively.
[d] The initial protein concentrations of different algal biomass of *D. primolecta* and *D. tertiolecta* remained at 1.67 g/L and 1.53 g/L, respectively.

studies demonstrated that carbohydrates could be effectively utilized by hydrogen-producing bacteria and further converted to hydrogen through fermentation [11,12]. In addition, protein is necessary to provide nitrogen for microorganism growth [12]. To quickly identify the potential of Dunaliella biomass as a fermentation substrate, the organic composition of microalgal biomass was measured (Table 2).

The algal cells of *D. primolecta* and *D. tertiolecta* contain relatively high contents of carbohydrate (more than 20%) and protein (more than 30%), and thus have the potential to be used as fermentation substrates (Table 2). The percentages of carbohydrate of algal residues after lipid extraction from *D. primolecta* and *D. tertiolecta* were increased to 26.47% and 37.10%, respectively (Table 2), which suggested that algal residue may be more suitable as a fermentation substrate than algal cells. A biorefining method that integrates sustainable production of either oil-derived or sugar-derived biofuels is an economically attractive way to reduce the cost of producing third-generation biofuels from microalgal biomass [26]. As shown in Table 2, *Dunaliella* was also shown to be suitable for production of microalgal biodiesel with high lipid content (26.39% and 32.85% for *D. primolecta* and *D. tertiolecta*, respectively). Therefore, from the perspective of hydrogen production potential or comprehensive utilization, it may be more advantageous to make full use of lipid-extracted algal residue.

Hydrogen production from different algal biomass

In order to further verify the application potential of lipid-extracted *Dunaliella* algal residue in hydrogen production by *T. eurythermalis* A501, fermentation assays by algal residues were performed and compared with dry algal cells and wet algal cells. The SHYs of wet algal cells of *D. primolecta* and *D. tertiolecta* were 36.84 mL/g VS and 25.34 mL/g VS, respectively, more than six times higher than that of dry algal cells (Fig. 1a and d). This difference may be due to higher humidity of wet algal cells. The fermentation substrate was sterilized before the dark fermentation process and the increase of humidity in wet algal cells may enhance the sterilization effect and accelerate the thermal degradation process, thus resulting in higher SHY than dry algal cells [27,28]. This result is consistent with a previous study by Batista et al. [29], who observed that wet algal cells were more favored as a substrate for *Enterobacter aerogenes* than dry algal cells.

More importantly, it is evident that lipid-extracted algal residues performed very effectively as a substrate for hydrogen fermentation. Under the same initial biomass concentration (Fig. 1a and d), the SHYs of algal residues of *D. primolecta* and *D. tertiolecta* were 155.74 mL/g VS and 117.02 mL/g VS, which were 4.2 times and 4.6 times higher than that of wet algal cells, respectively. This result confirmed our speculation that algal residue may be more suitable for fermentation because of higher carbohydrate and protein contents (Table 2). Nobre et al. [30] also reported that higher SHY was obtained with lipid-extracted algal residue rather than algal cells and attributed the reason to a higher effective fermentable compound concentration in residue due to the oil extraction.

On the other hand, the C/N ratio of substrate is also an important factor affecting the efficiency of hydrogen production. Maintaining an appropriate C/N ratio can not only ensure the growth of hydrogen-producing bacteria, but also prevent the nitrogen inhibition effect caused by excessive nitrogen sources (proteins) in substrates [31]. Sun et al. [32] reported that protein-rich *Chlorella pyrenoidosa* and carbohydrate-rich rice residue could be used as co-ferment substrates at various mix ratios to optimize hydrogen production. Xia et al. [33] reported that when the C/N ratio of the substrates mixed with *Arthrospira platensis* and *Laminaria digitata* was 26.2, the SHY of anaerobic sludge reached a maximum of 83.9 mL/g VS. A wide range of C/N ratios has been reported, and differences in reaction conditions and hydrogen-producing bacteria may result in differences in optimal C/N ratio [31]. In this study, when comparing the SHYs of algal biomass from two species of *Dunaliella* under the same initial substrate concentration, the SHY of *D. primolecta* was always higher, regardless of whether the form was dry algal cells, wet algal cells or algal residue (Fig. 1a and d). This indicated that a higher ratio of protein to carbohydrate (lower C/N ratio) may be more conducive to the fermentation process of *T. eurythermalis* A501.

Furthermore, we compared the SHYs of different algal biomass under the same carbohydrate concentration (Fig. 1b and e) and the same protein concentration (Fig. 1c and f). From Fig. 1b and c, the SHYs of algal residues of *D. primolecta* were 159.30 mL/g VS and 162.31 mL/g VS, respectively. From Fig. 1e and f, the SHYs of algal residues of *D. tertiolecta* were 171.03 mL/g VS and 144.30 mL/g VS, respectively. Even with the same concentration of carbohydrate or protein in the substrate, lipid-extracted algal residues still performed very effectively as a substrate compared with wet or dry algal cells. Lakaniemi et al. [34] reported that the SHY was 12.6 mL/g VS when untreated anaerobic sludge was combined with microalgal cells of *D. tertiolecta*. Our results further demonstrated the superiority of *T. eurythermalis* A501 using *Dunaliella* algal residue to produce hydrogen.

Table 2 – Characterization of algal cells and lipid-extracted algal residues of *D. primolecta* and *D. tertiolecta*. The data is the mean of two repeats (mean ± SD).

Parameter	*Dunaliella primolecta*		*Dunaliella tertiolecta*	
	Algal cell	Lipid-extracted algal residue	Algal cell	Lipid-extracted algal residue
VS (%, w/w)	78.56 ± 0.02	80.83 ± 0.12	87.71 ± 0.28	84.77 ± 0.23
Proteins (%, w/w)	33.41 ± 026	39.78 ± 0.68	30.64 ± 0.05	31.57 ± 0.35
Lipids (%, w/w)	26.39 ± 0.41	n.d.	32.85 ± 0.18	n.d.
Carbohydrates (%, w/w)	20.99 ± 0.56	26.47 ± 0.21	20.38 ± 0.37	37.10 ± 1.13

n.d.: not detected.

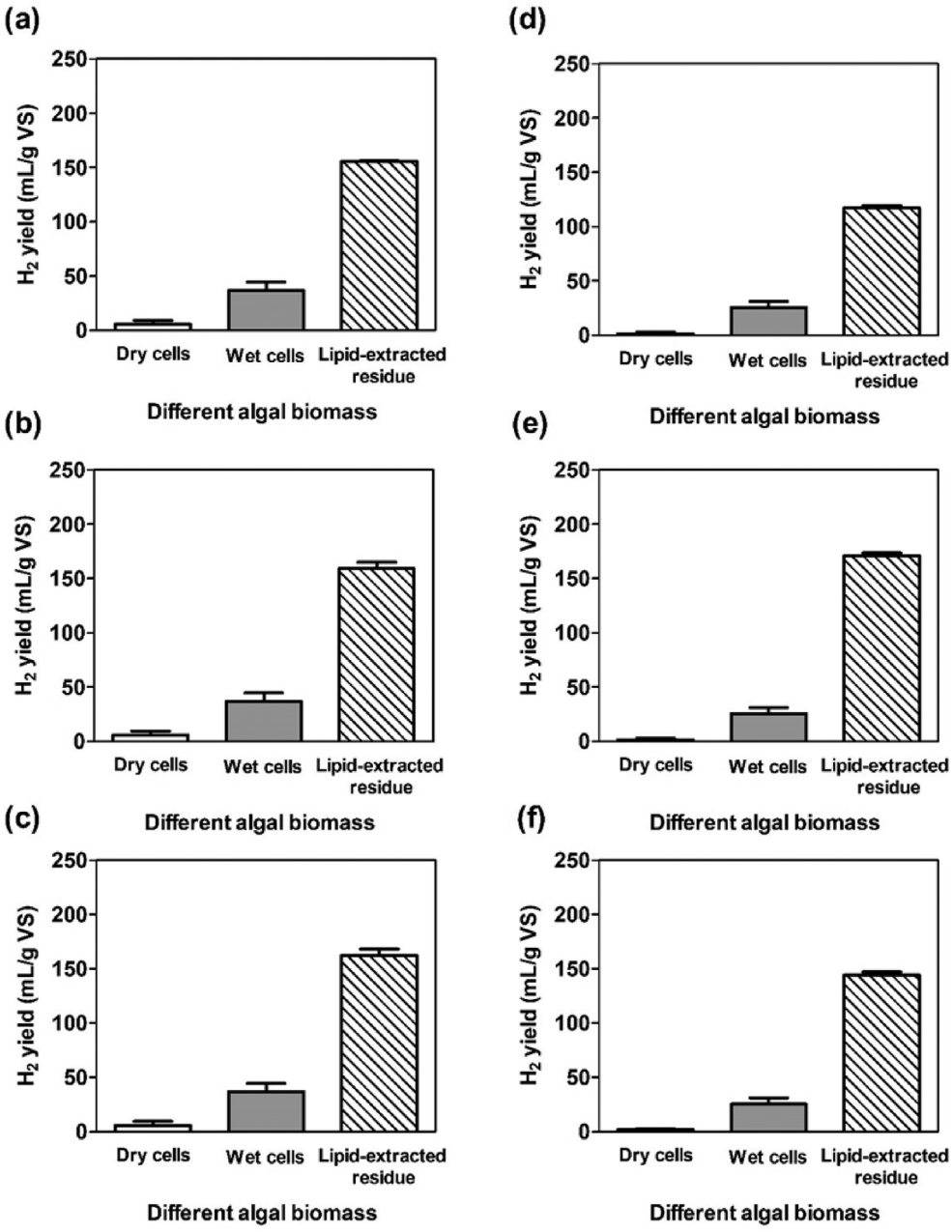

Fig. 1 – SHYs from different algal biomass of *Dunaliella primolecta* (a, b, c) and *D. tertiolecta* (d, e, f) by *Thermococcus eurythermalis* A501. a, d: Different algal biomass under the same initial biomass concentration; b, e: different algal biomass under the same initial carbohydrate concentration; c, f: different algal biomass under the same initial protein concentration.

There were several reasons for the obvious increase of hydrogen production when lipid-extracted algal residue was used as a substrate. Considering all three conditions, the reason may be related to a higher fragility of cellular structure. The process of repeated extraction of oil components may increase the specific surface area and intracellular accessibility of microalgal cells [35], rendering intracellular carbohydrate and protein more readily useable by hydrogen-producing bacteria. Moreover, it was reported that fermentation of long-chain fatty acids (which are included in lipid content) to shorter chain fatty acids is thermodynamically non-spontaneous unless methanogenesis is involved [36]. Therefore, the presence of a large amount of oil in dry and wet algal cells is not efficiently utilized by hydrogen-producing bacteria, resulting in lower SHYs of dry and wet algal cells compared to algal residues.

In a biorefinery context, using algal residue to produce hydrogen can promote the comprehensive utilization of microalgal resources. Moreover, the results indicated that the SHY was also significantly increased when algal residue was used as a substrate. This can be advantageous, considering that it is possible to reduce costs with an integrated biorefinery approach while achieving high SHY. Therefore, lipid-extracted algal residue was used as a substrate for the following optimization experiments.

Optimization of initial algal concentration for hydrogen production

Many previous studies have shown that substrate concentration is one of the core influencing factors of dark fermentation [37,38]. In our study, the effects of algal residue concentrations on hydrogen production are shown in Fig. 2.

When residue concentration of *D. primolecta* and *D. tertiolecta* increased from 0.625 g/L to 2.5 g/L, there were no significant changes in SHYs (Fig. 2). At a concentration of 2.5 g/L, the SHYs of *D. primolecta* and *D. tertiolecta* algal residues achieved 209.04 mL/g VS and 178.65 mL/g VS, respectively. With the residue concentration further increased to 10 g/L, the SHYs of *D. primolecta* and *D. tertiolecta* algal residues gradually decreased to 97.47 mL/g VS and 94.18 mL/g VS, respectively.

Lower yield obtained at higher substrate concentration was also observed in previous studies. Batista et al. [39] reported that when initial biomass concentration of *S. obliquus* increased from 2.5 to 50 g/L, the production of hydrogen decreased from 56.5 to 10.8 mL/g VS. This may be related to product inhibition. In a fixed headspace reactor, the increasing partial pressure of hydrogen is an important factor affecting dark fermentation. At high partial pressure of hydrogen, the reactions related to acetogenesis are thermodynamically non-spontaneous [40], while volatile fatty acids (VFAs) will gradually accumulate as by-products as dark fermentation progresses [41]. The increased concentration of VFAs will enhance the ionic strength in the medium and the inhibitory effect of un-dissociated acids, thus reducing the metabolic activity of the hydrogen producer. For example, Ginkel and Logan [42] observed that hydrogen production was inhibited when the concentration of self-produced undissociated acids accumulated reached 19 mM. Moreover, the increase of hydrogen pressure will also affect the enzymatic activity of microorganisms, thus inhibiting the further production of hydrogen [43]. Fiala and Stetter [44] reported that the accumulation of hydrogen would strongly inhibit the growth activity of *Pyrococcus furiosus*, which belongs to the order Thermococcales. At higher hydrogen pressure, *P. furiosus* will switch metabolic pathways to reduce further hydrogen production [45].

On the other hand, keeping a relatively high organic loading rate is also one of the reference indexes when selecting the optimal initial substrate concentration. In industrial production, maintaining a high organic loading rate can reduce the cost of equipment operation in the fermentation process while increasing the total SHY [31]. The SHYs were basically at the same high level when the initial substrate concentration ranged from 0.625 g/L to 2.5 g/L (Fig. 2). Increasing concentration will lead to increased organic loading rate, which is more advantageous. Therefore, the initial algal residue concentration of 2.5 g/L was selected for further research.

Optimization of initial gas-liquid volume ratio for hydrogen production

Since the volume ratio of gas to liquid in the fermentation system can directly affect the partial pressure of hydrogen, it is also a parameter that needs to be optimized in fermentation reaction [46]. Therefore, the effect of the initial volume ratio of gas to liquid on the fermentation performance was investigated (Fig. 3).

When the initial volume ratio of gas to liquid increased from 1:5 to 2:1, there was a continual increase in the SHYs (Fig. 3). At the initial ratio of 2:1, the SHYs of *D. primolecta* and *D. tertiolecta* algal residues both increased by 1.7-fold, as compared to the SHYs at the initial ratio of 1:5. Higher SHY achieved at higher ratio of gas to liquid may be related to the larger headspace volume of reactor, which lowers the partial pressure of hydrogen [47,48]. Bastidas-Oyanedel et al. [43] reported that in a mixed culture, the gas composition in the reactor headspace can affect hydrogen related metabolic pathways. The enzymatic activity related to hydrogen synthesis such as NADH hydrogenase was thermodynamically controlled by the partial pressure of hydrogen. Similarly, when the concentration of hydrogen increases, the reduction of oxidized ferredoxin is favored by the ferredoxin hydrogenase, thus shifting the metabolism to the direction of hydrogen reduction [49].

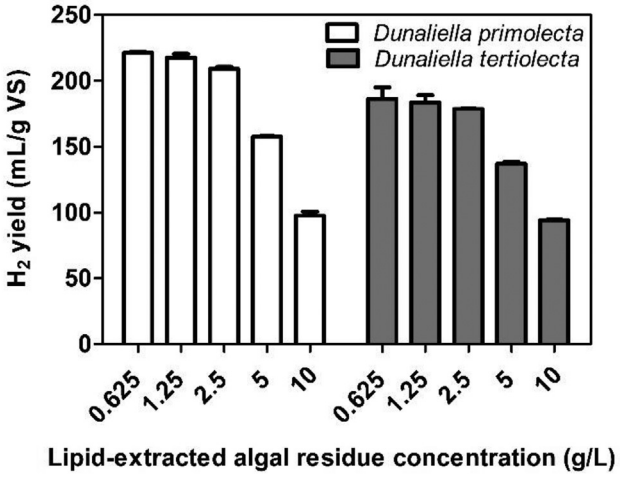

Fig. 2 – Effects of algal residue concentrations on SHY of *Thermococcus eurythermalis* A501.

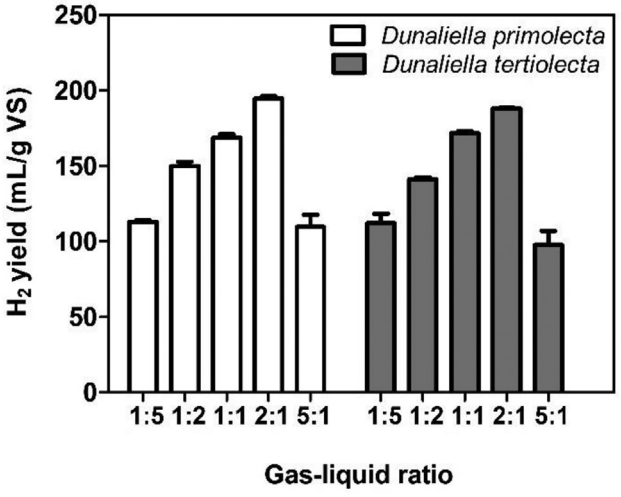

Fig. 3 – SHYs of *Thermococcus eurythermalis* A501 under different initial volume ratios of gas to liquid.

However, as the initial ratio of gas to liquid further increased to 5:1, the SHYs of *D. primolecta* and *D. tertiolecta* algal residues significantly decreased to 109.74 mL/g VS and 97.41 mL/g VS, respectively (Fig. 3). When the reactor volume is fixed, the further increase of gas-liquid ratio will lead to the decrease of the volume occupied by the fermentation broth, which is not conducive to the growth of hydrogen-producing bacteria.

Overall, the highest SHYs were achieved when the initial volume ratio of gas to liquid was 2:1 (Fig. 3). Therefore, it was decided to maintain the gas-liquid ratio of 2:1 in subsequent experiments.

Biohydrogen production kinetics

In order to further analyze the parameters in the process of dark fermentation and verify the hydrogen production potential of *T. eurythermalis* A501, kinetic analysis was carried out using a modified Gompertz equation (Eq. (1)). The conditions set in the simulation were the optimal conditions, that is, the algal residue concentration was 2.5 g/L and the initial volume ratio of gas to liquid was 2:1. The hydrogen generation kinetic fitting results of *D. primolecta* and *D. tertiolecta* are shown in Fig. 4.

With the extension of fermentation time, the dynamic curve first went through a lag phase, and then hydrogen production increased rapidly until the hydrogen accumulation finally reached a stable phase (Fig. 4). The equation fits the experimental data well, since the R^2 is higher than 0.99 (Table 3). The estimated kinetic parameters H_m, R_m and λ are in Table 3. The maximum SHYs with *D. primolecta* and *D. tertiolecta* as substrates for *T. eurythermalis* A501 were 201.423 mL/g VS and 184.038 mL/g VS, respectively. These values are quite high when compared with previous studies. Yang et al. [50] obtained a maximum SHY of 45.5 mL/g VS using lipid-extracted *S. obliquus* microalgal residues as a substrate for fermentation by an anaerobic digested sludge. Nobre et al. [30] also reported a SHY of 97.7 mL/g VS using oils and pigments extracted *Nannochloropsis* sp. microalgal biomass as feedstock through dark fermentation by *Enterobacter aerogenes*.

Short lag periods were also observed during fermentation (6.569 h and 5.652 h when using *D. primolecta* and *D. tertiolecta* as substrates, respectively), indicating that *T. eurythermalis* A501 has strong adaptability to the operating fermentation conditions. To further estimate the time required to complete the fermentation process, the kinetic parameter t_{95}, which represents the time required to complete 95% of the hydrogen production reaction [25], was also estimated by equation (2):

$$t_{95} - \lambda = \frac{H_m}{R_m e}(1 - ln(-ln0.95)) \quad (2)$$

The values of t_{95} of *D. primolecta* and *D. tertiolecta* were 18.807 h and 16.045 h, respectively (Table 3). This is relatively short when compared with previous studies, including one where more than 70 h fermentation time was needed by *Clostridium acetobutylicum* using *A. platensis* as feedstock [51]. This is a beneficial result considering that short lag period and fermentation time can reduce energy expenditure and increase hydrogen production efficiency.

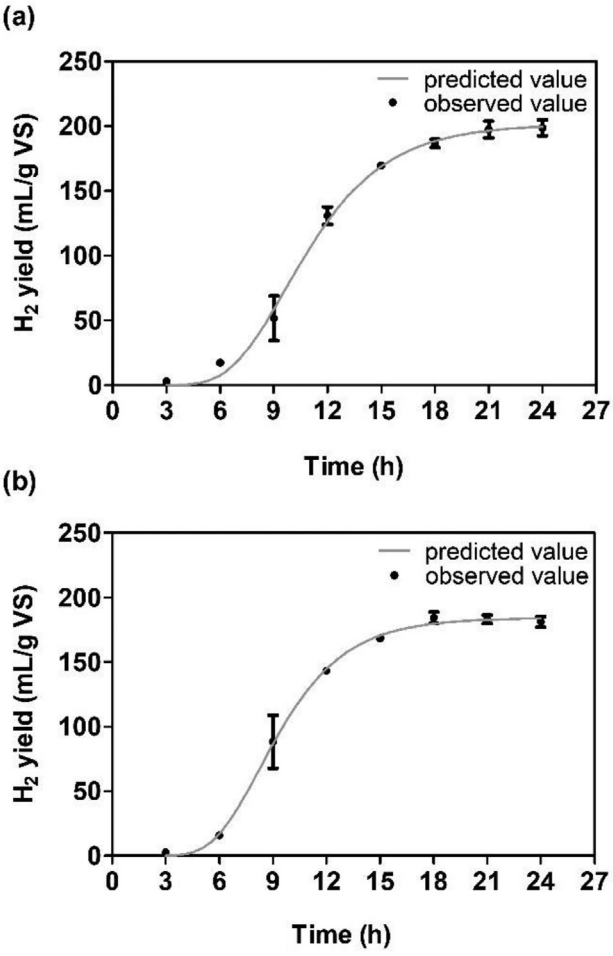

Fig. 4 – Biohydrogen production kinetics of lipid-extracted algal residues of *Dunaliella primolecta* (a) and *D. tertiolecta* (b) by *Thermococcus eurythermalis* A501. Kinetic analysis conditions were as follows: microalgal residue concentration 2.5 g/L, initial volume ratio of gas to liquid 2:1.

High SHY and short lag phase obtained by *T. eurythermalis* A501 using *Dunaliella* algal residue as a substrate can be related to the advantages of thermophiles. This is consistent with a previous study by Kumar et al. [52], who showed that the lag phase is greatly shortened under thermophilic

Table 3 – Fitting parameters of dark fermentation from *Thermococcus eurythermalis* A501 combined with *Dunaliella* algal residue substrates. Kinetic analysis conditions were as follows: microalgal residue concentration 2.5 g/L, initial volume ratio of gas to liquid 2:1.

Parameter	*Dunaliella primolecta*	*Dunaliella tertiolecta*
H_m (mL/g VS)	201.423 ± 4.720	184.038 ± 1.887
R_m (mL/g VS/h)	24.040 ± 2.071	25.864 ± 1.216
λ (h)	6.569 ± 0.362	5.652 ± 0.175
t_{95} (h)	18.807	16.045
R^2	0.996	0.999

conditions, and therefore more suitable for hydrogen production than under mesophilic conditions. In addition, *Dunaliella* algal biomass, which lacks a cell wall, may also promote substrate utilization by *T. eurythermalis* A501, thus increasing the SHY.

Effects of autoclave pretreatment on hydrogen production

It is necessary to sterilize the culture medium before a fermentation reaction when the hydrogen-producing microorganism is in pure culture. In this study, *Dunaliella* biomass did not undergo any pretreatment, except the autoclaved process necessary for fermentation medium (with addition of microalgal biomass). This study used a new hyperthermophilic archaeon, *T. eurythermalis* A501 as hydrogen-producing catalyst, which may have the advantage of preventing contamination due to the high temperature (85 °C) maintained by its fermentation process. In this case, the effect of autoclave pretreatment was investigated (Fig. 5).

The SHYs of *D. primolecta* and *D. tertiolecta* without autoclave sterilization were 192.35 mL/g VS and 183.02 mL/g VS, respectively (Fig. 5). The SHYs were only slightly lower than that with sterilization, indicating that the high SHY was almost unaffected by autoclave treatment. This can be explained by the high temperature maintained during fermentation. In addition to preventing contamination, the purpose of autoclaving *Dunaliella* biomass is to destroy cell structure and hydrolyze the released polysaccharides into monosaccharides [53,54]. *Dunaliella* have no cell walls, meaning they do not require drastic pretreatment. Moreover, the oil extraction process also destroyed the structure of *Dunaliella* cells, leading to easier release of intracellular compounds. During dark fermentation, maintaining a high temperature can also promote the utilization of algal residue by *T. eurythermalis* A501. The results suggested that the temperature of the fermentation process may be high enough that the algal residue is fully utilized by *T. eurythermalis* A501, and thus high SHYs were obtained.

Dark fermentation can be inoculated with either single or mixed culture. Compared with single culture, mixed culture such as anaerobic sludge is easier to use because of its simple operation and because sterilization is not required [55]. Sterilization on an industrial scale is economically inefficient. However, special treatments are still needed for the initial inoculum of mixed culture, so as to select hydrogen-producing microorganisms and inhibit the activity of other competitors or hydrogen consumers. This was confirmed by the work of Cai and Wang, who observed that the hydrogen production effect of pre-treated intertidal sludge was significantly better than that of untreated sludge [56].

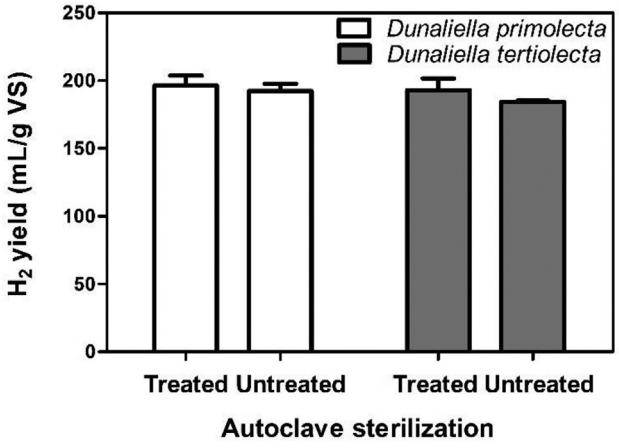

Fig. 5 – Effect of autoclave sterilization on SHY of *Thermococcus eurythermalis* A501.

At present, the most common treatment method is heat shock [57], but during the treatment process, the diversity of hydrogen-producing bacteria will decline (lack of mesophilic hydrogen-producing bacteria that do not form spores), and the activity of hydrogen consumers cannot be completely inhibited, which will eventually decrease hydrogen production [58,59]. In this study, high SHY was still obtained without sterilization when *T. eurythermalis* A501 used *Dunaliella* algal residues as a substrate. This is favorable, considering that the hydrogen production mode of *T. eurythermalis* A501 combined with lipid-extracted *Dunaliella* algal residues can achieve high SHY while reducing sterilization costs. Moreover, the SHY obtained with this method lacking any pretreatment was quite high, considering that the range of hydrogen production by fermentation of microalgal biomass without any pretreatment in previous studies is 0.37–97.7 mL/g VS [60].

It should be noted that the high temperature required by *T. eurythermalis* A501 in the fermentation process still requires a high energy input, which may also increase the cost of dark fermentation. For possible future industrial use, additional heat demand required in thermophilic fermentation may be achieved at a large-scale via the use of waste heat with efficient heat exchange and recovery units. Ljunggren and Zacchi [61] reported that the use of heat recovery devices can reduce heat demand by 88%, thus greatly reducing the cost of thermophilic fermentation. On the other hand, they also found that the addition of yeast extract as a nutrient to the fermentation medium significantly increased the total cost. In our study, *T. eurythermalis* A501 produced hydrogen in the basal TRM medium (without yeast extract or tryptone) with *Dunaliella* algal residue as the sole substrate. Moreover, high value chemicals such as VFAs were also produced when hydrogen is produced by dark fermentation. The fermentation effluent can be used as a feedstock for further biogas production (combined with anaerobic digestion or photo fermentation) or biomass production (microalgal culture) in a biorefinery way, thus further enhancing comprehensive utilization and feasibility of this model [62–64].

Conclusions

In this work, lipid-extracted *Dunaliella* residue was found to be a potential fermentation substrate utilized by *T. eurythermalis* A501 that can promote comprehensive utilization of microalgal resources in an integrated biorefinery approach. Substrate concentration and initial volume ratio of gas to liquid were both key factors in the fermentation process. The highest hydrogen yield of 192.35 mL/g VS with *D. primolecta* algal residues was obtained under optimal conditions without any pretreatment. The results indicated the superiority of *T.*

eurythermalis A501 in fermentation with lipid-extracted *Dunaliella* biomass, which can be used to provide insights into the development and utilization of deep-sea thermophiles resources for dark fermentation research.

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